Spoilage bacteria in ewe milk and the sheep dairy farm environment

A thesis presented in partial fulfilment of the requirements for the degree of

Doctor of Philosophy

in

Veterinary Science/Microbiology

at Massey University, Manawatū, New Zealand.

Alexis Nils Risson

2023

MASSEY UNIVERSITY
TE KUNENGA KI PŪREHUROA
UNIVERSITY OF NEW ZEALAND

GRADUATE RESEARCH SCHOOL

Note for Examiners of Doctoral Theses
Explanation of COVID-19 Impacts

The Doctoral Research Committee recognises the impacts of Covid-19 on research, particularly for doctoral candidates, and we appreciate the efforts made by supervisors and candidates to ensure timely completion of the doctoral thesis. We know that in some cases this has meant the project has needed to be changed in some way, including its final presentation. For students whose work has been impacted, we invite supervisors to provide a note for examiners explaining the circumstances.

Instructions for Supervisors:

The note is designed to enable you to communicate to examiners your desire for them to take account of certain factors in their assessment of a thesis to address delays and disruptions experienced by a thesis student as a result of the Covid-19 pandemic.

The attached form should be used to provide **an explanation** to the examiners on what to consider in their evaluation. It should detail how the project was altered or how the final product of the thesis has been affected as a result of the disruption. Statements should be clear and succinct for the benefit of the examiners and in fairness to the student and others in the student cohort.

The form should be signed by the student, the supervisor and the Head of Academic Unit, or nominee, and included in the information that is sent out with the thesis.

For doctoral candidates, the completed form should be inserted into the front of the thesis before the abstract by the candidate when submitting their digital thesis for examination in the Student Portal. At the completion of the examination, the amended form which excludes any confidential comments to the examiners, should be included in the appendices.

Please be sure to indicate whether a student has received a suspension of studies due to Covid-19 and/or an extension, as it is important to note if students have already had some special consideration.

Approved by DRC 10/Feb/2021
DRC 21/02/03

MASSEY UNIVERSITY
TE KUNENGA KI PŪREHUROA
UNIVERSITY OF NEW ZEALAND

GRADUATE RESEARCH SCHOOL

Note for Examiners
Explanation of COVID-19 Impacts

Thank you for taking the time to examine this thesis, which has been undertaken during the Covid-19 pandemic. The New Zealand Government's response to Covid-19 includes a system of Alert Levels which have impacted upon researchers. Our University's pandemic plan applied the Government's expectations to our research environment to ensure the health and safety of our researchers, however, research was impacted by restrictions and disruptions, as outlined below.

For a six-week period from March 26 to April 27 2020, New Zealand was placed under very strict lockdown conditions (Level 4 – Lockdown), with students and staff unable to physically access University facilities, unless they were involved in essential research related to Covid-19. All field work ceased and data collection with humans was restricted to online methods, if appropriate. The restrictions were partially lifted on April 27, but students and staff were not generally allowed back into University facilities until May 13.

Ongoing disruptions have also been encountered for some students due to uncertainties over the potential for future Covid-19-related restrictions on activities, and a Covid-19 cluster outbreak based in Auckland in New Zealand on 12 August 2020 led to the imposition of rolling Level 2 (Reduce) and Level 3 (Restrict) conditions until 23 September 2020. Auckland campus based students remained on Level 2 until 7 October 2020. This Alert Level system continues to be utilised throughout 2021.

These changing Alert Levels have meant that some research students had experimental, clinical, laboratory, field work, and/or data collection or analysis interrupted, and consequently may have had to adjust their research plans. For some students, the impacts of Covid-19 stretched far beyond the lockdown period in April/May 2020, as they may have had to significantly revise their research plans.

Overseas travel is not permitted by the University and restrictions have been placed on the New Zealand borders which are closed to non-New Zealand citizens and permanent residents. This meant that international students who were based offshore at the time of lockdown, were unable to return to New Zealand. A small number of offshore students were provided permission to return to New Zealand in early 2021. Many students have also suffered from anxiety and stress-related issues, and have had financial impacts, meaning their research progress has been significantly delayed.

This form, as completed by the supervisor and student, outlines the extent that the research has been affected by Covid-19 conditions.

Approved by DRC 10/Feb/2021
DRC 21/02/03

Please consider the factors listed below in your assessment of the work.

This statement has been prepared by the candidate's supervisor in consultation with the student and has been endorsed by the relevant Head of Academic Unit.

Student Name:	Alexis Risson	ID Number:	[redacted]
Supervisor Name:	Dr Anne Midwinter	Date:	15-Mar-23
Thesis title:	Spoilage bacteria in ewe milk and the sheep dairy farm environment		

Considerations to be taken into account. Note: This statement will remain in the final copy of the thesis which will be available from the Massey University Library following the examination process.
[*Enter key considerations here for the examiners. This can include but is not limited to change of scope, scale, topic, focus; limitations in relation to data collection, access to necessary literature or archival materials, laboratories, field sites; disruptions as a result of lockdown and various alert levels, medical or health considerations etc*]

The national lockdown in March/April 2020 prevented access to the laboratory and delayed sample analysis. As I was returning from a trip overseas, I had to self-isolate for 15 days which also delayed my ability to work on my experimental work and on my thesis during this time, due to difficulties of accessing necessary resources. Bacterial isolates that were being studied at the time of the nation-wide lockdown had to be stored and were revived at a later date to resume analysis, which caused delays and resulted in the loss of few isolates. Upon regaining access to the laboratory, there were delays in obtaining reagents, kits, and some basic consumables as a result of high demand and supply-chain disruptions caused by the pandemic. As a consequence of these interruptions to my research, and of personal issues stemming in part from this situation, I successfully applied for a suspension of studies for 6 months.

Approved by DRC 10/Feb/2021
DRC 21/02/03

Confidential for Examiners Only: [*Please enter any other considerations which are confidential for examiners only and should not be placed in the final thesis version submitted to Library following the examination process]*

Signed, confirming this is a fair reflection of the impact of Covid-19 on this research.

Student 

Supervisor Anne Midwinter Digitally signed by Anne Midwinter DN: cn=Anne Midwinter, c=NZ, email=a.c.midwinter@massey.ac.nz Date: 2023.03.15 09:46:42 +13'00'

Head of Academic Unit (or nominee) Naomi Cogger Digitally signed by Naomi Cogger DN: cn=Naomi Cogger, c=NZ, o=Massey, ou=School of Veterinary Science, email=n.cogger@massey.ac.nz Date: 2023.03.21 18:05:32 +13'00'

Approved by DRC 10/Feb/2021
DRC 21/02/03

Abstract

Milk spoilage bacteria are capable of impacting the stability, processability, and overall quality of dairy products. These contaminants are highly diverse and can be found at various stages of the dairy production, but the majority originate from the dairy farm environment where they contaminate raw milk. On bovine dairy farms, research has identified several routes of transmission of spoilage microorganisms into raw milk, as well as some of the factors that modulate this process. This knowledge has aided the design, development and implementation of interventions aiming to limit the on-farm contamination of raw milk and thereby improve the microbiological quality of cow dairy products. In contrast, the microbial communities associated with the ovine dairy production have been largely overlooked. This lack of understanding of the ecology and transmission of spoilage bacteria on sheep dairy farms and in dairy products hinders attempts at reducing the farm-borne contamination of ewe milk by spoilage organisms.

In this thesis, I set out to identify and characterise the microbial communities associated with the ovine dairy production, with a focus on bacteria capable of impacting ewe milk quality.

In the first part, I compiled, developed, and subsequently used an extensive set of culture methods targeting the main types of bacteria implicated in dairy spoilage, namely spore-forming bacteria, and *Pseudomonas* species. These methods were applied to survey the microbial communities present in the farm environment of dairy ewes that were grazed or housed. For the first time, this approach provides a detailed overview of the ecology of spoilage bacteria found on sheep dairy farms and enables the identification of numerous bacterial species with spoilage potential. The diversity and principal reservoirs of spoilage bacteria were found to vary between the two farms, possibly as a result of their farming practices.

In the second part, I performed source-tracking of the contaminants isolated from milking cups using phylogenetic tools such as DNA fingerprinting and whole-genome sequencing. According to this approach, sources of milking cup, and thus possibly raw milk contamination were found to vary between the two farms. In the barn environment, the silage-faeces axis was identified as the main source of spoilage bacteria in the wider farm environment and in milking cups. There was also some contribution, albeit minimal, from bedding materials and animal drinking water as potential intermediaries. In the pasture environment, the transmission of bacteria in the environment was low, but the origin of the contaminants found in milking cups was multiple, dependent on the bacteria considered, and included soil, pasture, animal faeces and drinking water. In addition, the phylogeny

of *Thermoactinomyces* spp., which were found to be particularly abundant in both housing and grazing environments, was studied in more depth using whole-genome sequencing.

Finally, I surveyed the microbiological quality of ewe raw milk samples and sheep dairy products produced in New Zealand using the culture methodology developed for the farm studies. This work provided a glimpse of the diversity and abundance of the spoilage microbiota of sheep milk and dairy products produced in New Zealand. It was found that the microbiological quality of sheep dairy products was high, however, the presence of a highly diverse range of spoilage bacteria highlights their potential to impact the quality of a variety of dairy products.

Acknowledgements

I would like to start by thanking my supervisory panel, Dr Anne Midwinter and Dr Tanu Gupta, for their invaluable assistance and mentorship during my PhD journey. Tanu, thank you for your guidance and for everything you have taught me in terms of theoretical and practical scientific knowledge but also with regards to collaboration with stakeholders. Although it did cause us a few headaches, I have thoroughly enjoyed our discussions about the project. Anne, I am particularly grateful for the help provided in reviewing this thesis and for your support throughout the ripples (and tsunamis) of my PhD journey. I was lucky to have your support, understanding, and kindness at times when I really needed it. My thanks also go to Dr Craig Prichard for his help in bridging our science with the real world during the early steps of the project, and in facilitating contact and collaboration with the sheep dairy industry.

A very special thanks goes to Faith Palevich, research associate and laboratory manager at the Hopkirk Institute, for all your help, support, and kindness. Faith, you have been my unofficial laboratory advisor during this project, and I am extremely thankful for all the discussions we had. Your insights in microbiology and molecular biology have been invaluable for my research and the extensive knowledge you have passed on to me has increased my confidence in the laboratory. I would also like to thank Kwang Subharat for the help provided for sample analysis, and particularly around the disruptions caused by the pandemic. Thank you to Paul Mclean and Ruy Jauregui from the AgResearch Data Science team for help in the processing and assembly of the genome sequences.

I am grateful to the Food Systems Integrity team and AgResearch for providing me with the environment, equipment, and funding for the first three years of my PhD, as part of the Strategic Science Investment Fund (SSIF). I would also like to thank Massey University and the School of Veterinary Sciences for the research funding provided in the form of postgraduate research grants, postgraduate travel grants and equipment grants. I am also grateful to Massey University for providing me with financial assistance as part of the COVID-19 hardship grant. Many thanks for the sheep dairy farmers involved in the project for facilitating access to their farms and for helping with sample collection.

I am grateful for the support of the Lab Services team at the Hopkirk Institute, Tania Buwalda and Arvina Ram. I have thoroughly enjoyed working alongside you and your support, encouragement, kindness, and friendship have been invaluable during the writing of my thesis. I am also very grateful

to Natalie Parlane and the Animal health team at the Hopkirk Institute for welcoming into the team during this time.

Thank you to everyone at the Hopkirk Institute and in the wider Massey University Manawatu campus with whom I have shared good times, lunch breaks, discussions, conferences and drinks. A special thanks to my fellow students at the time and friends: Amila, Rose, Eden, Zack, Sandy, Paul, Reed, Davide, Marie, and many others, for their support and the times we have shared inside and outside the office. I will always treasure these memories.

I am very grateful for the love and support of my family and friends in France. Despite being half a world away, you have kept me going and encouraged me during these challenging times.

My deepest thanks go to my biggest supporter, talented researcher, best friend and partner, Olivia, and to my most vocal supporter, our cat Milo. Olivia, your sharp data visualisation, and statistics skills have been invaluable in making the figures of my thesis and you were always here to help me if I needed. You have always been there for me during the good or the hard times, and I would have not been able to do this without your love, encouragements, and support throughout my PhD. I look forward to sharing more of my life with you, I love you.

I would like to dedicate this thesis to my late grandparents, Mayette and Papy. You have been an incredible support throughout my life and have always supported my early scientific curiosity. I miss you enormously, but I am sure that you would be proud of me and my achievements.

Table of Contents

List of Figures

List of Tables

List of Appendices

Glossary

16S rRNA: Gene coding for the RNA component of the 30S small subunit of the prokaryotic ribosomal RNA

23S rRNA: Gene coding for the RNA component of the 50S large subunit of the prokaryotic ribosomal RNA

AnO2: Anaerobic conditions

APC: Aerobic plate count

APC35: Aerobic plate count at 35°C

APC65: Aerobic plate count at 65°C

ARDRA: Amplified ribosomal DNA restriction analysis

BAB: Butyric acid bacteria

BC: Bray-Curtis dissimilarity metrics

BIGSdb: Bacterial Isolate Genome Sequence Database

BLAST: Basic Local Alignment Search Tool

BO/BN: Bedding “old”, or bedding ‘new”

CC: Colony counts on CFC agar

CFC: Cetrimide Fucidin Cephalosporin agar

cfu: Colony forming units

CMG: Cooked meat glucose broth

dDDH: digital DNA-DNA hybridization

DPA: Dairy product A

DPB: Dairy product B

EC number: Enzyme Commission number

ELISA: Enzyme-linked immunosorbent assay

ERIC-PCR: Enterobacterial repetitive intergenic consensus PCR

ESL: Extended shelf-life

EYA: Egg yolk agar

F1-F5: Farm 1 - Farm 5

FO/FN: Faeces "old"/faeces "new"

GBDP: Genome Blast Distance Phylogeny

GGDC: Genome-To-Genome Distance Calculator

GIT: Gastrointestinal tract

H': Shannon diversity index

HSPs: high-scoring segment pairs

HTST pasteurisation: High temperature short time pasteurisation

INSDC: International Nucleotide Sequence Database Collaboration

IO/IN: Infrastructure "old"/infrastructure "new"

ITS: Internal transcribed spacer

LPSN: List of Prokaryotic names with Standing in Nomenclature

MA: Milk agar

MALDI-TOF: Matrix Assisted Laser Desorption Ionization - Time of Flight

MLST: Multilocus sequence typing

MPI: New Zealand Ministry for Primary Industries

MPN: Most probable number

MPST: Multiprotein sequence typing

MSC: Mesophilic spore count

NCBI: National Center for Biotechnology Information

NGS: Next generation sequencing

NMDS: Nonmetric multidimensional scaling

PD: Phylogenetic distance

PSC: Psychrotrophic spore count

qPCR: quantitative PCR

RAPD-PCR: Random Amplified Polymorphic DNA PCR

RCA: Reinforced clostridial agar

RDP: Ribosomal Database Project

rep-PCR: Repetitive-element PCR

SO/SN: Silage "old"/silage "new"

s.l.*: *sensu lato

s.s.*: *sensu stricto

SBA: Columbia sheep blood agar

SFP: Shahidi-Ferguson perfringens agar

SPR: Superconvergent patch recovery

TSC: Thermophilic spore count

TYGS: Type Strain Genome Server

UHT: Ultrahigh temperature

UPGMA: Unweighted pair group method with arithmetic mean

UV: Ultraviolet

WGS: Whole genome sequencing

WO/WN: Water "old"/water "new"

Chapter 1 - General Introduction

Culturally anchored in the European basin and Middle East where many world-famous cheeses are manufactured, sheep milk production has recently gained interest in other regions including the United States of America (Thomas et al., 2014), Australia (Bencini and Murray, 2012), and New Zealand (Peterson and Prichard, 2015). Most of the sheep milk production originates from the Mediterranean region (Europe and Africa, 27.4 and 23.5%, respectively) or Asia (48.2%) and is primarily used for the manufacturing of specialty cheeses (and and Agriculture Organization of the United Nations, 1997). Ewe milk accounts for a mere 1.3% of the global milk production, far behind the 81% share represented by cow milk, but its superior nutrient profile makes it a premium and highly nutritious product (Gantner et al., 2015, Vyssotski et al., 2017). Not only does sheep milk typically contain higher concentrations of essential nutrients such as protein, fat, and certain minerals than cow milk (Table 1.1), but it also boasts unique bioactive compounds (e.g., conjugated linoleic acid, antioxidants) and a more favourable fatty acid profile. Ewe milk has demonstrated better digestibility and lower allergenicity than cow milk (Vojdani et al., 2018, Roy et al., 2021), indicating it to be a prime choice for the manufacturing of products destined for consumption by higher-risk populations (i.e. elderly, infants) and by some individuals with intolerance to cow milk (Vojdani et al., 2018, Shrestha et al., 2021). Furthermore, the high solids content of sheep milk makes it particularly adapted for use in the manufacturing of hard and semi-hard dairy products (e.g., cheeses, yoghurt but also dairy powders), considering a higher yield of dairy products is achieved per litre of ewe milk, as compared to cow milk (Gantner et al., 2015, Chia et al., 2017). Because of these nutritive advantages, sheep milk is considered a high-quality food that can sell for as high as three times the price of cow milk (Pulina et al., 2018, Beef+Lamb New Zealand, 2019).

Table 1.1 Comparison of the general nutrient profile of sheep and cow milk

Parameter	Sheep milk	Cow milk
Fat content	Higher fat content (typically 6-7%)	Lower fat content (typically 3-4%)
Protein content	Higher protein content (typically 5-6%)	Lower protein content (typically 3-4%)
Lactose content	Similar lactose content (typically 4-5%)	Similar lactose content (typically 4-5%)
Minerals	Higher levels of certain minerals such as calcium and zinc	Lower levels of certain minerals compared to sheep milk
Vitamins	Higher levels of certain vitamins such as A, B12, C, and folic acid	Lower levels of certain vitamins compared to sheep milk
Casein profile	Higher proportion of casein protein, with more diversity in types	Predominantly contains alpha-s1 casein and beta-casein A1 and A2
Fatty acid composition	Higher concentration of short-chain (C_2-C_5) and medium-chain (C_6-C_{12}) fatty acids, higher concentration of conjugated linoleic acid	Higher concentration of long-chain (C_{13}-C_{21}) fatty acids

Data collated from (Bramanti et al., 2003, Balthazar et al., 2017, Chia et al., 2017, Pietrzak-Fiećko and Kamelska-Sadowska, 2020, McCoard et al., 2023)

Upon his second visit to Aotearoa New Zealand in 1773, James Cook introduced a pair of Merino sheep that managed to survive for only a few days after ingesting native toxic plants. Though inauspicious, this event would be the first step towards the development of one of the biggest sheep farming industries in the world. Sheep farming in New Zealand began close to the years 1850 and

focused on the production of wool and meat. At that time and up until the 1980s, the success of the industry explains why sheep became and remain symbolically associated with New Zealand. It was only in 1992 that sheep dairy farming started with the introduction of East Friesian genetics, a versatile breed combining traits for dairy, meat and wool production (Allison, 1995). Although this first start-up phase was unsuccessful for the industry, a few producers remained active and sold their production to local cheesemakers, and to Australia. The second breath of the New Zealand sheep dairy industry came in the years 2010s, with a shift of focus to the manufacturing of dairy powders destined for export (Peterson and Prichard, 2015). In 2014, there were five sheep dairy operations in New Zealand, ranging from a modest flock of 70 ewes to one of the largest sheep dairy operations in the world with 20,000 ewes, operated by Blue River Dairy in the South Island. Locally, the industry struggled to find a durable market space, but several products were found on and off the shelves of retailers (cheeses, yoghurt), though there was strong competition for shelf space with local bovine dairy giant, Fonterra.

Recent successes of the sheep dairy industry exports have prompted the creation of new and dedicated processing power (e.g., government and industry co-funded milk drying plant in the Waikato) as well as the development of collaborations between research entities, government and the industry to improve different facets of the New Zealand sheep dairy production (e.g., "Boosting exports of the emerging New Zealand Dairy Sheep industry" a collaborative research project involving several New Zealand universities and Crown Research Institutes). The goals of these initiatives is to support the growth of the nascent sheep dairy industry in New Zealand and to boost the commercial value of exports, including through research on the nutritive properties of sheep milk (Ahlborn et al., 2020, Burrow et al., 2020) and on the optimisation of farming practices (e.g., farming systems, rearing practices) or sheep genetics (Nieper et al., 2017, Cristobal-Carballo et al., 2019). In New Zealand, sheep dairy farms have largely adopted the pasture-based farming system of their bovine counterparts, but housing is also frequently used, either periodically or continuously, to protect dairy ewes from environmental challenges and sickness. The development of pasture-based sheep dairy farming in New Zealand has also benefitted from environmental regulations restricting farming of dairy cows on specific areas, including close to large bodies of water, to limit contamination of waterways due to high nitrogen leaching (Wilcock et al., 2007, AgResearch Limited, 2021).

The quality of dairy products is dependent on their composition, both nutritional and microbial. Though the former is receiving particular attention in the context of the New Zealand production, there is a clear lack of information on the spoilage microbiota of sheep milk and dairy products. Dairy spoilage bacteria originate from the farm environment and may gain access to the processing

environment and dairy products after contaminating raw milk. The presence of spoilage bacteria in milk and dairy products can impact the quality, processability and stability of dairy products, and may, when left unchecked, cause significant financial and reputational losses. Cow milk, dairy products and the bovine dairy farm environment have been the focus of most studies investigating the prevalence of dairy spoilage bacteria. However, sheep dairy farms and dairy products have been largely overlooked and remain poorly characterised. A better understanding of the identity and dynamics of the microbial communities present in the sheep dairy farm environment and in raw milk may assist the identification of bacterial hotspots and routes of raw milk contamination. This data can be subsequently used to adapt, design, develop and implement interventions or farm practices that can improve the microbiological quality of ewe milk. As the developing New Zealand sheep dairy industry remains particularly experimentative around its farming systems and practices, there is potential to consider the microbiological implications of farm practices in this selection.

1.1 Research questions and objectives

The general objectives of this thesis were to carry out explanatory descriptive research in order to characterise the ecology of the dairy-associated spoilage microbiota of New Zealand sheep dairy farms, and simultaneously highlight potential microbial reservoirs and sources of milk contamination. To this end, the following research questions were formulated and investigated in the research chapters:

What is the prevalence and diversity of dairy spoilage bacteria on sheep dairy farms? (Chapters 4 & 5)

Do farming systems impact the dairy farm microbiota (Chapters 4 & 5) and the transmission of bacteria in the environment? (Chapter 6)

What are the main sources of raw milk contamination on sheep dairy farms? (Chapter 6)

What is the microbiological quality of ewe milk and sheep dairy products in New Zealand? Are these food commodities at risk of microbial spoilage? (Chapter 7)

Specific objectives for each research chapter are as follow:

Chapters 4 & 5

- Compile, adjust, and use an extensive set of culture-based methods to obtain a holistic view of the main types of bacteria involved in dairy spoilage.
- Survey the abundance, prevalence and ecology of the five main types of dairy-associated spoilage bacteria on two sheep dairy farms with different farming systems.
- Perform a first assessment of the potential environmental sources contributing to milk contamination during grazing or housing of the animals.
- Collect farm bacterial isolates for future characterisation.

Chapter 6

- Assess the use of DNA fingerprinting as a phylogenetic tool for environmental source-tracking.
- Identify and confirm sources of raw milk contamination by spoilage bacteria on sheep dairy farms, depending on farming systems.
- Use whole-genome sequencing to improve the phylogenetic resolution of selected bacteria.

Chapter 7

- Perform a first survey of the microbiological quality of sheep milk and dairy products produced in New Zealand
- Characterise and study the prevalence of spoilage bacteria in sheep milk and dairy products.
- Highlight the potential transfer of bacteria from the farm environment to raw milk and dairy products.

1.2 Aim and structure of the study

The general objectives of this thesis were to carry out explanatory descriptive research in order to characterise the ecology of the dairy-associated spoilage microbiota of New Zealand sheep dairy farms, and thereby highlight potential reservoirs and sources of milk contamination. The thesis is divided into four research chapters that each aim to address specific research questions (Figure 1.1). This preliminary study will help address the lack of microbiological data pertaining to sheep dairy

farms and thereby support the design of future interventions aiming to improve the quality of sheep milk and dairy products by limiting the contamination of raw milk at the farm level.

Chapter 2 introduces spoilage bacteria associated with the dairy production and describes their ecology throughout the dairy production, and particularly on dairy farms, according to available literature.

Chapter 3 compiles the methodology used over the course of this work.

Chapters 4 and 5 contain in-depth descriptive studies of the microbial communities present on two sheep dairy farms operating under different farming systems (housed vs grazed animals), with a focus on dairy-associated spoilage bacteria. The goals of these studies were to use culture methods and a polyphasic identification approach to isolate, identify and assess the spoilage potential of the farm microbiota and highlight reservoirs of spoilage bacteria in sheep dairy farm environments. In addition, the isolation and characterisation of milking cups contaminants enabled a preliminary investigation of the possible environmental sources of milk contamination according to different farming systems.

In **Chapter 6**, DNA fingerprinting and whole-genome sequencing were used for subtyping and source tracking of selected spoilage bacteria isolated from the milking cups of the two sheep dairy farms. The aim of this study was to identify environmental sources of the bacteria isolated from milking cups and highlight their possible transfer across the environment, in order to identify farm-level routes of milk contamination.

Chapter 7 describes the microbiological content and spoilage microbiota of a set of ewe milk samples and sheep dairy products produced in New Zealand. The goal of this investigation was to provide a first-hand assessment of the spoilage microbiota of sheep milk and dairy products to identify potential quality threats present in raw materials and end-products. This work also enables a preliminary comparison of the microbiological content of raw milk produced on several different farm with varying farming systems and practices.

Chapter 8 provides a general discussion of the findings and conclusions of the thesis. Research perspectives uncovered by my work with potential application for the New Zealand sheep dairy industry are also discussed.

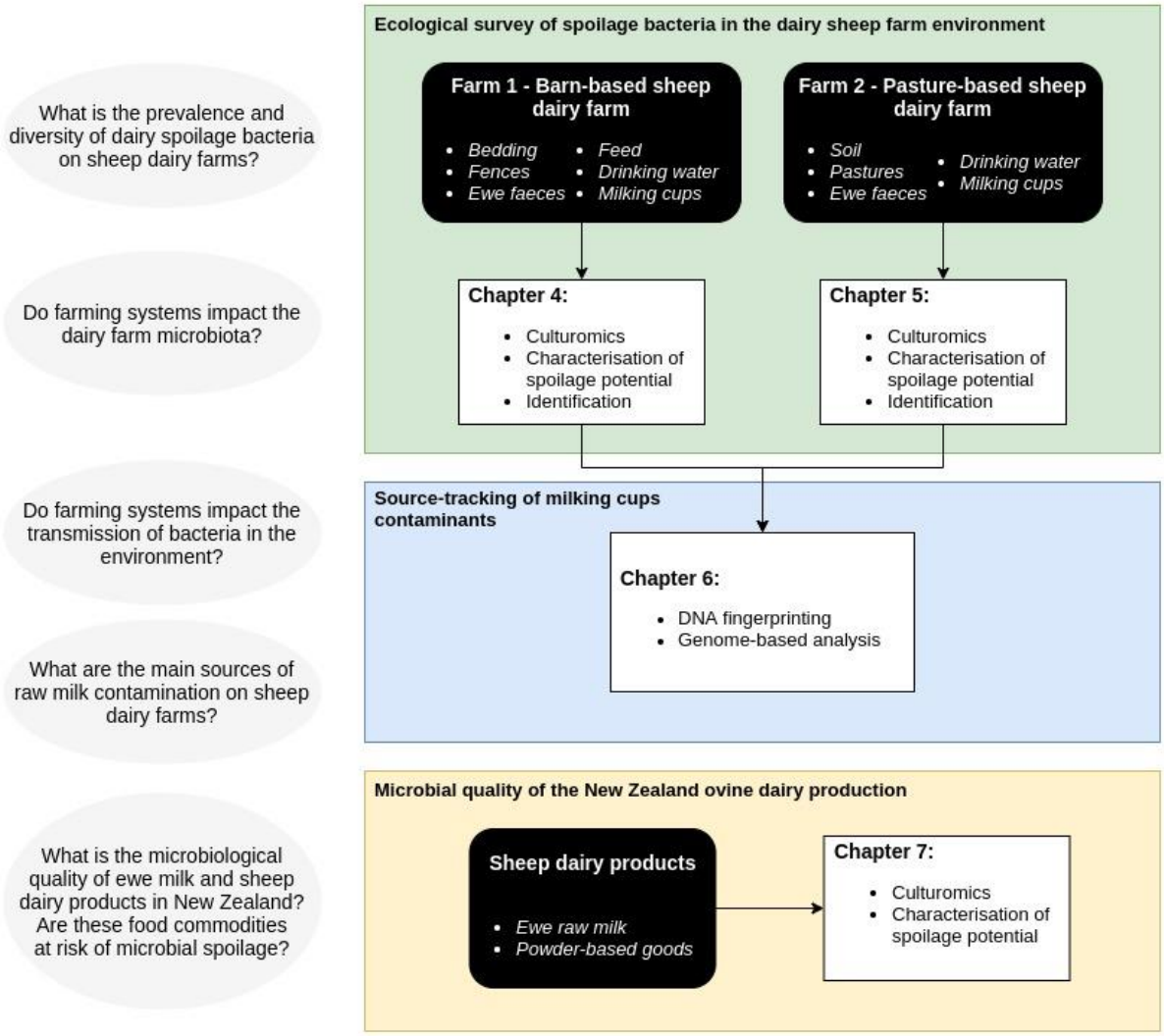


Figure 1.1. Structure of the thesis and research questions

Chapter 2 - Literature Review: The dairy farm microbiology and sources of raw milk contamination by spoilage bacteria

2.1 Introduction

Dairy products represent highly nutritious and easily accessible food commodities that are consumed worldwide in a variety of different forms. Due to its high nutrient and water contents, milk can support the growth of a wide range of microorganisms, including pathogenic or spoilage bacteria that can subsequently impact the safety and quality of manufactured dairy products. Dairy spoilage bacteria encompass those species of bacteria capable of impacting milk composition after evading the preventive actions currently in place to shield dairy quality from microbial spoilage (e.g., raw milk refrigeration, dairy processing). After proliferating at low temperatures during the storage of unprocessed raw milk, vegetative cells of psychrotrophic bacteria, mainly *Pseudomonas* spp., may reach remarkably high concentrations in raw milk and produce compounds associated with dairy spoilage. Tremonte et al. (2014) reported an increase from 8 to 11 $\log_{10}$ cfu/L of *Pseudomonas* cells in bovine raw milk after 72 h of refrigerated (4°C) storage. Conversely, the ecological niches occupied by spore-forming bacteria in dairy production consist of post-processing dairy products in which the local microbiota, apart from thermoduric microorganisms, has been largely removed from raw milk by processing, leaving spore-formers to grow in a nutrient-rich environment with little to no competition (Ivy et al., 2012, Martinez et al., 2017). As these two types of spoilage bacteria can impact the quality of dairy in spite of processing, the microbiological quality of raw milk is therefore a key determinant in the quality of processed dairy products such as pasteurised milk, cheese, dairy beverages, or milk powders.

The contamination of raw milk by spoilage bacteria is a complex process that is multifactorial, and which spans from the dairy farm all the way to the processing facility (McHugh et al., 2020). As a result, a wide array of sources may be implicated in the contamination of raw milk, including the farm and milking environments (Vissers et al., 2007c, Martin et al., 2019), farm management practices (Miller et al., 2015a, Murphy et al., 2019), as well as raw milk storage and transport conditions (Zweifel et al., 2005). Although poor conditions of raw milk storage and transport can lead to significant bacterial growth (De Jonghe et al., 2011, Tribst et al., 2019), it has been demonstrated by studies in bovine dairy farm environments that milking represents the main point of entry of environmental bacteria into raw milk (Vissers et al., 2007c, Verdier-Metz et al., 2009). Current knowledge available on the ecology and population dynamics of bacteria on dairy farms has been mainly procured through studies conducted in bovine dairy farm environments, while ovine dairy farms have been largely understudied. However, similarities between the general functioning and structure of dairy operations farming different species of livestock suggest that some of the findings obtained on bovine dairy farms may be translatable to ovine dairy farms. For example, feeding of silage has been associated with high spore counts in raw milk from cows (Vissers et al., 2007b), sheep (Burtscher et al., 2020), and goats (Reindl et al., 2014). Yet, differences pertaining to the structure and management of dairy farms (e.g., farming system, feed regime, milking regime) resulting from physiological and morphological differences between ruminants, and particularly between large and small ruminants, may lead to variations in the diversity and transmission of bacteria present on dairy farms.

The recent development of the sheep dairy industry, both worldwide and in New Zealand, calls for a better understanding of the microbiota of sheep dairy products, milk, and dairy farms. This data may assist the design and development of strategies aiming to safeguard the quality of the sheep dairy production from microbial alterations. In this literature review, I begin by defining the main groups of bacteria capable of impacting the quality of dairy products, as well as the mechanisms through which this process takes place, and the consequences it may have on the contents and attributes of dairy products. I then describe some of the methods currently available to assess the microbiological quality of milk and dairy products, with particular emphasis on the detection of spore-forming and psychrotrophic bacteria. Subsequently, the interplays of farming systems and farming practices on the quality of raw milk are described, primarily using available research pertaining to bovine dairy farms, but also to ovine and caprine dairy farms when available. Literature findings obtained from bovine dairy farms are also simultaneously discussed for their applicability on sheep dairy farms.

2.2 The microbiota of raw milk and dairy products

Raw milk provides a highly nutritious growth medium that can support the survival and growth of numerous species of bacteria. The microbiota of raw milk is known to be highly diverse, with studies reporting up to 2,000 different genera being identified in bovine raw milk using next generation sequencing [NGS, (Parente et al., 2020)]. Several works have highlighted the presence of a core microbiota in milks from different animal species, including cows (Parente et al., 2020), goats (McInnis et al., 2015), and sheep (Biçer et al., 2021), comprising members of the phyla *Bacillota* [formerly *Firmicutes*, (Oren and Garrity, 2021)], *Actinomycetota* (formerly *Actinobacteria*), *Pseudomonadota* (formerly *Proteobacteria*) and *Bacteroidota* (formerly *Bacteroidetes*). According to NGS approaches, the core microbiota of sheep raw milk includes the taxa *Lactobacillus*, *Corynebacterium* and *Staphylococcus*, with many other genera being also frequently detected, such as *Bacillus*, *Clostridium*, *Enterococcus*, *Jeotgalicoccus*, *Pseudomonas*, *Psychrobacter*, *Stenotrophomonas* and *Streptococcus*, to cite a few (Quigley et al., 2013, Esteban-Blanco et al., 2020, Biçer et al., 2021). Similar microbial populations structure have been reported in milk from other species of dairy livestock, but higher abundance of some bacterial taxa were found to be associated with the dairy production from specific animals species, including *Bacteroides*, *Lachnospiraceae* and *Ruminococcaceae* in cow milk, or *Lactobacillus*, *Bacillus* and *Escherichia* in ewe milk (Quigley et al., 2013, Oikonomou et al., 2020). The bacteria present in raw milk originate in the majority from the farm environment, and they may contaminate raw milk during milking after attaching on the mammary surface or entering the milk ducts of dairy livestock (Vissers et al., 2007c). From the extensive literature available on bovine dairy production, it is clear that the diversity of bacteria found in raw milk is also highly variable across dairy farms as a result of varying farm features [e.g., farming system, farm management practices, season, location and local microbiota (Miller et al., 2015a, Skeie et al., 2019)]. Due to a lack of global research focus on sheep dairy production, the interplay between features of sheep dairy farms and the microbial composition of raw milk produced on these farms remains particularly unclear. The paucity of studies focusing on the characterisation of the milk microbiota from sheep and other small ruminants has only recently begun to be addressed (Esteban-Blanco et al., 2020, Biçer et al., 2021) and additional data, including that obtained across different farming systems and geographical locations, will be required to match the breadth of data available on the microbiota of bovine raw milk. Similar to what could be achieved in the bovine dairy production (Derakhshani et al., 2018), this data may enable the testing of hypotheses on the interplay of the milk microbiota and milk quality.

2.2.1 Psychrotrophic bacteria

The microbiota of raw milk is highly diverse, but only a fraction of these bacteria are capable of proliferating during its refrigerated storage and transport, at temperatures below 6°C. The growth of psychrotrophic bacteria during raw milk storage can irreversibly impact the contents and physicochemical properties of raw milk, and thus that of any subsequently manufactured dairy products, even after the producing organisms have been removed by processing (Matéos et al., 2015). Psychrotrophic bacteria, which are cold-tolerant and differ from cold-requiring (< 15°C) psychrophilic bacteria, include species able to proliferate at temperatures close to 0°C but with optimal growth taking place between 15 to 30°C (Gounot, 1986). During storage and transport, the microbial diversity of raw milk shifts as a result of refrigeration, and psychrotrophic bacteria including *Pseudomonas*, *Acinetobacter*, *Arthrobacter* and *Lactococcus* rapidly become predominant at the expense of mesophilic populations from the taxa *Streptococcus*, *Enterococcus*, *Staphylococcus* and *Enterobacteriaceae* (Nunez et al., 1984, von Neubeck et al., 2015, Ribeiro Junior et al., 2018). Specifically, *Pseudomonas* spp. are frequently identified as the most abundant bacteria in raw milk that has been refrigerated, even to temperatures < 4°C (Hantsis-Zacharov and Halpern, 2007, Bruzaroski et al., 2020), with concentrations reaching up to 11 $\log_{10}$ cfu/L in some instances (Tremonte et al., 2014). Members of the *P. fluorescens* lineage demonstrate high fitness at low temperatures, allowing them to outcompete other psychrotrophic bacteria such as *Bacillus s.l.* and *Paenibacillus* in milk (Gram et al., 2002, Simões et al., 2008). The *Pseudomonas* genus represents one of the most versatile and diverse bacterial taxa (Silby et al., 2011), and members of this genus are known to produce a wide range of enzymes and metabolites, including several compounds involved in dairy spoilage, such as spoilage enzymes and pigments. Blue and grey discoloration of dairy products such as fluid milk and cheese has been attributed to bacteria members of the *Pseudomonas fluorescens* group, including *Ps. azotoformans*, *Ps. putida* and *Ps. koreensis* (Evanowski et al., 2017, Carrascosa et al., 2021). Yuan et al. (2018b) identified *Pseudomonas* spp. such as *Ps. fluorescens*, *Ps. putida*, *Ps. lurida* and *Ps. azotoformans* as some of the most potent producers of spoilage enzymes (i.e., proteases and lipases) across psychotrophic isolates obtained from bovine raw milk, alongside genera such as *Acinetobacter*, *Chryseobacterium*, *Flavobacterium* and *Yersinia* (Yuan et al., 2018b). Several of these bacteria were also found to produce cold-active or thermoresistant spoilage enzymes (Alquati et al., 2002, Yuan et al., 2018b) that may affect the nutritional quality and the processability of raw milk and dairy products that are subject to pasteurisation or sterilisation-grade dairy processing, such as ultra-high temperature (UHT) milk

processing (Marchand et al., 2017). Conventional heat treatments used for dairy processing such as pasteurisation are very effective at reducing viable populations of vegetative cells to well below detectable limits (Juffs and Deeth, 2007). However, the presence of thermotolerant enzymes in milk, or post-pasteurisation contamination by Gram-negative or Gram-positive psychrotrophic bacteria [which may result from inadequate hygiene or clean-in-place procedures (Martin et al., 2018)], can cause monetary losses stemming from reduced quality and shelf-life of milk and dairy products (Marchand et al., 2017, Reichler et al., 2018). Members of the *Pseudomonas* genus are known to produce a variety of thermostable enzymes, including the AprX protease (Matéos et al., 2015, Marchand et al., 2017).

2.2.2 Thermoduric spore-forming bacteria

Heat processing of food products has enabled significant improvements of their safety and shelf-life due to the removal of pathogenic and spoilage organisms (Pasteur, 1865). Because these processes remove most, but not all of the bacteria present in food, they have also simultaneously led to the creation of new post-processing ecological niches that have become colonised by thermoduric bacteria which survive pasteurisation and other processing treatments. Thermoduric bacteria capable of surviving heat processing include Gram-negative and Gram-positive non-spore-forming bacteria such as *Kocuria*, *Microbacterium*, *Micrococcus*, *Enterococcus*, *Streptococcus* and *Arthrobacter* (Robertson, 1927, Chauhan et al., 2013). In addition, the endospores of Gram-positive spore-forming bacteria, such as those from the taxa *Bacillus*, *Clostridium*, *Geobacillus* and *Paenibacillus* are highly thermoduric (Mah et al., 2009, Berendsen et al., 2016). Taxonomically, spore-formers are a polyphyletic and highly eclectic group of bacteria that includes members of several families part of the phylum *Bacillota*, and more precisely of the orders *Bacilli* (spore-forming aerobes or facultative anaerobes) and *Clostridia* (spore-forming obligate anaerobes). Spore-forming bacteria can produce endospores (also called spores) that are resistant to a wide variety of stresses, such as antimicrobials, extremes of temperature, pH, water activity, radiation and nutrient unavailability, ultimately reverting to a vegetative state when conditions become favourable (De Vos et al., 2009). Because of their unique resistance to stresses, spore-forming bacteria have been found in highly inhospitable environments, including thousand-year-old sediments (Nilsson and Renberg, 1990), clean rooms used for spacecraft assembly (Venkateswaran et al., 2003, Vaishampayan et al., 2010), the Earth stratosphere (Shivaji et al., 2006), Arctic terrains (Yukimura et al., 2009) and highly acidic or alkaline geothermal pools (Cavello et al., 2020). The unique endurance of the spores produced by these

bacteria allows them to transit to and through, and be present in, a large variety of environments, either actively growing as vegetative cells and interacting with their environment, or simply persisting as endospores.

In food production, the phenotypic cell features of endospores and vegetative cells of spore-forming bacteria are key determinants of their ecological niches of predilection. Acid-tolerant thermophilic spore-formers such as *Alicyclobacillus* spp. are involved in the spoilage of high-acidity canned goods, including tomato products and fruit juices (Groenewald et al., 2009). The growth of psychrophilic anaerobic spore-formers of the *Clostridium* genus on refrigerated meat has been associated with reduced quality and even spoilage of vacuum-packed products (Broda et al., 2000). The breadth of species of spore-forming bacteria that may be involved in the spoilage of dairy products is only a match to the extensive range of dairy products that are produced, and to the diversity of processing treatments used for their manufacturing. After entering raw milk during milking from various farm sources including soil, feed, bedding materials and the animals, spore-forming bacteria can persist in milk and dairy products as spores. The stringency of processing, as well as the environmental temperatures encountered throughout processing and storage, are determining factors for the diversity of spore-forming bacteria that may remain in finished dairy products.

2.2.2.1 Anaerobic spore-forming bacteria

In the dairy production, anaerobic environmental conditions enabling the growth of obligate anaerobic spore-formers such as *Clostridium* spp. are found in hard and semi-hard cheeses undergoing maturation. The survival of mesophilic *Clostridium s.l.* spores throughout the cheesemaking process, including after milk pasteurisation and/or bactofugation (short-time centrifugation of milk destined to remove bacterial spores before cheese manufacturing), is a quality concern. Spore germination and subsequent growth in cheese may lead to the development of organoleptic defects, notably through gas production which can result in late blowing (Klijn et al., 1995). *Clostridium* species implicated in the development of late blowing in cheese manufactured with either ovine or bovine, raw or pasteurised milk comprise lactate-fermenting butyric acid bacteria (BAB), such as *Clostridium tyrobutyricum*, *C. butyricum*, *C. beijerinckii* (Goudkov and Sharpe, 1965, Le Bourhis et al., 2007). Proteolytic or saccharolytic bacteria such as *C. perfringens* and *C. sporogenes* may also grow during cheese maturation and simultaneously help facilitate the development of organoleptic defects and late blowing by increasing nutrient availability for use by BAB (Pahlow et al., 2003, Zheng et al., 2020). Amongst 223 *Clostridium* isolates recovered from

ewe milk cheeses with late-blowing defects, Garde et al. (2011) identified *C. sporogenes* (78.9% of the isolates), *C. beijerinckii* (10.3%), *C. tyrobutyricum* (9.0%) and *C. butyricum* (1.8%) as the main contaminants. Several clostridia such as *C. botulinum*, *C. perfringens* and *Paeniclostridium sordellii* are also known for their ability to produce toxins that are involved in the onset of bacteraemia, enteric disorders and other diseases in both animals and humans (Songer, 1996, Uzal and Songer, 2008, Muylaert et al., 2010). The presence of *Clostridium s.l.* is thus undesirable in milk, and particularly in dairy products intended for consumption by higher-risk populations [i.e., the elderly, immunocompromised, infants (Barash et al., 2010)]. However, unlike in meat- and vegetable-based, temperature-abused cooked meals that may become anaerobic and permit germination and growth of *Clostridium* spp. spores (Grass et al., 2013), dairy products have not been identified as risk factors or vectors for the transmission of clostridial diseases to the general population (McHugh et al., 2017, Ministry for Primary Industry Regulation and Assurance Branch, 2019).

2.2.2.2 Aerobic spore-forming bacteria

Because the manufacturing process of dairy products is typically undertaken under high aeration, aerobic spore-forming bacteria such as those of the genera *Bacillus s.l.*, *Paenibacillus* and *Geobacillus* represent the most common contaminants of dairy products. Spore-forming bacteria present in raw milk and dairy products may originate from the farm environment, but there is evidence that some aerobic spore-forming bacteria have evolved to adapt to the conditions of dairy processing and gained genotypic or phenotypic growth advantages tailored to processing environments (Burgess, 2016, Ostrov et al., 2019). The unique phenotypic diversity found across aerobic spore-forming bacteria contributes to their ubiquity throughout dairy production and manufacturing and also determines which species of bacteria may be found in specific dairy products. Spore-forming bacteria implicated in the quality of dairy products can be broadly assigned to three groups, psychrotrophic, mesophilic, and thermophilic spore-formers, based on their ability to grow at low, moderate, or high temperatures, respectively.

Psychrotrophic spore-forming bacteria have been implicated in the spoilage of refrigerated dairy products such as milk processed with high temperature short time (HTST) pasteurisation (Ranieri and Boor, 2009), or extended shelf life (ESL) milk (Doll et al., 2017). In the absence of significant post-processing contamination by Gram-negative bacteria [e.g., *Pseudomonas* spp. (Reichler et al., 2018)], psychrotrophic spore-formers represent the main limiting factor for the shelf-life of pasteurised fluid milk (Ranieri and Boor, 2009). After surviving pasteurisation or other heat-treatments as endospores,

psychrotrophic spore-formers may subsequently germinate and grow during refrigerated storage (Huck et al., 2008). The microbial threshold below which pasteurised milk remains acceptable for consumption, 7 $\log_{10}$ cfu/L, is generally reached after 12 to 30 days in dairy products contaminated by psychrotrophic spores for HTST pasteurised milk, depending on the temperature of storage (Ranieri and Boor, 2009, Ziyaina et al., 2018), and at least 30 days for ESL milk (Doll et al., 2017). In the USA, it is estimated that 7 billion tons (about 30%) of fluid cow milk are discarded each year by retailers and consumers due to spoilage or perceived spoilage by psychrotrophic bacteria (Buzby et al., 2014), with about half these losses attributed to psychrotrophic spore-forming bacteria (Martin et al., 2021). The main psychrotrophic spores in raw milk are those of *Bacillus* and *Paenibacillus*. *Bacillus* spp. account for most of the contaminants present in freshly pasteurised milk but *Paenibacillus* spp. become predominant throughout refrigerated transport and storage, owing to higher fitness (e.g., shorter generation times) at low temperatures (Huck et al., 2008). Psychrotrophic bacteria such as *B. licheniformis*, *B. pumilus s.l.* (which includes *B. safensis*, *B. pumilus*, *B. aerophilus*, *B. stratosphericus*, *B. zhangzhouensis*, *B. australimaris* and *B. altitudinis*) and psychrotrophic members of *B. cereus s.l.* (such as the spoilage bacteria *B. mycoides* or pathogenic bacteria *B. cereus s.s.*) are the principal *Bacillus* contaminants of pasteurised milk (Ranieri and Boor, 2009). Surveys on pasteurised milk in the USA have identified *Paenibacillus odorifer* as the predominant *Paenibacillus* spp. in pasteurised milk, with other such as *Paenibacillus graminis* and *Paenibacillus amylolyticus* being also frequently isolated (Ivy et al., 2012, Beno et al., 2020). *Bacillus* spp. and *Paenibacillus* spp. can secrete several types of potent dairy spoilage enzymes including lipases, protease, β-galactosidases and lecithinases (De Jonghe et al., 2010, Ivy et al., 2012, Ziyaina et al., 2018).

During the manufacturing of dairy powders, high processing temperatures select for the growth of (obligate or facultative) thermophilic spore-formers members of *Geobacillus*, *Anoxybacillus* and thermotolerant *Bacillus* species. These bacteria are not pathogenic but high concentrations of thermophiles in dairy powders (generally > 4 $\log_{10}$ cfu/g) are an indicator of poor hygiene during manufacturing and can also have repercussions on product quality. This is because thermophilic spore-formers can proliferate in the high heat sections of dairy powders manufacturing chains when temperatures exceed 50°C and after their competition has been removed by heat processing (Murphy et al., 1999). Several thermophilic spore-formers are known to produce compounds associated with reduced food quality and spoilage during high-temperature manufacturing, including hydrolytic enzymes and acids (Kalogridou-Vassiliadou, 1992, Sadiq et al., 2018). But the potential for dairy-associated thermophilic spore-formers to cause actual spoilage is low due to the low water activity of

dairy powders and the fact that they are stored at temperatures that are too low to support the growth of obligate thermophiles (< 37°C). Still, extensive growth of thermophilic bacteria during powder manufacturing, and particularly over long processing runs (Murphy et al., 1999), can lead to increased biofouling and the production of powders that fail to achieve the increasingly stringent microbial requirements set by regulatory entities. Biofilm formation by thermophilic spore-formers on the surface of dairy processing equipment, also called biofouling, occurs at temperatures between 40–65 °C (Flint et al., 1997, Murphy et al., 1999, Burgess et al., 2010). Attachment of both spores and vegetative cells on stainless steel surfaces of the processing pipeline can initiate the development of biofilm, which can form at several locations along the dairy powder processing pipeline (e.g., during evaporation, on plate heat exchangers, in warm centrifugal separators). The establishment of biofilms on the surface of processing equipment can, when coupled with inadequate cleaning-in-place procedures, lead to ongoing contamination of a dairy manufacturing plant and its production, including across batches of products (Bremer et al., 2006). The source of thermophilic spores in dairy powders mainly consists of the processing environment, while raw milk contributes levels of thermophilic spores often less than 100 cfu/ml (McGuiggan et al., 2002). The diversity of mesophilic and thermophilic spore-forming bacteria in raw milk and dairy powders also differs (Miller et al., 2015b), and the species that are most abundant in dairy powders, *Anoxybacillus flavithermus* and *Geobacillus stearothermophilus*, are rarely isolated from raw milk. Low amounts of these thermophilic bacteria can enter the processing environment from external sources, including through raw milk, and may subsequently colonise the environment and become enriched during processing where environmental temperatures enable their growth. Strains of *Geobacillus* and *Anoxybacillus* associated with dairy processing have demonstrated signs of adaptation to the dairy processing environment, which provides further support to this hypothesis (Burgess, 2016).

Mesophilic spore-formers are a highly diverse group of bacteria that have been isolated from a wide array of dairy products, including fluid milk (Ivy et al., 2012), yoghurt and sour cream (Mehta, 2018), condensed milk (Martinez et al., 2017) and dairy powders (McHugh et al., 2018). This is because mesophilic spore-formers bacteria, which grow at moderate temperatures, are also often simultaneously psychrotolerant (i.e., psychrotrophic, such as *B. pumilus s.l.* and *B. cereus s.l.*) or thermotolerant (i.e., facultative thermophiles, such as *B. licheniformis* and *B. subtilis*). Due to their unique versatility, the spores of mesophilic spore-forming bacteria are often the most abundant spores in the farm environment and in raw milk (Martin et al., 2019). Several species of mesophilic and thermophilic spore-formers are also capable of forming highly heat resistant spores that may survive UHT processing or commercial sterilisation (Scheldeman et al., 2005, Scheldeman et al., 2006).

2.3 Dairy quality and spoilage bacteria

Sensory quality is one of the most important aspects of a food product, and it is determined by a combination of attributes pertaining to its appearance, texture and flavour. The microbiological and physicochemical composition of raw milk, the processing treatments used for the manufacturing of dairy products, as well as interplays between these aspects, can affect the different phases of production and hence the final quality of the product. In particular, unsuitable initial microbial composition, or inadequate processing may result in microbial alteration of milk, and in the development of undesirable attributes, or in the absence of desirable physicochemical and organoleptic characteristics (Pirisi et al., 2007). Dairy products that are manufactured using milk from small ruminants such as goat and sheep have a unique nutrient composition that significantly differs from that of cow milk, and which is associated with aromas that are specifically sought after, including in cheeses and dairy powders (Zabaleta et al., 2017, Teng et al., 2018). Both the protein and lipid content of sheep milk are about twice as much that of cow milk (Roy et al., 2020), and though this points to the nutritive superiority of the former, it also underline a possible greater susceptibility to alterations by contaminating bacteria. The degradation of the main components of milk, proteins and lipids, is associated with the development of unpleasant aromas that are mainly due to the release of small peptides and free fatty acids, respectively (Shipe et al., 1978, Zabaleta et al., 2017). In addition, there is a range of microbial metabolic pathways, or secreted compounds that may affect the appearance, texture and flavour of dairy products. The presence of defects in the final product may cause financial or reputational losses and hence have economic impacts on the dairy industry.

2.3.1 Spoilage enzymes

Apart from water, the most abundant components of milk are proteins and lipids, therefore it is not surprising that the main enzymes associated with dairy spoilage are proteases and lipases. There are several types of native (animal) proteases and lipases present in raw milk (Albenzio et al., 2009), but extracellular microbial enzymes (also called exoenzymes) are also produced and secreted in milk by the contaminating microbiota, and contribute significantly to the development of organoleptic alterations. Adequate dairy handling (e.g., refrigeration) and processing (e.g., pasteurisation or other heat-treatments), contribute to either limit microbial and enzymatic activity, or destroy the bacteria and enzymes present in milk, respectively. However, cold-tolerant microbial enzymes may still be

active at low temperatures (Hantsis-Zacharov and Halpern, 2007), while thermotolerant enzymes have been shown to retain activity following heat treatments, and to remain in dairy products where they may cause spoilage, even in the absence of their producing organism (Datta and Deeth, 2001). Due to human error or processing failures, post-processing contamination, and sometimes to the lack of a well-defined handling quality structure in the dairy supply chain, unsuitable temperatures during storage and manufacturing may also lead to increased microbial and enzymatic activity. When left unchecked, this can result in loss of product quality, reduced shelf-life and premature spoilage (Vilar et al., 2012, Doll et al., 2017, Martin et al., 2021).

2.3.1.1 Proteolytic enzymes

Peptidases (EC 3.4), also called proteases, are a broad family of proteins capable of hydrolysing peptidic bonds in proteins. In bovine dairy production, serine proteases (EC 3.4.21), both native (e.g., plasmin protease) and of microbial origin (e.g., subtilisin-like enzymes), are the predominant extracellular proteases implicated in milk quality (Ma et al., 2003, Matéos et al., 2015). Unpleasant aromas that may arise as a result of the proteolysis of milk and dairy range from bitter, rancid or even putrid, and are caused by the release of small peptides after hydrolysis of caseins and whey proteins (Shipe et al., 1978). The concentration of tyrosine in milk has been used as an organoleptic indicator because this amino acid is released upon the hydrolysis of caseins and hence it can be used to assess overall casein integrity in milk and the extent of proteolysis (Juffs, 1975). Sheep milk carries more caseins and whey proteins than cow milk, and particularly higher levels of β- and κ-caseins (Roy et al., 2020), thus suggesting that it may more susceptible to microbial proteolytic activity. Extensive degradation of the caseins, and particularly of κ-caseins which are key for maintaining stability of the milk emulsion (Courthaudon et al., 1999), can also affect the physicochemical properties of milk. This can result in spontaneous curdling or destabilisation of the milk matrix and gelation, as well as prevent adequate coagulation during cheese manufacturing or cause viscosity defects in fluid milk (D'Incecco et al., 2022). The activity of microbial proteases can be evaluated qualitatively using plate-based assays (i.e., hydrolysis of milk agar), or quantitatively using biochemical assays based on spectrophotometry [hydrolysis of azocasein or reaction between free α-amino acids and trinitrobenzene sulfonic acid (Santos et al., 1996, De Jonghe et al., 2010)].

2.3.1.2 Lipolytic enzymes

Lipolytic enzymes (EC 3.1) are a class of enzymes capable of degrading different types of lipids such as glycerides and phospholipids. Extracellular microbial lipolytic enzymes that are relevant for the quality of milk and dairy products include predominantly lipases (e.g., triacylglycerol lipases), which hydrolyse medium- and long-chain fatty acids in emulsion, and esterases (e.g., tributyrin esterase) which hydrolyse short-chain fatty acids in solution (Brand et al., 2000, Ali et al., 2012). Phospholipases such as lecithinase, can also degrade phospholipids in milk that are involved in stabilisation and homogeneity of the milk fat globule membrane (Gallier et al., 2010). The by-products of lipolytic activity are free fatty acids, many of which carry strong aromas with low detection thresholds. these compounds may be desirable in small amounts, and characteristic of products such as cheeses, but become undesirable in other dairy products such as fluid milk, or at higher concentrations (Santos et al., 2003b). Long-chain fatty acids (C14 to C20, i.e., myristic, palmitic, oleic, and stearic acids) are the main lipids found in ewe milk, accounting for up to 80% of the total lipid content (Shipe et al., 1978, Park et al., 2007). The hydrolysis of long-chain fatty acids generates compounds that can generate soapy off-flavours, although with high sensory detection thresholds (Mattes, 2009). Medium-chain fatty acids (C6 to C12) are also more abundant in ewe milk as compared to cow milk, particularly caproic, caprylic, capric, and lauric acids (Mollica et al., 2021). Branched-chain fatty acids such as 4-ethyloctanoic acid (C10), and to some extent 4-methyloctanoic acid (C8) and 4-methylnonanoic acid (C9), are the main contributors to some of the typical odours and flavours associated with sheep products; the ‘muttony’ or ‘sheepy’ aromas (Teng et al., 2018). Microbial lipolysis of butanoic, caproic, caprylic and capric acid from triglycerides leads to the release of the corresponding free fatty acids in milk, which carry strong and unpleasant aromas with low detection thresholds and that have been characterised as acidic, sweaty, soapy or rancid (Park et al., 2007, Su et al., 2020). The activity of microbial lipolytic enzymes can be evaluated qualitatively using plate-based assays [i.e., hydrolysis of tributyrin for esterases or olive oil for true lipases (Kouker and Jaeger, 1987)]), and quantitatively using biochemical assays based on titration of free fatty acids (De Jonghe et al., 2010) or via spectrophotometry [hydrolysis of p-nitrophenyl palmitate (Teh et al., 2013)].

2.3.1.3 Production of spoilage enzymes

The production of extracellular enzymes by bacteria is a key ecological feature that enables them to process and/or consume complex molecules for survival, pathogenesis, symbiosis, or bioremediation

(Hentschel et al., 2000, Bhandari et al., 2021, Da Silva et al., 2021). Because of the variety of ecological niches that may be inhabited by different bacteria, the frequency and range of production of extracellular enzymes, and thus by extension the spoilage potential of bacteria, is highly diverse. *Pseudomonas* species produce a variety of extracellular hydrolases, including many lipases and proteases (Sorhaug and Stepaniak, 1997). Specifically, members of the *Ps. fluorescens* lineage such as *Ps. fluorescens* s.s, *Ps. fragi*, *Ps. jessenii*, *Ps. lundensis*, and *Ps. gessardii* produce potent and cold-active lipases and proteases that can impact raw milk and dairy quality (Rajmohan et al., 2002, Yuan et al., 2018b). Although *Pseudomonas* are sensitive to pasteurisation and other heat treatments, they can also produce thermostable enzymes that retain activity following heat processing, such as the AprX serine protease which has been extensively studied for its impact on the quality and integrity of UHT milk (Zhang et al., 2019). Spore-forming bacteria such as *Bacillus s.l.*, *Paenibacillus* and *Geobacillus* also produce a panoply of extracellular proteases and lipases that are highly diverse (Lucking et al., 2013, Sadiq et al., 2016b), many of which are used industrially for their potency, for example in the formulation of cleaning products (Sellami-Kamoun et al., 2008), to process by-products (Li et al., 2019), or in the leather and baking industries (Chandra et al., 2020).

The production and secretion of microbial proteases and lipases is an intricate and highly regulated process that can take place during the exponential or the stationary phase of growth depending on the species of bacteria. (Priest, 1977, Makhzoum et al., 1995). Enzyme production is stimulated by a variety of compounds, including proteins, lipids, amino acids, and polysaccharides (Makhzoum et al., 1995, Chen et al., 2003), many of which are found at high levels in milk. The presence of these activator compounds lead to the expression of genes coding for extracellular proteases or lipases, which may be located on the same polycistronic operon in some bacteria (e.g., *aprX* and *lipA* genes in *Ps. fluorescens*) leading to their joint transcription (Woods et al., 2001). The secretion of enzymes is also a temperature-dependent process that is often optimal at mesophilic temperatures around 20°C (Merieau et al., 1993), although it can also take place in psychrophilic (Yadav et al., 2016) or thermophilic conditions depending on the species of bacteria (Yunitsyna et al., 2019). In dairy production, some bacteria are capable of producing multiple types of enzymes that are active at different temperatures (He et al., 1991, Sadiq et al., 2016b, Yuan et al., 2018b). Across a set of psychrotrophic bacteria isolated from raw milk, multiple authors identified that the frequency of lipase and protease activities were similar at mesophilic (e.g., 28°C) or psychrophilic temperatures (7°C), though the activity of the enzymes was higher at mesophilic temperatures (von Neubeck et al., 2015, Yuan et al., 2018b). During their late exponential or stationary phases of growth, *Pseudomonas* spp. produce lipases and proteases that are in majority cold-tolerant or cold-active, (Makhzoum et al.,

1995, Yuan et al., 2018b). Conversely, the production of enzymes by *Bacillus* and other spore-formers matches the growth rate of these bacteria, and takes place predominantly during the earlier stages of exponential growth (Priest, 1977). Due to the particularly diverse growth capabilities found across spore-formers, these enzymes may be psychrophilic, psychrotrophic, mesophilic or thermophilic (Kohlmann et al., 1991, Abol Fotouh et al., 2016, Farooq et al., 2021).

2.3.2 Other notable spoilage-associated mechanisms

The main fermentative process that is associated with economically significant organoleptic defects in dairy products is lactate fermentation, which is performed by anaerobic BAB of the genus *Clostridium s.l.* (e.g., *C. tyrobutyricum*, *C. butyricum*, *C. sporogenes*) in cheeses and leads to the production of gas and butyric acid (D'Incecco et al., 2018). Gas production in hard and semi-hard cheeses may result in the development of slits, cracks and structural defects, while the release and accumulation of butyric acid is associated with unpleasant rancid aromas, which are characteristic of late blowing (Garde et al., 2011). The spores of lactate-fermenting BAB may survive pasteurisation and bactofugation, particularly when they are initially present in high concentrations, and can be responsible for the onset of late blowing after germinating during cheese ripening (Pahlow et al., 2003, Garde et al., 2011). The feeding of silage was found to increase the incidence of late blowing in bovine, ovine and caprine milk cheeses, and because of the high regional economic importance of these dairy products, the use of silage may be restricted or forbidden to achieve low counts of clostridial spores in milk (Pirisi et al., 2007, Burtscher et al., 2020). Other aerobic spore-forming bacteria such as *B. licheniformis* may also be capable of gas production due to different fermentative processes, but they generally do not impact dairy products (Niu et al., 2018, Niu et al., 2020).

Discoloration of dairy products can be attributed to the biosynthesis of pigments by microbial contaminants. *Pseudomonas* species are capable of producing a variety of differently coloured pigments including pyoverdine or fluorescein, pyorubin, pyomelanin, pyocyanin, and indigoidine (Quintieri et al., 2019a). Several *Bacillus* spp. also produce pigments that are thought to be involved in protection against external stresses and in the sporulation process (Moeller et al., 2005), but which have not been associated with dairy products quality (Reichler et al., 2019). In *Pseudomonas*, pigment synthesis is thought to be orchestrated to counteract the increased oxidative stress that pseudomonads undergo at low temperatures, modulate the transition from planktonic to biofilm state, to act as antimicrobials against other microorganisms or as signalling molecules and virulence factors (Martin et al., 2011, Quintieri et al., 2021). Production of indigoidine was shown to confer higher fitness to a

strain of *Roseobacter* spp., when grown in a co-culture with other microorganisms (Cude and Buchan, 2013). Fluorescent siderophores called pyoverdines may be produced on the surface of cheeses by *Pseudomonas* spp. and cause discolorations (Quintieri et al., 2019b, Reichler et al., 2019). These fluorescent siderophores can modulate the production of microbial lipases and proteases by impacting cellular iron levels (Wilderman et al., 2001, Brown and Luke, 2010).

2.4 Detection and identification of dairy-associated spoilage bacteria

2.4.1 Culture-independent approaches

2.4.1.1 PCR-based approaches

Quantitative PCRs (qPCRs) based on genes that are highly conserved across bacteria are designed to enumerate bacterial species or genera that may represent pathogens or beneficial and spoilage organisms (Furet et al., 2004, Suchodolski et al., 2012, Kable et al., 2019). qPCRs have been extensively used for the monitoring of total bacteria in dairy products such as fluid milk, dairy powders and cheeses (Agrimonti et al., 2019), including throughout the different stages of processing (Kable et al., 2019). Targeting of the 16S rRNA gene for qPCR analysis is particularly common because this gene is ubiquitously found across bacteria and highly conserved at the species level (Ranieri et al., 2012, Chauhan et al., 2013). However, the number of copies of the 16S rRNA gene found in bacterial genomes varies across species and may introduce bias in the enumeration determined by the qPCR assay (Rueckert et al., 2005b, Rueckert et al., 2005a).To broaden the application of qPCR in dairy monitoring and research, improvements of the specificity of the assay, and the optimisation of DNA extraction from biologically complex samples containing bacterial spores, are required.(Knüpfer et al., 2020, Pancza et al., 2021).

2.4.1.2 Next generation sequencing

With recent technological advances associated with a reduction in cost and time required for laboratory and *in silico* analysis, the use of high-throughput DNA sequencing has been rapidly developing in food science to study the microbiome and metagenome of food products. Food

metagenomics and metataxonomics have helped elucidate the population dynamics of contaminating microorganisms throughout food production (Quigley et al., 2016), and identify pathogenic or spoilage bacteria and their genetic determinants in dairy products (Walsh et al., 2017). A common metataxonomic approach is the use of 16S rRNA gene amplicon sequencing, which captures a global overview of the microbiota of a sample while overcoming typical biases associated with bacterial culturability. Amplicon sequencing has been extensively used to study the microbiota of bovine raw milk (Rasolofo et al., 2010) and of dairy products such as cheeses (Dreier et al., 2022) or dairy powders (McHugh et al., 2020), but also to study the microbiota of bedding used on bovine dairy farms (Neher et al., 2022). Because this approach employs short-read sequencers (e.g., Illumina's MiSeq), DNA fragments of maximum size 600 bp are generated and may not allow for reliable species-level identification without comparison of the entire 16S rRNA gene. Several protocols combining short-read sequencing and post-sequencing assembly procedures have been developed to try and obtain full-length copies of the 16S rRNA gene using short-read sequencers in order to improve bacterial identification (Burke and Darling, 2016, Abellan-Schneyder et al., 2021). Long-read sequencing technologies such as Oxford Nanopore Technology significantly improve the resolution of metataxonomics and metagenomics investigations by generating full-length sequences of the 16S rRNA gene (~1,500 bp) or the 16S-ITS-23S rRNA operon region (~4,300 bp) (Karst et al., 2021, Seol et al., 2022), however long-read sequencing has lower throughput than short-read sequencing.

Shotgun metagenomics is the untargeted sequencing of all microbial genomes present in a sample. Shotgun sequencing can be used to profile the taxonomic composition of microbial communities at the species, and even subspecies levels (Milani et al., 2020), but also to investigate the functional potential of a sample's metagenome (Donia et al., 2014). The financial cost of metagenomics is significantly greater than that of 16S rRNA metataxonomics but the depths of sequencing that are reached with this method are superior to the point that metagenomic data can be used to recover whole genomes sequences (You et al., 2022). In dairy microbiology research, shotgun metagenomics have been used to identify pathogenic bacteria in a fermented dairy beverage (Walsh et al., 2017) or spoilage microorganisms in cheese (Quigley et al., 2016). You et al. (2022) screened the metagenome of fermented dairy products (e.g., kefir, yoghurt, koumiss) for the presence of genes related to carbohydrate-active enzymes, secondary metabolite clusters, and bacteriocins as potential spoilage factors. Using the MetaBAT 2 genome reconstruction software (Kang et al., 2019), You et al. (2022) were also capable of assembling 153 genome sequences from the metagenomic data generated in their study, including that of isolates representing yet undescribed bacterial species.

Recently, the combined application of multiple omic studies has helped understand the molecular basis of different biological processes pertaining to human health (Karczewski and Snyder, 2018), agriculture (Ichihashi et al., 2020), and food production (Zhuang et al., 2022). The potential of this field of research is tremendous as it can be used to identify molecular markers linked with biological food-associated processes by uncovering regulatory units across several omics layers [e.g., DNA, RNA, proteins, metabolites, etc. (Wells-Bennik et al., 2016)]. Before these high-throughput, high-resolution approaches may be validly applied to dairy products, future developments will be needed to assess and address the reliability of the results generated by NGS and other omics when studying dairy products because of the biological and molecular complexity of milk-based matrices (i.e., a mixture of animal and microbial cells, enzymes, metabolites).

Despite overcoming challenges associated with bacterial culturability and having superior resolution than culture-dependent approaches, high-throughput sequencing methodologies fail to accurately detect members of the spoilage microbiota of dairy products, and particularly spore-formers. This may be explained in part by the fact that the spores of spoilage-associated spore-forming bacteria are often present at lower abundance and may not be detected over the vegetative cells of non-spore-forming bacteria. Furthermore, the *Bacillota* (formerly *Firmicutes*) are under-detected by metagenomic and metataxonomic approaches because their cells, and particularly their spores, are resilient to many traditional methods of DNA extraction (Filippidou et al., 2015, Knüpfer et al., 2020). In order to study the diversity of mesophilic spore-formers in whey powders using shotgun metagenomics, McHugh et al. (2018) used an enrichment step on agar to facilitate spore germination and lysis of the cells, as well as to remove DNA from thermosensitive bacteria and bovine cells. The use of NGS also leads to destruction of the sample which prevents the obtention of future information on bacterial isolates, including with their regards to their spoilage or pathogenic phenotypes.

2.4.2 Culture-based approaches

One of the key advantages of culture-based methods is that they can be used to isolate microorganisms from a wide range of environmental (e.g., dairy farm), animal (faeces, milk) and food (i.e., dairy products) samples that may considerably vary in terms of microbial load or sample type and texture. Samples containing high concentrations of bacteria may be diluted accordingly while those with low concentrations of bacteria can be concentrated, including by membrane filtration or centrifugation (Gupta and Brightwell, 2017, Stelma, 2018, Ohkubo et al., 2019). Culture-based methods have been extensively used for the isolation of food spoilage bacteria because these organisms are capable of

growth in a nutrient-rich food matrix, and thus they are also likely to grow on nutrient-rich media in laboratory conditions. Beyond variations associated with location, the microbial diversity obtained from a given matrix can vary significantly based on the conditions of culture used to isolate bacteria. Therefore, it is important to carefully select and define the methods of culture prior to any investigation in order to ensure that the isolated bacteria are relevant in the context of the research and its goals. The type of media used, any pre-treatment, as well as the temperature and duration of incubation can significantly impact the growth and thus the diversity and quantity of bacteria that are detected by culture-based methods (Kent et al., 2016, Murphy et al., 2021).

2.4.2.1 Isolation of psychrotrophic non-spore-forming bacteria

Selective isolation of psychrotrophic bacteria requires extended incubation times (typically at least a week) at temperatures lower than 10°C. Yuan et al. (2017) incubated bovine raw milk samples on plate count agar at 7°C for 7 days and identified the predominant psychrotrophic bacteria as members of the genera *Pseudomonas*, *Acinetobacter*, *Flavobacterium* and *Sphingobacterium*. Enrichment of samples at psychrotrophic temperatures, either with or without the addition of liquid media, may also be performed to improve the detection of bacteria of interest (e.g., *Pseudomonas*) or of lower-abundance populations (Zhang, 2020). Because of their relevance for dairy quality and the need to accurately detect *Pseudomonas* spp. different selective media have been designed for their isolation (Mead and Adams, 1977, Flint and Hartley, 1996). Cephaloridine fucidin cetrimide (CFC) agar (Mead and Adams, 1977) has been extensively used for the enumeration and isolation of *Pseudomonas* spp. from milk and dairy products (Dogan and Boor, 2003, Weber et al., 2019, Bruzaroski et al., 2020). This growth media contains three antibiotics to which *Pseudomonas* are naturally resistant, but which inhibit the growth of most other bacteria. However, in addition to *Pseudomonas* spp., (Tryfinopoulou et al., 2001) isolated *Enterobacteriaceae*, *Proteus* and *Shewanella* from fish using CFC medium. These naturally resistant bacteria may thwart the detection of *Pseudomonas* spp. in samples where they are particularly abundant, including in the natural environment.

2.4.2.2 Isolation of spore-forming bacteria

The isolation of spore-forming bacteria is carried out using a heating step prior to incubation on agar media, which is intended to destroy vegetative cells and leave only bacterial spores to be cultivated. The stringency of the heat treatment determines the type of bacteria that will be isolated according to

the heat resistance of their spores (Wehr et al., 2004). There are 3 main types of heat treatments used for the isolation of bacteria in dairy microbiology, pasteurisation grade, spore pasteurisation grade, and ultra-pasteurisation grade. Pasteurisation-grade heat treatments (e.g., 63°C for 30 min, also called the laboratory pasteurisation count) mimic the conditions of pasteurisation and enable the enumeration and isolation of thermoduric bacteria capable of surviving commercial pasteurisation, which includes both non-spore-formers (e.g. *Micrococcus*, *Enterococcus* and *Streptococcus* spp.) and spore-formers (Elmoslemany et al., 2009a, Jiménez-Sobrino et al., 2018). In order to exclusively isolate spore-forming bacteria, higher temperatures are used to remove most vegetative cells, and retain only spores. Spore pasteurisation, which is carried out at 80°C for about 10 min, is the most commonly used method to isolate spore-forming bacteria. This method has been used to isolate a particularly wide array of bacteria, including many species of aerobic mesophilic, thermophilic, psychrotrophic and anaerobic spore-formers implicated in dairy quality (Scheldeman et al., 2005, Buehner et al., 2014, Doll et al., 2017). Variations of the time or temperature of incubation are frequently reported (Vissers et al., 2007d, Ribeiro Júnior et al., 2016, Martin et al., 2019). Ultra-pasteurisation grade heat treatments are generally carried out at 100°C for 30 min to isolate highly heat resistant spores that may survive industrial sterilisation such as *B. licheniformis*, *Heyndrickxia sporothermodurans* (formerly *B. sporothermodurans)* and *B. subtilis* (Scheldeman et al., 2006). This method has been used to screen milk and dairy products as well as farm environmental samples such as feed for the presence of highly heat resistant spores (Scheldeman et al., 2005). Harsher heat-treatments (106°C for 30 min) have also been used to specifically isolate those species of spore-formers capable of producing specially thermoresistant spores (Kent et al., 2016, Martin et al., 2019). Following heat-treatment and before plating, spore suspensions may be enriched, with or without the addition or growth media, in order to facilitate the recovery of lower-abundance populations (e.g., psychrotrophic or thermophilic spore-formers (Watterson et al., 2014)), or to remove the interfering microbiota through selective enrichment [e.g., inhibition of facultative anaerobes (Gupta and Brightwell, 2017)].

2.4.2.2.1 Aerobic spore-forming bacteria

Most aerobic spore-forming bacteria implicated in dairy spoilage have non-fastidious growth requirements, indicating they may grow on a wide variety of media (Nickerson and Bulla, 1974). However, the isolation of dairy-associated spore-forming bacteria is often carried out using nutrient-rich media to maximise the recovery of bacteria and provide growth conditions similar to those found in milk and dairy products. A particular wide array of media has been used to isolate aerobic spore-

forming bacteria, irrespectively of the temperature of incubation, including plate count agar, brain heart infusion agar, milk agar and blood agar (Kent et al., 2016, Gupta and Brightwell, 2017, Murphy et al., 2021). The selection of the type of media used for the isolation of aerobic spore-former is often dependent on local availability as well as the researchers' preference and financial capacity, though efforts have been made to normalise these detection methods in order to facilitate comparison across investigations (Frank and Yousef, 2004). Indeed, recent studies have showed that the conditions selected for the isolation of spore-forming bacteria can significantly affect both the amount and diversity of bacteria that are isolated (Kent et al., 2016, Murphy et al., 2021). A heat treatment at 80°C for 10 to 12 min was shown to allow for better recovery of thermophilic and mesophilic spore-formers in dairy powders, as compared to a more stringent heat treatment at 100°C for 30 min (Watterson et al., 2014, Kent et al., 2016). (Murphy et al., 2021) found that plate count milk agar performed best for the recovery of thermophilic and mesophilic spores from dairy products, as compared to other media such as brain heart infusion agar, standard methods agar, or tryptic soy agar supplemented with starch. Variations in the diversity of bacteria isolated from different types of agar media also indicate that interstudy comparisons are limited.

Selective growth media are rarely used for the isolation of aerobic spore-forming bacteria, with the exception of *B. cereus s.l.* that have received specific attention due to their ability to cause dairy spoilage, and disease in humans (Marston et al., 2008, Chon et al., 2014). Media selecting for *B. cereus s.s.* have high sensitivity but often display low selectivity as they rely on phenotypic characteristics found in *B. cereus s.l.* but also across other *Bacillus s.l.* (e.g., resistance to polymyxin or lecithinase activity), and require expertise for accurate enumeration of *B. cereus s.l.* (Marston et al., 2008).

Differential growth of dairy-associated aerobic spore-forming bacteria is predominantly achieved by modifying the temperature of incubation. There are three main ranges of temperatures that are used to recover aerobic spore-forming bacteria, and they correspond to the three main types of temperatures encountered throughout dairy production: cold, ambient, and hot.

The isolation of psychrotrophic spore-formers is undertaken under growth conditions similar to those used for the isolation of psychrotrophic bacteria, namely at temperatures < 10°C and for a duration of at least a week. Martin et al. (2019) recorded up to 5.04 $\log_{10}$ cfu/g of psychrotrophic spores after incubation of milk and farm environmental samples at 6°C for 7 days on brain heart infusion agar. Because psychrotrophic spore-formers are capable of growth at both cold and ambient temperatures, these bacteria are also frequently isolated after psychrotrophic enrichment followed by plating and

incubation at temperatures close to 30°C (Ranieri and Boor, 2009, Doll et al., 2017). Doll et al. (2017) recovered psychrotrophic spore-formers such as *B. cereus s.l.*, *B. pumilus s.l.*, *Paenibacillus odorifer*, *P. xylanexedens* and *P. tundrae* from cow milk after psychrotrophic enrichment of milk samples at 6°C for 21 days, followed by incubation on agar media at 30°C for 6 days.

Thermophilic spore-formers, which can proliferate in the high-heat sections of dairy powder manufacturing plants, are typically isolated at high temperatures [> 50°C (Burgess et al., 2010)]. These conditions allow for the recovery of both thermotolerant spore-formers *Bacillus* spp. and obligate thermophilic spore-formers such as *Geobacillus* and *Anoxybacillus* species. Miller et al. (2015b) isolated thermophilic spore-formers present in cow milk and dairy powders using a combination of direct plating at 55°C or enrichment at 55°C followed by plating. The bacteria that were isolated with these methods included *B. licheniformis*, *Anoxybacillus* spp., *Geobacillus* spp., and *Aeribacillus pallidus* (Miller et al., 2015b). Unlike obligate thermophilic bacteria such as *Anoxybacillus*, *Geobacillus*, *Aneurinibacillus* and *Aeribacillus*, which can grow upwards of 65°C (Kikue et al., 2019), thermotolerant *Bacillus s.l.* do not typically proliferate above 60°C (Burgess et al., 2014). Zhao et al. (2013) observed accumulation of thermotolerant species of *Bacillus* and *Brevibacillus* after enrichment of biofouling samples at 55°C, while enrichment of the same sample at 65°C resulted in the accumulation of obligate thermophilic *Geobacillus* and *Anoxybacillus* species. Obligate thermophilic spore-formers are present in particularly low concentrations in raw milk (McGuiggan et al., 2002), and thus potentially in the dairy farm environment, while thermotolerant spore-formers are typically more abundant as they are also able to grow at ambient temperatures. Hence, the detection of obligate thermophiles, which are the main contaminants of dairy powders, may be improved in raw milk by using higher incubation temperatures limiting the growth of thermotolerant bacteria.

The enumeration of mesophilic spore-formers is often regarded as the total spore count of a sample, namely because this incubation method allows the recovery of the most comprehensive set of bacteria across spore detection methods. At mesophilic incubation temperatures, obligate mesophilic spore-formers (growth at 20-40°C) but also both psychrotolerant (growth at < 30-35°C) and thermotolerant mesophiles (growth at > 30-35°C) can be cultured. Temperatures neighbouring 30-35°C and an incubation time of 24 to 48 h are typically used for the isolation of mesophilic spore-formers, although an array of variations has been reported. In the USA, Frank and Yousef (2004) defined optimal conditions of incubation for the isolation of dairy-associated mesophilic spore-formers as 32°C for 48h on nutritive agar (e.g., plate count agar). These conditions were subsequently used across

numerous other studies in the USA, which facilitated interstudy comparisons (Miller et al., 2015b, Kent et al., 2016, Martin et al., 2019, Murphy et al., 2019). Both lower and higher temperatures have been used for the isolation of mesophilic spore-forming bacteria, such as 30°C (Scheldeman et al., 2005), 35°C (Gupta and Brightwell, 2017) or even 37°C (Scheldeman et al., 2005, Buehner et al., 2014). Many incubators are set by default at 37°C, which is a temperature historically used to culture bacteria, including many pathogens or human commensals such as *Escherichia coli*. However, some spore-forming bacteria such as psychrotolerant mesophiles may not be able to proliferate at temperatures above 30-35°C, hence the use of relatively high temperatures for the isolation of mesophilic spore-formers could skew the detection towards obligate mesophiles and thermotolerant mesophiles.

2.4.2.2.2 Isolation of *Clostridium s.l.*

Accurate enumeration and isolation of *Clostridium s.l.* associated with the dairy production are notoriously challenging due to the simultaneous presence of facultative anaerobic bacilli (e.g., *Bacillus s.l.*, *Paenibacillus*) which have similar growth conditions to that of obligate anaerobic clostridia and also form thermoduric spores (Weenk et al., 1995, Doyle et al., 2018). In dairy microbiology, the enumeration of the spores of butyric acid bacteria from dairy products and the farm environment is carried out using the most probable number (MPN) approach (Driehuis, 2013, Brändle et al., 2017). After appropriate dilutions, the sample is distributed in vials of media containing lactic acid which are sealed, incubated at 37°C, and monitored for gas development that is characteristic of the growth of BAB. Estimates of clostridial spore counts can be subsequently inferred according to the results of the MPN methodology. This approach is labour- and time-consuming and in addition, non-clostridia spore-forming bacteria capable of gas production such as *B. licheniformis* and *Paenibacillus* spp. may also be enumerated alongside clostridia (Driehuis et al., 2016). The isolation of BAB can be carried out after MPN enumeration by plating positive reaction tubes on reinforced clostridial agar [RCA (Arias et al., 2013)], though other bacteria may also be isolated with this method and interfere with the isolation of *Clostridium s.l.* (Driehuis et al., 2016). After plating of silage, faeces and cow milk MPN tubes positive for gas production on RCA, Driehuis et al. (2016) recovered *B. licheniformis*, *B. pumilus*, *Paenibacillus* spp. and *Aneurinibacillus* spp. in addition to *Clostridium beijerinckii*, and *C. tyrobutyricum*. Despite the presence of interfering bacteria, RCA is widely used for the isolation of clostridia from sheep or cow milk and cheeses (Julien et al., 2008, Garde et al., 2011, Arias et al., 2013, Turchi et al., 2016). Colony morphology can be a useful tool for the selection

of clostridial colonies on agar media to be further identified and characterised (Barash et al., 2010). On selective media containing sulphate (e.g., Shahidi Ferguson perfringens [SFP] agar (Shahidi and Ferguson, 1971)), sulphate-reducing clostridia, but also few other facultative anaerobic bacilli, can be conveniently distinguished as they form typical large black colonies. Several other types of selective media have been designed to isolate different *Clostridium s.l.*. of particular relevance, including *C. botulinum* (Dezfulian et al., 1981), *Clostridioides difficile* (George et al., 1979), *C. butyricum* (Popoff, 1984), or *C. perfringens* (Shahidi and Ferguson, 1971). These media have varying levels of selectivity and often fail to suppress the growth of facultative anaerobes and/or of other non-target clostridia. Anaerobic enrichment of samples containing clostridial and bacilli spores can help limit interference by facultative anaerobes that are often more abundant prior to enrichment. Xiao et al. (2020) formulated a media containing glucose, sucrose and maltose to perform selective enrichment of butyric acid clostridia from geological samples. Gupta and Brightwell (2017) carried out anaerobic enrichment of bovine dairy effluent samples using cooked meat glucose media and subsequently plated the resulting suspension on SFP agar for anaerobic incubation at 35°C for 24 to 48h. This approach enabled the exclusive isolation of *Clostridium s.l.* and prevented the growth of facultative anaerobic bacteria (Gupta and Brightwell, 2017). It was later demonstrated that selective growth was achieved via growth inhibition of non-*Clostridium* bacteria by clostridial compounds produced during cooked meat glucose broth enrichment (Pahalagedara et al., 2020, Pahalagedara et al., 2021). Current methods available for the isolation and study of dairy-associated clostridia require improvements, particularly on the selectivity of the isolation media in order to provide accurate enumeration and measure of relative abundance of clostridial populations present in milk, dairy products and on dairy farms.

2.4.2.3 Identification of spoilage bacteria

As opposed to NGS approaches which combine computation of relative abundance with identification, traditional culture-based methods require molecular or biochemical evaluation in order to identify bacterial isolates. In dairy microbiology, one of the most common identification method of identification is (partial) sequencing of the 16S small subunit rRNA gene, an approximately 1.5 kb DNA sequence that is highly conserved at the genus and species level and which can be used to infer taxonomies and phylogenies (Lane, 1991). However, bacterial identification is often performed using short [e.g., 418 bp (Rothman et al., 2002)] or medium size (e.g., 700 bp (Miller et al., 2015b) fragments of the 16S rRNA gene that may lead to ambiguous identification statuses, as some species

of bacteria share more than 99% of their full-length 16S rRNA gene sequence (Shivaji et al., 2006). One of the strengths of 16S rRNA gene-based identification comes from its popularity and widely adopted use, both of which have fuelled extensive development of curated databases with entries for thousands of different bacterial species. Such tools include NCBI's Basic Local Alignment Search Tool [BLAST, (Altschul et al., 1990, McGinnis and Madden, 2004)], SILVA (Pruesse et al., 2007) and the Ribosomal Database Project (RDP, (Cole et al., 2014) to cite a few, although many others exist and are still being developed to this day (Sikolenko and Valentovich, 2022). Sequencing of the 16S rRNA gene is one of the preferred methods of identification in dairy microbiology and has shown suitable resolution for the identification of spore-forming bacteria and some *Pseudomonas* spp., even when using short DNA fragments (Huck et al., 2007a, Reichler et al., 2018). Clusters of species that are particularly closely related and share high similarity (> 98-99%) in their 16S rRNA gene sequence, such as *B. cereus s.l.* or *Pseudomonas fluorescens s.l.*, may require the use of alternative identification methods with better phylogenetic resolution that may be obtained using a polyphasic approach or via whole-genome sequencing.

Chiefly in clinical diagnostic and research, matrix-assisted laser desorption ionisation time-of-flight (MALDI-TOF) mass spectrometry has been extensively used for reliable and fast identification and typing of bacteria using pure cultures (Dingle and Butler-Wu, 2013). Because of this, bacterial databases used for mass spectrometry spectra comparison are largely skewed towards clinical strains or bacterial species of clinical relevance and rarely include spoilage bacteria. Custom databases can be created by the users, but it is a time-consuming process which also requires prior identification of the isolates using an alternative and reliable method such as 16S rRNA gene sequencing. Vithanage et al. (2017) showed that MALDI-TOF mass spectrometry performed worse than 16S rRNA gene sequencing for the identification of Gram-negative pathogens (e.g., *Salmonella*) and spoilage bacteria (e.g., *Pseudomonas*) isolated from bovine raw milk. MALDI-TOF mass spectrometry is a promising technique for fast and reliable identification of dairy spoilage bacteria, however new, open-access MALDI-TOF bacterial databases with a focus on spoilage bacteria should be generated to permit its use in dairy microbiology.

2.4.2.4 Subtyping of spoilage bacteria

DNA-based subtyping methods enable differentiation of bacterial isolates beyond the species and subspecies level. These tools have been used to detect and track foodborne disease outbreaks (Swaminathan et al., 2001), and to identify sources of bacterial contamination throughout food

production systems, including dairy (te Giffel et al., 2002, Huck et al., 2008). Sequencing of the *rpoB* gene, which encodes the β subunit of bacterial RNA polymerase, has become a popular approach for simultaneous identification and subtyping of bacteria isolated with dairy production (Huck et al., 2008). When originally applied to a set of dairy isolates in the USA, this method was shown to produce phylogenies with higher resolution than those generated using 16S rRNA gene sequencing (Huck et al., 2007b). However, *rpoB* subtyping requires access to a reference database which must be itself built using another method for identification of the isolates, such as 16S rRNA gene sequencing. The Food Microbe Tracker (Vangay et al., 2013), a database set up and curated by Cornell University (Ithaca, USA), contains phenotypic and genomic data, including 16S rRNA and *rpoB* gene sequences, that have been generated from microbial isolates recovered over the course of numerous studies pertaining to food production, and particularly dairy production, in the USA. This database is continuously enriched with new data and contains entries for a wide array of dairy spoilage bacteria such as psychrotrophic spore-formers and *Pseudomonas* spp., two of the main causes of dairy spoilage in the USA.

Multilocus sequence typing (MLST) is another approach in which several genes are amplified and sequenced to achieve subtyping of bacterial isolates. Housekeeping genes such as the 16S rRNA, *gyrA*, *rpoB*, and *rpoD* are frequently included in MLST schemes, and the simultaneous comparison of these genes provide additional layers of phylogenetic resolution as compared to their individual study. MLST has been invaluable to refine our understanding of the intricate taxonomy and phylogeny of the *Pseudomonas* genus and particularly the *Pseudomonas fluorescens* complex (Garrido-Sanz et al., 2016), which members are frequently associated with dairy spoilage and often undistinguishable using partial 16S rRNA gene sequencing. However, MLST can becomes expensive and labour-intensive for large bacterial datasets, and as a result, cheaper, single-gene alternatives with suitable resolution are often sought. To this end, Reichler et al. (2021) evaluated the use of single-gene subtyping with seven different housekeeping genes (*glnS*, *gyrB*, *ileS*, *nuoD*, *recA*, *rpoB*, and *rpoD*) to identify, subtype and track *Pseudomonas* spp. associated with dairy spoilage. The authors ultimately determined that *ileS* had the most suitable traits for simple and affordable single-gene characterisation of *Pseudomonas* (Reichler et al., 2021).

The highest degree of phylogenetic resolution for subtyping is achieved using whole genome sequencing. Genomes of individual isolates are sequenced, subsequently identified, and compared with phylogenetic tools such as the Type Strain Genome Server (TYGS) pipeline (Meier-Kolthoff and Göker, 2019, Meier-Kolthoff et al., 2022). Whole genome sequencing has been recently increasingly used to track food pathogens and spoilage bacteria and identify the source of these

organisms throughout production (Chiesa et al., 2014, Chiaverini et al., 2021). However, high costs associated with the sequencing of whole genomes can be prohibitive when studying large sets of isolates.

Affordable alternative subtyping methods which rely on DNA fingerprinting have been used to subtype and track bacterial contaminants, including randomly amplified polymorphic DNA PCR [RAPD-PCR, (Welsh and McClelland, 1990, Williams et al., 1990)], repetitive element sequence-based PCR [rep-PCR, (Versalovic et al., 1998)], amplified ribosomal DNA restriction analysis (ARDRA, (Grimont and Grimont, 1986)], multilocus variable-number tandem-repeat analysis coupled with high-resolution melt analysis [MLV-HRMA, Seale et al. (2012)], and enterobacterial repetitive intergenic consensus PCR (ERIC-PCR, (Versalovic et al., 1991)]. Using defined or randomly generated primers the DNA of a pure culture is amplified by PCR, resulting in the amplification of DNA fragments with varying sizes that can be visualised on a gel electrophoresis to generate a fingerprinting pattern. DNA fingerprinting is easily scalable and implemented, including on a wide array of bacterial species, and has been successfully used at different stages of the dairy production to probe bacterial phylogenies and perform source attribution of potential contaminants (te Giffel et al., 2002, Rueckert et al., 2004, Gopal et al., 2015). However, the reproducibility and thus reliability of these techniques has been criticised as they may be highly sensitive to experimental variations during the PCR step (e.g. primer mismatch) and to variations during in-silico analysis [e.g. adequate band detection (Tyler et al., 1997)].

2.5 Factors and sources of milk contamination by spoilage bacteria on dairy farms

The dairy farm environment is home to a complex, diverse and heterogeneous set of bacteria that dwell in different matrices. Because of the intrinsic relationship between different components of this ecosystem, bacteria can be transmitted in the environment, cycle, and travel across the farm, ultimately ending up in raw milk. The dairy farm contamination cycle (Figure 2.1) illustrates the transmission of bacteria in the farm environment and highlights the principal routes of bacterial contamination. Primary sources of spoilage bacteria on dairy farms include the microbial communities present in the natural environment (e.g., soil) or in association with its macroflora (e.g., plants, animals) and materials derived from these sources (e.g., manufactured feed, bedding). Bacteria present in these sources may come in contact with the animals and gain access to their digestive tract

upon feeding, and/or subsequently to the surface of their udders, which can lead to contamination during milking. Because of the close relationship between the different components of the farm, bacteria can also easily cycle throughout the farm, leading to their dispersal across the environment and potential accumulation in specific sources.

Over the past few years, numerous studies have improved our understanding of the interplays between dairy farm-associated sources and factors (e.g., farm environment, management practices) and the microbiological quality of raw milk. Most of these efforts have been undertaken on bovine dairy farms while ovine dairy farms have been largely understudied. The species of animal farmed can impact farm practices and lead to differences in the diversity and dynamics of the bacteria present on farm, yet the functioning of dairy farms farming different animals remains highly similar and thus with possible similarities in both the diversity of bacteria that are found, and their transmission dynamics across the farm.

The following section describes the involvement of the principal known routes and sources of milk contamination that have been identified on bovine dairy farms, as well as on ovine dairy farms, when available.

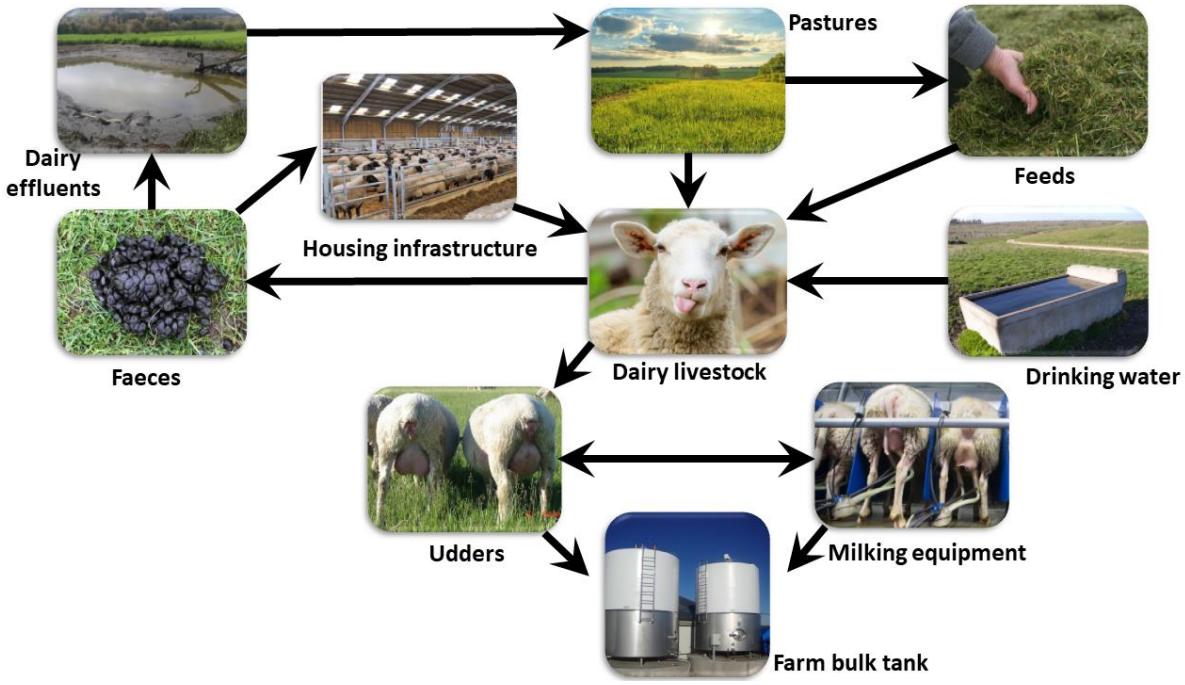


Figure 2.1. Contamination cycle picturing the transmission of bacteria across dairy farms.

2.5.1 Feed

The diet of dairy livestock plays a pivotal role in shaping their gut microbiota, and also that of their faeces (Wu et al., 2007). Bacterial spores are particularly successful at crossing throughout the gastrointestinal tract of livestock, and may gain access to the udders of the animals, and subsequently raw milk, after cross-contamination with faecal particles that may contain high concentrations of these bacteria [e.g., > 5.80 $\log_{10}$ cfu/g (Martin et al., 2019)]. The feed-faeces axis has been identified as an important source of bacteria in raw milk from different animal species, particularly when feed contains high concentrations of bacteria or when animals have poor hygiene (Vissers et al., 2007d, Arias et al., 2013, Martin et al., 2019). Using predictive modelling, Vissers et al. (2006) identified silage as the main source of BAB spores in bulk tank milk on bovine dairy farms. The same authors also identified that feeding of silage with high spore counts (> 5 $\log_{10}$ cfu/g) should be prohibited, as simulations showed it consistently led to the production of raw milk with more than 3.00 $\log_{10}$ cfu/L of BAB, which has a high frequency of late-blowing defects when used for cheese manufacturing (Vissers et al., 2006, Pirisi et al., 2007, Vissers et al., 2007b). The microbiota of different feed types may be particularly varied, which can modulate the bacteria that are present in the faeces of dairy livestock, on their udders, and possibly in raw milk. As reported by Doyle et al. (2017), microbial communities in faeces and teat skin swab samples from cows kept outdoors (and fed by grazing) or housed indoors (and fed with silage) clustered separately from each other following principal coordinate analysis, indicating different community structures. The microbial composition of feed is affected by feed characteristics such as initial microbial and nutrient content, as well as any processing, manufacturing and storage conditions to which feed may be exposed before it is consumed by dairy livestock.

2.5.1.1 Pasture grazing

Pasture grazing of dairy livestock is a widely used practice that allows for the animals to feed on natural grasses and forages. In addition to maximising the use of natural resources and thus limit costs associated with feeding, grazing can provide many benefits for the health and well-being of the animals, including increased physical activity and exposure to natural environments (Wilkinson et al., 2020). Moreover, the grazing of dairy livestock is well-perceived by consumers and it has been associated with desirable and distinctive physicochemical and flavour profiles in sheep milk and dairy products (Gutiérrez-Peña et al., 2021). However, pastures harbour a particularly diverse microbiota and thus can represent a source of udders and raw milk contamination by spoilage bacteria. When

dairy cows were grazed, their teat microbiota was shown to be highly similar to that of pasture and soil but not to that of housing-associated sources (Falardeau et al., 2019).

The epiphytic microbiota of pasture grasses is dominated by lactic acid bacteria, *Pseudomonas*, and *Enterobacteriaceae* (Pahlow et al., 2003). *Pseudomonas* species are commonly found in the rhizosphere and phyllosphere of plants (Kupferschmied et al., 2013, Bender et al., 2016, Ogunade et al., 2018). As such, their occurrence in milk has been frequently associated with the grazing of dairy cows (Doyle et al., 2017). On the contrary, spore-forming bacteria generally account for less than 1% of the microbiota of pastures, unlike in processed feeds in which they can be found in high concentrations (Martin et al., 2019, Flossdorf, 2021). However, spore-forming bacteria are particularly abundant in soil where they may reach spore concentrations greater than 5.00 $\log_{10}$ cfu/g (Vissers et al., 2007b, Martin et al., 2019) and persist for long periods of time (Davies et al., 1996, Garbeva et al., 2003). Several species of aerobic spore-forming bacteria, including dairy-associated spore-formers such as *Priestia megaterium*, *B. cereus s.l.* and *Paenibacillus* spp. can also form symbiotic or parasitic relationships with the local macroflora [i.e., plants or insects (Ortíz-Castro et al., 2008, Grady et al., 2016, Kalaycı Kara et al., 2021)] and live out their full vegetative-sporulation-germination life cycles. Overall, the diversity of the bacterial microbiota found in the phyllosphere of plants species is similar to that found in their rhizosphere as a result of the proximity and close association between these matrices, both of which facilitate the exchange of bacteria (Saito et al., 2007). Similarly, soil cross-contamination of grasses can increase the concentration of bacteria found on pasture and may thereby exacerbate the uptake of bacteria during feeding and their shedding in the faeces of the animals.

Soil ingestion is common in grazed animals, and particularly in sheep which tend to graze on vegetation patches closer to the ground. It remains unknown if soil ingestion is capable of significantly altering the microbiota of livestock faeces, however, most bacterial spores have the potential to cross the gastrointestinal tract (GIT) of the animals unscathed (Wu et al., 2007), or may even settle in and colonise it (Lee et al., 2019). In a study conducted by Healy and Ludwig (1965) in New Zealand, sheep were recorded ingesting up to 220 g of soil per day (10 – 220 g) which led to increased wearing of teeth. Soil ingestion is highly variable across farms and seasons as a result of varying factors influencing the exposure of the animal to soil and soil particles, including state and composition of the pasture (soil structure, plant species), stocking rate, and weather. In the above-mentioned study, Healy and Ludwig (1965) recorded yearly soil ingestion values 2 to 20 times lower in sheep grazing native pastures as opposed to those grazing ryegrass clover pastures. Ingestion of soil was also seasonal and the greatest amount of ingested soil were recorded during the wet season,

as rain increases the dispersal of soil particles onto pasture and may also lead to, or favour the development of, acute pasture damage. Christiansson et al. (1999) also identified the wet season as associated with increased *B. cereus s.l.* contamination of raw milk produced by grazed dairy cows. Risk factors identified by the authors included high pasture soil water content, low water evaporation, pasture damage, and muddy walkways, all of which increased contact between soil and the animals (Christiansson et al., 1999). Dairy ewes are significantly lighter (roughly seven times) than dairy cows, and hence may not be as prone to cause extensive pasture damage, even in wet climate (Drewry et al., 2000). Nonetheless, sheep dairy farmers should ensure adequate stocking rate, grazing rotation and preserving overall pasture health to limit contact between the animals and soil.

The ingestion of bacteria by grazing animals may also be increased when biological amendments containing high concentrations of bacteria are used to fertilise pastures (Davies et al., 1996, Jackson et al., 1997). These amendments can include compost, manure, dairy effluents and other farm slurries, all of which typically contain high concentration of spore-forming bacteria (Partanen et al., 2010, Gupta and Brightwell, 2017). Following the application of biological amendments, dormant bacterial spores may persist on pastures and in soil (Davies et al., 1996, Jackson et al., 1997), and could be ingested by the animals upon grazing, even months after application. Additional processing of these amendments before use may reduce their bacterial spore content (Stanford et al., 2015). Farmers may also consider sensible use of inorganic fertilisers which generally contains significantly lower bacterial loads than biological amendments.

2.5.1.2 Silages

Grass and crop silages represent an integral part of the diet of dairy livestock, particularly during winter when access to grazeable pasture may be limited, or when the animals are housed throughout the year (King et al., 2018). Poor quality silage can contain high concentrations of spore-forming bacteria and has been repeatedly documented as a source of bacterial spores in raw milk, including those of facultative anaerobic spore-forming bacteria such as *Paenibacillus* and *Bacillus* spp. (te Giffel et al., 2002, Borreani et al., 2019), but particularly of obligate anaerobic *Clostridium* species (Driehuis, 2013, Driehuis et al., 2016). In some sheep dairy flocks dedicated to the production of specialty cheeses (e.g., Roquefort, Saint-Nectaire), feeding of silage is often restricted or prohibited to avoid the development of cheese defects associated the presence of high counts of clostridial spores in raw milk (Pomiès et al., 2014, French Ministry of Agriculture and Food, 2017). The spore microbiota of silage, its dynamics during ensiling, and its contribution to the contamination of raw

milk have been extensively studied, but little is known about the contribution of silage as a source of non-spore-forming bacteria. *Pseudomonas*, which are frequently found in fresh forages, have also been detected in finished silage (Zheng et al., 2017, Keshri et al., 2019). Therefore, the production of high-quality silage is central to minimising the transmission of spoilage bacteria to raw milk.

The presence of high concentrations of bacterial spores in silage is typically caused by the growth of spore-forming bacteria during the ensiling process or by the uptake of large amounts of their spores during forage harvesting. Spores present in soil and on forage plants contaminate silage during harvesting and can remain viable throughout ensiling (Vissers et al., 2007b, Borreani et al., 2019). Vissers et al. (2007b) found that the soil content of herbage should be less than 1% in order to secure pre-ensiling BAB spore counts of 3 spores/g of silage which was identified as a threshold to produce silage of good microbiological quality (i.e., containing < 3 $\log_{10}$ BAB spores/g after ensiling). During ensiling, depletion of the available oxygen and rapid acidification of the silage by lactic acid bacteria normally inhibit the growth of aerobic and anaerobic spore-forming bacteria, respectively (Pahlow et al., 2003). Inadequate conditions of ensiling can thwart the fermentations carried out by lactic acid bacteria (lactic and acetic acids fermentations), and allow for transient growth of spoilage microorganisms (e.g., spore-formers, moulds, yeasts) and associated undesirable fermentations (e.g., propionic or butyric acid fermentations) at different steps of the ensiling process (Kung et al., 2018). Butyric acid fermentation, also called clostridial fermentation in the ensiling context, is the most common cause of silage quality loss and spoilage. The main clostridia that have been implicated in clostridial fermentation of silage include *C. tyrobutyricum*, *C. beijerinckii*, *C. butyricum* and *C. perfringens* (Driehuis, 2013). Although the precise dynamics remain unclear and likely interchangeable across some species, the growth of these clostridia during clostridial fermentation of silage is sequential. Species such as *C. perfringens*, *C. butyricum* and *C. sporogenes* are thought to initiate clostridial fermentation through proteolysis in silages with low water-soluble carbohydrate content (Zheng et al., 2017, Li et al., 2020). Clostridial fermentation is then subsequently performed by lactic acid fermenters such as those of the *C. butyricum* and *C. tyrobutyricum* groups which convert strong lactic acids into weaker butyric acids, also leading to energy and dry matter losses in silage (Pahlow et al., 2003). Several other clostridia have been recovered from spoiled silage (e.g., *Garciella*, *Paraclostridium*, *Terrisporobacter*), however their potential deleterious effect on silage fermentation or implication in clostridial fermentation, if any, remain unclear (Zheng et al., 2020). Clostridial silage contains high spore concentrations of clostridial species implicated in cheese spoilage, has low palatability, low nutritious value, and can represent a health hazard for the animals due to the presence of undesirable compounds, and also potentially of pathogenic bacteria (Driehuis,

2013, Driehuis et al., 2018). Factors that have been associated with the onset of clostridial fermentation during ensiling include those related to the composition of the ensiled forage (e.g., dry matter content, water-soluble carbohydrates, crude proteins, buffering capacity, bacterial microbiota), and those related to forage harvesting and processing (e.g., harvest timing, chopping and wilting, silage silo or bale sealing, use of silage inoculants). By ensuring that both the crop to be ensiled and the ensiling practices are compatible and suitable, farmers can preserve the microbiological and nutritional quality of the silage produced on farm and limit its contribution to the bacterial contamination of raw milk.

When silage is exposed to air upon opening the silo or after its removal from bales (i.e., feed-out period), fermentation acids and other substrates are oxidised by aerobic bacteria, yeasts and moulds, leading to a decrease in metabolisable energy and to the outgrowth of these microorganisms. In a study by Borreani and Tabacco (2010) the infiltration of air in the peripheral areas of corn silage stack led to the growth of moulds and aerobic spore-forming bacteria which degraded organic acids and raised the pH. Due to extensive consumption of oxygen caused by the growth of aerobic bacteria, anaerobiosis was restored in some peripheral areas of the silage, and subsequently led to the outgrowth of clostridial spores to levels up to 3 $\log_{10}$/g higher as compared to the well-preserved, well-fermented silage core (Vissers et al., 2007b, Borreani et al., 2019). It is recommended that silage should be kept a maximum of 7 hours under aerobic conditions to limit aerobic deterioration and the outgrowth of undesirable microorganisms (Borreani and Tabacco, 2010).

A successful strategy to ensure the efficiency of the ensiling process and thus the stability of silage during feed-out is the use of silage additives, either probiotics (lactic acid bacteria) or prebiotics (acids, salts). This complex topic and the extensive research involved has been recently reviewed by (Muck et al., 2018). A recent consensus has been the use of silage inoculants containing strains of heterofermentative *Lentilactobacillus hilgardii* and *Lentilactobacillus buchneri* that were shown to improve the acid fermentation profile and aerobic stability of corn silage whilst also preventing the onset of clostridial fermentation, including in low dry matter (wet) silages (Assis et al., 2014, Drouin et al., 2019). In alfalfa (lucerne) silage, which is prone to clostridial fermentation due to the forage high buffering capacity and low concentration of water-soluble carbohydrates (Coblentz and Muck, 2012), the addition of sucrose and *Lactobacillus plantarum* L20JPL65 was shown to prevent artificial, *C. perfringens*-induced clostridial fermentation and preserve silage quality (Zheng et al., 2020).

2.5.1.3 Other feed types

An increasingly diverse array of feeds and supplements are being supplied to livestock as farmers seek cheaper and year-round available alternatives to silages and grains-based supplements (Lardy et al., 2018). These alternative feeds are mainly comprised of ecological leftovers that include industrial by-products (e.g., palm kernel extract, beet pulp), feed produced on land unsuitable for human food production (e.g., weeds, cattle turnips) and harvest by-products such as cull vegetables, crop co-products and grain screenings (Lardy et al., 2018). Feeding of these products that are non-palatable for humans as cheaper alternative feeds to dairy livestock has been identified as crucial to achieve food security and sustainability of the livestock-associated production by minimising the wastage of nutrients throughout our food systems (Mottet et al., 2018). While these alternative feeds generally exhibit satisfactory palatability for livestock and boast high nutrient levels (e.g., fibers, amino acids, phenolic compounds, vitamins), it is important to note that their microbiological instability and perishability may become significant concerns when they are not pelletised.(Wang et al., 2014). Brewers' spent grain, also called wet brewers' grain, is a by-product of the brewing industry that is obtained after pressing and filtering the liquid fermentation product of grain. Because of its high microbial and water activity, this feed has a short shelf-life though it may be stored refrigerated, dried or even ensiled to increase storability and preserve its energy and protein content (Schneider et al., 1995, Santos et al., 2003a). There is a clear lack of representation and characterisation of the microbial populations found in alternative feeds (Flossdorf, 2021). Both *Bacillus* spp. and *Pseudomonas* spp. have been isolated from palm oil (Oguntimehin and Adeleke, 2022), and may be also present in other palm co-products. Spore-forming bacteria are also particularly abundant in grain where they persist despite desiccative stress [e.g., wheat, barley (Valerio et al., 2012)] and are the blight of breweries, bakeries and mushroom farms. In a study evaluating the interplays between farm practices and the contamination of ewe milk by gas-producing clostridia in Spain, Arias et al. (2013) recorded that 60% of the farms incorporated brewers' spent grain as part of the diet of the ewes. Using logistic regression, the authors identified this practice to result in a 3.90 times higher probability of obtaining raw milk of poor microbiological quality [i.e., containing > 3 $\log_{10}$ cfu/L of BAB according to MPN count (Pirisi et al., 2007)], as compared to the farms that did not use brewers' spent grain.

Newly introduced and imported feed types should be tested for their microbiological contents prior to incorporation in the diet of livestock to ensure that they will not increase the uptake and shedding of bacteria by the animals, and thus possibly indirectly increase the contamination of raw milk. There are several other factors that should also be considered when contemplating using a new type of feed, including its composition and suitability in the diet of the animals and for milk production, its

storability, local access and any costs associated with this, but also its safety for the animals (Lardy et al., 2018).

2.5.2 Bedding

Although generally reserved for the winter season, housing of dairy livestock is becoming more common throughout the year as a result of shrinking agriculture land, the need to protect the animals against predators, disease and harsh weather, or simply to ease management (Harrison, 1980, Turchi et al., 2016). During housing and upon lying down, contact between the udders of the animals and bedding materials can lead to the attachment of faecal and bedding bacteria on the udders, which may subsequently result in their transfer into raw milk during milking. Pregnant dairy ewes were found to spent more time lying down and had more lying bouts when they were housed as compared to grazed dairy ewes (Williams et al., 2021), suggesting that housing conditions may favour soiling of the udders. Bedding materials can contain particularly diverse and complex microbial communities that originate from the primary materials used for their manufacturing, as well as from any contamination acquired during storage and use (Murphy et al., 2019). In the bovine dairy farm environment, Ray et al. (2022) recorded a significant shift in the microbial composition of bedding samples during use, which was found to be dependent on the type of unused bedding material and its initial microbial composition. In organic non-manure bedding materials (i.e., straw, wood), bedding usage increased the relative abundance of *Bacillota* and *Bacteroidetes*, however, the microbial composition of unused and used recycled manure solids was not significantly different (Ray et al., 2022*)*. Fresh, unused bedding materials generally contain significantly less bacteria than used bedding materials, as the latter are enriched by a supply of microorganisms and nutrients carried by animal excrements and which promotes bacterial growth (Murphy et al., 2019, Ray et al., 2022). Godden et al. (2008) highlighted the growth of the environmental pathogens *Klebsiella pneumoniae* and *Enterococcus faecium* after spiking of artificial bedding microcosms composed of clean sand, recycled sand, digested manure solids, or wood shavings. Dairy spoilage bacteria, and particularly spore-formers, have been frequently isolated from bedding materials used on bovine dairy farms but their presence in the sheep dairy farm environment lacks characterisation. Bava et al. (2017) recovered 4.20 $\log_{10}$ cfu/g of the spores of *C. tyrobutyricum*, *C. butyricum*, *C. beijerinckii*, and *C. sporogenes* in straw bedding sampled on bovine dairy farms. Murphy et al. (2019) recorded up to 6.44 ± 0.82 and 5.75 ± 1.06 $\log_{10}$ cfu/g of the spores of mesophilic and thermophilic aerobic spore-forming bacteria, respectively, in various types of unused and used bedding materials collected on bovine dairy farms

(e.g., sand, limestone, wood shavings, manure solids and straw). *Pseudomonas* have been identified as one of the predominant genera of bacteria found in unused and used bedding materials utilised on bovine dairy farms in the microbiome study by (Ray et al., 2022). However, the specific contribution of bedding materials to the transmission of *Pseudomonas* across the farm environment and to raw milk, if any, remains unclear and will require future assessment.

Adequate selection and management of bedding materials have been identified as key factors involved in preserving the well-being, comfort and health of the animals, including against mastitis-causing pathogens, but also in safeguarding the quality of the dairy production against contamination by spoilage bacteria (Singh et al., 2020). Furthermore, bedding choices and bedding management may also affect feed intake, milk production, or milking parlour throughput (Fetrow, 2000). However, investigations on the contribution of bedding materials to the contamination of raw milk by spoilage bacteria have reported contrasting findings. On an Irish bovine dairy farm, Doyle et al. (2017) showed that bedding materials were not a significant source of raw milk contamination, and that their contribution was further reduced with the application of a suitable premilking routine: water wash, discarded first milk flow, and application of a chlorhexidine disinfectant followed by drying. Other authors have also identified bedding materials to be only a minor source of raw milk contamination (Falardeau et al., 2019), however these single-farm studies may be reflective of adequate bedding management and milking practices on these farms. Several other studies have identified a clear link between the microbial composition of bedding materials and that of raw milk (Murphy et al., 2019, Patel et al., 2019). Murphy et al. (2019) carried out an extensive survey of mesophilic and thermophilic bacterial spore counts in raw milk and in 13 types of bedding materials utilised on 190 bovine dairy farms with different bedding management practices in the USA. Using statistical modelling, the authors were able to highlight the transfer of bacterial spores in bedding to raw milk by identifying positive relationships between spore counts in unused bedding materials and in used bedding materials, as well as between spore counts in used bedding materials and in raw milk (Murphy et al., 2019). These studies suggest that the contribution of bedding materials to the contamination of raw milk can be highly variable across farms and is predominantly modulated by factors such as the type of bedding materials and the bedding management practices of the farm.

2.5.2.1 Bedding materials

Materials that are selected to be used as bedding need to fulfil requirements of animal comfort (e.g., impact on udder and foot health, absence of pathogens, ability to remain dry) and farmer comfort

(e.g., cost, storability, ease of management). However, the microbiological content of bedding materials is rarely taken into account for this decision, nor is their potential to support bacterial growth, provided that the material considered satisfy basic microbiological safety requirements. The ability of bedding materials to support bacterial growth, as well their initial levels of contamination, can vary drastically across types of materials and particularly between inorganic and organic bedding materials.

Inorganic materials that are used as bedding include sand, recycled sand, limestone and rubber mats. These materials have a limited initial microbiota, low nutrient content (total C < 1% and total N < 0.1%) and are slightly basic (pH > 8) which prevents extensive growth of bacteria (Godden et al., 2008). However, during use, animal excretion will provide a supply of nutrients and microbes that can enrich inorganic bedding materials and increase their bacterial load. Murphy et al. (2019) recorded the lowest concentrations of mesophilic and thermophilic bacterial spores in unused inorganic bedding materials including new sand (mesophilic spore count [MSC] = 3.06 ± 0.84 $\log_{10}$ cfu/g; thermophilic spore count [TSC] = 2.36 ± 0.61 $\log_{10}$ cfu/g) and limestone (MSC = 2.15 ± 0.64 $\log_{10}$ cfu/g; TSC < 2.00 $\log_{10}$ cfu/g) as compared to unused organic bedding materials (i.e, wood, straw, manure solids; average MSC = 4.67 ± 1.66 $\log_{10}$ cfu/g; TSC = 4.06 ± 1.52 $\log_{10}$ cfu/g). The used inorganic bedding materials contained up to 2.5 $\log_{10}$/g more spores than the unused bedding, and in addition, unused reclaimed (recycled) sand bedding also contained 1.5 $\log_{10}$ spores/g more than unused new sand bedding, highlighting the persistence of bacterial spores during the sand recycling process and their carry-over to reclaimed bedding (Murphy et al., 2019). Sand is considered a gold standard for bedding materials on bovine dairy farms due to its moisture wicking ability, microbial growth-inhibiting properties, ability to maintain cow cleanliness and comfort as well as its recyclability (Cook, 2003, Norring et al., 2008).

Straw and other organic materials are generally preferred to sand on sheep farms as they stimulate natural behaviour as well as promoting animal growth and feed assimilation (Jaborek et al., 2016), and thus possibly milk production. Organic bedding materials are also easier to source, manage and dispose of than inorganic materials which require local recycling facilities, a suitable disposal location, or third-party disposal as they do not decompose naturally. Because of their high nutrient content and often substantial initial microbiota, organic bedding materials such as recycled manure solids, straw and other alternative organic bedding materials have been identified as risk factors associated with higher contamination of raw milk by spore-forming bacteria (Martin et al., 2019, Murphy et al., 2019). Fresh organic bedding materials which were found to carry the highest concentrations of spore-forming bacteria included recycled manure solids and paper (> 6 $\log_{10}$ cfu/g

spores (Martin et al., 2019, Murphy et al., 2019)]. Straw, woodchips or sawdust bedding have lower initial concentrations of spores, but they can support considerable bacterial growth due to high nutrient availability, which includes lignocellulosic compounds. *Pseudomonas*, which are bacteria frequently associated with plants, have also been identified as one of the predominant genera of bacteria found in organic bedding materials used on bovine dairy farms such as sawdust, straw and recycled manure solids (Ray et al., 2022).

Woodchips are frequently used as bedding for dairy livestock as they are convenient to access and manage, offer adequate animal comfort and hygiene, and also have moderate concentrations of bacteria. Miscanthus (*Miscanthus x giganteus*) is a popular alternative bedding material in the equine and poultry industries, but its use in ruminant systems has been limited to date. In New Zealand, miscanthus is used as a bioenergy crop, nitrogen-mineralisation crop, and as a bedding material for livestock, including small dairy ruminants. Advantages of miscanthus bedding include low ammonia retention, low dust, affordability, and availability during the drought period of other bedding types (Rauscher and Lewandowski, 2016). Both spore-forming bacteria (i.e., *Bacillus s.l.*, *Lysinibacillus* and *Clostridium*) and *Pseudomonas* (e.g., *Ps. brennerii*, *Ps. fluorescens*) have been isolated from miscanthus plants and seedlings (Cope-Selby et al., 2017), suggesting these bacteria may be carried over to miscanthus bedding. Future characterisation of the microbiota of miscanthus bedding, and particularly of its spoilage-associated microbiota, will need to be carried out to evaluate potential milk contamination risks associated with miscanthus bedding. Straw bedding, which is similar to miscanthus bedding, was shown to contain high concentrations of bacterial spores both before use (4.61 ± 1.38 $\log_{10}$ cfu/g) and after use on bovine dairy farms [5.88 ± 0.72 $\log_{10}$ cfu/g, (Murphy et al., 2019)]. However, the use of straw as bedding has also been linked to lower thermophilic spore counts in raw milk (Miller et al., 2015a). Recycled manure solids is a type of bedding that is increasingly used on bovine dairy farms, and which represents one of the most presently studied type of bedding as it was found to consistently contain high concentrations of bacteria [frequently > 7 $\log_{10}$ cfu/g and up to 10 $\log_{10}$ cfu/g (Green and Bradley, 2014)], including spoilage (Driehuis et al., 2014) and sometimes pathogenic microbes (Lasprilla-Mantilla et al., 2019). Recycled manure solids are obtained after mechanical separation of manure, followed by facultative composting before use in order to kill or reduce the counts of pathogenic organisms (Stanford et al., 2015, Liu et al., 2022). There are limited reports on the use of manure for sheep bedding (Cestonaro et al., 2017). Sheep are particularly susceptible to a range of enteric pathogens, therefore recycled manure may not be suited for use as bedding materials on sheep farms as it may increase the occurrence and incidence of enteric

disease and associated pathogens in the environment of sheep, such as parasites, *C. perfringens*, *Salmonella* or *Klebsiella* species (Lasprilla-Mantilla et al., 2019, Ray et al., 2022).

2.5.2.2 Bedding management practices

Bedding management practices play a significant role in determining the cleanliness of dairy animals and the microbiota of bedding materials. The rate of bedding replacement and replenishment are management factors that impact the quantity and diversity of bacteria dwelling in bedding materials (Murphy et al., 2019). Infrequent bedding replacement leads to the accumulation of animal excretions and mud, which modulate the water activity, pH and nutrient availability of bedding materials and can lead to extensive bacterial growth, bedding deterioration and increased transmission of bacteria. Complete sawdust bedding replacement was shown to have limited efficacy at controlling populations of *B. cereus s.l.* in the bedding of dairy cows (Magnusson et al., 2007b). Though levels of *B. cereus s.l.* were lowest in freshly replaced bedding, they were restored to their pre-replacement concentrations after four to six weeks of use. Bacterial populations in newly laid bedding, and particularly in organic materials, quickly proliferate during the first 24 h after bedding application upon exposure to the animals (Godden et al., 2008, Ray et al., 2022). Frequent replenishment of the bedding area with fresh bedding materials has been identified as key to limit the contamination levels of bedding materials and their potential contribution to the contamination of raw milk (Martin et al., 2019, Murphy et al., 2019). Murphy et al. (2019) observed that bovine dairy farms that topped up the bedding area more frequently with fresh bedding, or which raked or tilled the bedding area more frequently, had lower mesophilic and thermophilic spore counts in bedding, which were in turn associated with lower spore counts in raw milk. To ensure that the addition of fresh bedding does not exacerbate the concentration of bacteria in bedding materials, unused bedding should have low concentrations of bacteria and not spoil during storage. Fresh bedding materials should be monitored and selected for their low concentrations of bacteria, in addition, the use of adequate storage facilities that limit exposure of bedding to precipitations and external contamination should be emphasised in order to limit bacterial growth during storage of bedding. Bovine dairy farms that replaced bedding more frequently had lower spore counts in unused bedding as it was stored on-farm for shorter periods of time before new bedding was purchased (Murphy et al., 2019).

Further research is also required to evaluate the efficiency of additives to improve bedding functionality and limit bacterial growth. Bedding water activity and pH are two variables that can be easily modulated by adding conditioners such as zeolite, limestone but also acidic organic matter such

as peat (Magnusson et al., 2007b, Marin et al., 2018). Simultaneously, overly dry bedding should be avoided as its handling lead to significant aerosolization of dirt particles and bacteria which can contaminate bedding materials and the udders of the animals (Murphy et al., 2019). Bedding probiotics or prebiotics are also available in the market, but there is limited available research validating their efficiency (Lallemand Animal Nutrition, 2021). The use of bedding probiotics has potential for the future as they may allow to control both the amount and diversity of bacteria present in bedding materials and it could be used to select for the growth of non-pathogenic, non-spoilage bacterial species in bedding.

2.5.3 Milking

Most of the contaminants present in raw milk originate from the farm environment, but it is during milking that the contamination of milk takes place, as environmental bacteria present in dirt attached on the udders can be dislodged and flow alongside raw milk. Thus, maintaining udder hygiene is perhaps the most important factor that can limit the contamination of raw milk by environmental and animal-associated bacteria. Martin et al. (2019) identified udder hygiene, individual cow milk spore level, and if udders were clipped/flamed as variables associated with spore counts in bulk tank raw milk. Though the udders of dairy ewes do not require hair clipping, an equivalent variable that may warrant further investigation could be the presence and length or weight of fleece on dairy ewes during milking and its putative link to milk microbiological quality as it carries its own microbiota, which includes dairy spoilage bacteria (Mulcock and Horn, 1965, Jackson et al., 2002).

The milking environment is a dynamic microbial hub with high animal and worker activity, and which is itself composed of several microbial niches (e.g., infrastructure, milking equipment) providing suitable, damp conditions for the growth and transmission of bacteria. The maintenance of proper hygiene during milking is crucial to control the transfer of bacteria to raw milk. The hands of dairy workers have been identified as a source of raw milk contamination, including by *Pseudomonas* spp. (Vidal et al., 2017), emphasising frequent hand washing as well as regular use and replacement of gloves. Gloves also prevent transfer of the human skin microbiota to the udders and teats of dairy ewes, which may include mastitis-causing bacteria such as *Staphylococcus epidermis* or *Staph. aureus* (Roberts et al., 2018). Dairy workers play a central role in ensuring that milking unfolds smoothly and under hygienic conditions, thus, they should be provided with appropriate training as it can help limit raw milk contamination (Evanowski et al., 2020). Training workers also improves their understanding of the milk contamination process and allows them to identify contamination risks

factors during milking so that they may be addressed or recorded for future corrective action. For example, the presence of excessive dust in the milking parlour has been associated with increased contamination of sheep milk by spore-forming bacteria (Arias et al., 2013). Skilled milkers can ensure adequate stocking rate during milking and that the animals are not stressed and agitated, which can limit aerosolization of dust and other particles.

2.5.3.1 Pre- and post-milking interventions

Active removal of dirt particles present on the udders of dairy livestock prior to milking may limit the transfer of bacteria to raw milk. In addition, pre- and post-milking interventions are also particularly successful at reducing the incidence and transmission of mastitis in dairy herds (Oliver et al., 1993, Gleeson et al., 2018). Although washing would be intuitively associated with improved udder hygiene and lower bacterial load on the udders, reports of the efficiency of pre-milking interventions have been contrasting. Magnusson et al. (2006) and Evanowski et al. (2020) recorded a 37 to 96% reduction in milk anaerobic spore counts, or mesophilic and thermophilic spore counts, respectively, after the implementation of a pre-milking routine including staff training and/or washing and drying of the udders with soap and towels washed with chlorine. The use of soap and thorough drying of the udders have been identified as key practices ensuring clean and dry udders which limit milk contamination. Evidence from the literature suggests that the effectiveness of pre-milking washing routines is particularly high when the udders of dairy livestock are soiled, including during animal housing (Vissers et al., 2007d, Morton et al., 2014). After application of a pre-milking teat disinfectant and a milking cup liner disinfectant, Verhegghe et al. (2019) observed a small but statistically significant decrease in the psychrotrophic bacterial counts present on the teat orifices of dairy cows, while counts of mesophilic bacteria and proteolytic bacteria did not change. In a grazing set-up, pre-milking routines have limited efficacy (Morton et al., 2014), and may even be detrimental for milk microbiological quality (Doyle et al., 2017). Morton et al. (2014) found that an iodine-based pre-milking washing routine of the udders did not significantly reduce the incidence of mastitis in a herd of grazed dairy cows. Udders manipulation prior to or during milking (udder massaging, foremilk stripping, washing) can dislodge dirt present on the udders and increase contamination of the teat or lower udders surface, which can lead to higher milk contamination (Miller et al., 2015a). Because of the mixed effectiveness of pre-milking regimens on the contamination of raw milk by bacteria, strategic and periodic implementation of pre-milking routines should be considered by

farmers who are struggling with maintaining adequate animal hygiene, for example during wet weather or when the animals are housed.

The teat sphincter relaxes during milking and can remain open for up to two hours, leaving it prone to colonisation by pathogens and other bacteria (Zecconi et al., 2002). Multiple milking sessions per day or the use of an automatic milking station (which increases milking frequency) also increase the opening duration of the teat sphincter (Hogenboom et al., 2019), and strain on the mammary tissues, which can increase colonisation of the teat duct, mastitis and possible contamination of milk by bacteria. To avoid contamination of the milk duct post-milking, animals should be moved into a clean environment shortly after milking and avoid locations containing high concentrations of bacteria or favouring their transfer, such as bedding and the housing environment, or muddy pastures. Freshly milked animals should also be prevented from lying down shortly after milking in order to limit contact between surfaces and the relaxed teat opening and thereby limit teat contamination (Devries et al., 2010). Feed manipulation is an approach that has shown promising results to control the standing behaviour of dairy cows and prevent extensive lying post-milking (Devries et al., 2010, King et al., 2016). Specifically, Devries et al. (2010) found that cows fed between 30 min prior to, and 60 min after milking were more likely to stand than animals that were fed outside of these boundaries. In addition, cows with a first lying bout at least 60 min after milking were 3.2 times less likely to acquire an intramammary infection as compared to cows lying down in the first 60 minutes (Devries et al., 2010). The use of post-milking cleaning interventions (post-dipping) can limit contamination of the milk duct by bacteria and reduce the incidence of mastitis (Oliver et al., 1993). However, the efficacy of these practices in reducing the bacterial contamination of raw milk is limited (Miller et al., 2015a). Post-dipping disinfectants recommended for use on the teats of dairy ewes include chlorhexidine, iodine compounds and quaternary ammonium salts which are combined with moisturising compounds. Alternative plant-based compounds have also been evaluated for their efficacy as antimicrobial post-dips, such as terpinen-4-ol from the tea tree (*Melaleuca alternifolia*), though it caused a limited reduction in the incidence of mastitis cases in dairy ewes (Dore et al., 2019). A thorough, albeit perhaps partially outdated document compiled by Ontario Sheep Farmers (Menzies, 2013) provides information about the milking practices of sheep dairy farms and on the practical efficiency, advantages and costs of some of the interventions and practices discussed hereabove.

2.5.3.2 Storage of raw milk and milking equipment

In the dairy industry the storage of raw milk plays a vital role in maintaining its quality before collection and processing. Generally, a maximum threshold temperature of 7°C is recommended, creating an environment that impedes the proliferation of most bacteria and prevents significant physicochemical alterations (Tribst et al., 2019, Malta et al., 2021). However, it's important to note that psychrotrophic organisms can remain active even at these lower temperatures, proliferating and producing thermostable spoilage enzymes that may impact the quality of dairy products even after processing (Matselis and Roussis, 1998, Marchand et al., 2017). On sheep dairy farms in New Zealand where the daily volume of milk production often does not warrant daily collection, raw milk may be stored on-farm for several days before pickup. Despite recently updated milk cooling guidelines for dairy farms in New Zealand specifying the need to cool milk to <10°C within 4 hours of milking commencement and maintain it at <6°C thereafter (Ministry for Primary Industries, 2022), specific regulations for sheep dairy farms engaged in multi-day storage are absent. Nevertheless, New Zealand farmers maintain a proactive approach, setting a target refrigeration temperature between 2-4°C to safeguard the quality and safety of stored raw milk from microbial activity.

Integrity and hygiene of the milking equipment, as well as water quality play an important role in controlling the bacterial contamination of raw milk. Following its passage through the milking pipeline, raw milk may become contaminated by bacteria present in the milking equipment, contributing to an increase in its microbial content. This stage in the dairy production process highlights the importance of maintaining stringent hygiene practices and regularly cleaning and sanitising the milking equipment to mitigate the risk of microbial contamination and uphold the quality standards of the raw milk. In their study, Tonamo et al. (2020) recorded APC that were up to 4 $\log_{10}$ CFU/ml higher in bulk tank milk, as compared to milk from individual ewes. Equipment wear and tear leads to the development of cracks and crevices in rubber parts, which may become contaminated and colonised by bacterial biofilms (Teixeira et al., 2005). Bava et al. (2011) recorded a positive correlation between the bacterial load of teat liners and that of bulk tank milk on bovine dairy farms. According to Weber et al. (2019), frequent colonisers of bovine dairy farm milking pipelines included members of the genera *Bacillus*, *Kocuria*, *Microbacterium*, *Staphylococcus*, *Acinetobacter*, *Chryseobacterium*, and *Pseudomonas*. Frequent and adequate washing of the milking equipment is required to prevent the build-up of biofilm and protect milk from excessive contamination borne out of the milking pipeline. The frequency and stringency of milking equipment washes is farm- and herd-dependent but it should include, at minimum, a daily cold water rinse and hot acid sanitiser detergent wash, as well as bi-weekly alkaline washes (Reinemann et al., 2003).

Increased contamination of raw milk by thermoduric bacteria has been linked to infrequent acid washes (Elmoslemany et al., 2010), and high alkalinity of the alkaline wash (Elmoslemany et al., 2009b). The efficacy of acid and alkaline washes is also highly dependent on water quality and temperature. Low temperature of the water used for milking equipment cleaning (< 42°C) has been associated with increased contamination of the milking equipment and raw milk by *Pseudomonas* (Feldmann et al., 2006) and other psychrotrophic bacteria (Bava et al., 2011), as well as thermoduric bacteria (Elmoslemany et al., 2009b). High water hardness decreases the efficiency of acid and alkaline washes and recommendations have been made on the use of water softeners (Elmoslemany et al., 2009b). The water source can also represent a key source of milking equipment contamination and its microbial composition should be monitored (Elmoslemany et al., 2009b) and a water purifier installed (Elmoslemany et al., 2010). Common contaminants of water supplies include coliforms, Gram-negative bacteria, and particularly *Pseudomonas* (Chambers, 2002), which excel at biofilm formation in the pipeline of livestock water drinking systems (Maes et al., 2019).

The implementation of interventions aiming to improve hygiene of the animals, the milking process, or that of the milking equipment should be tailored in order to optimise the efficacy of these interventions. Specific conditions and features of the farm (e.g., weather, farming system, milking system) should be carefully considered before the implementation of pre- or post-milking routines, which should also be driven by results of microbiological monitoring (e.g., high contamination of milking equipment surfaces, water supply) or visual observations (e.g., soiled udders).

2.6 Conclusions

Spoilage bacteria are contaminating organisms capable of impacting the stability, processability and overall quality of dairy products. The two principal causes of product quality decrease and spoilage are spore-forming bacteria and *Pseudomonas* spp. that originate from the farm environment and gain access to the processing environment and dairy products via raw milk. Variations in growth capabilities and spoilage potential across these taxa indicate that different bacterial species may impact the quality of dairy products according to their manufacturing conditions. In addition, heterogeneous growth requirements indicate that a comprehensive set of culture methods is required to accurately depict these populations.

Though the microbiota of cow milk and bovine dairy farms has been extensively studied, there is a clear paucity of data pertaining to the microbiota of sheep milk and ovine dairy farms. Additional

knowledge is required to improve our understanding of the diversity and dynamics of the spoilage microbiota of sheep dairy farms and products, in order to design and implement interventions and farm practices that may limit the contamination of ewe milk, similar to what has already been done for the bovine dairy production.

Chapter 3 - Materials and Methods

This chapter lays out the methodology used over the course of this study. Section 3.1 describes the general management practices of the different farms that were sampled and provides an overview of the methods employed across Chapter 4, 5 and 7 in the context of the isolation and characterisation of bacteria from farm samples, milk and dairy products. Section 3.2 describes the methodology used across Chapter 6, which focuses on the farm-level source attribution of these isolates using DNA fingerprinting and whole-genome sequencing.

3.1 Farm and products studies

3.1.1 Sampling and farm information

Five sheep dairy farms with flock size ranging between 800 to 3,000 ewes were sampled during this study. Farming systems varied between farms and are described in more details below. General farm management, such as the provenance of feed, rearing practices and milking practices, was similar between Farm 1, 2 and 3 while the general farm management of Farm 4 & 5 was similar. Meteorological data is only presented if environmental samples were analysed (i.e., not if only raw milk and/or milking cups were analysed). A general overview of the collection methods and samples collected can be viewed in Table 3.1. The total number of farm samples was n = 150.

Table 3.1. Farms sampling information

Sample type	Sample replicates (n = 150)		Sampling equipment
Farm 1[1] (n = 86)			
	Old[2] (n = 34)	**New[3] (n = 42)**	
Bedding	n = 8	n = 12	Whirl-Pak sampling bags (Whirl-Pak, Madison, USA)
Silage	n = 10	n = 10	Whirl-Pak sampling bags (Whirl-Pak)
Faeces	n = 8	n = 12	Faecal sampling jars (Thermo Fisher Scientific, Auckland, New Zealand)
Trough water	n = 4	n = 4	1 L Schott Duran glass bottles
Fence swabs	n = 4	n = 4	Ez-Reach sponge (World Bioproducts, Woodinville, WA, USA)
Milking cups swabs	n = 10		Whirl-Pak Speci-Sponge (Whirl-Pak)
Farm 2[4] (n = 51)			
Soil	n = 12		Whirl-Pak sampling bags (Whirl-Pak)
Grass	n = 12		Whirl-Pak sampling bags (Whirl-Pak)
Faeces	n = 12		Faecal sampling jars (Thermo Fisher Scientific)
Trough water	n = 4		1 L Schott Duran glass bottles
Milking cups swabs	n = 10		Whirl-Pak Speci-Sponge (Whirl-Pak)
Raw milk	n = 1		1 L Schott Duran glass bottles
Farm 3[1] (n = 1)			
Raw milk	n = 1		1 L Schott Duran glass bottles
Farm 4[1] (n = 1)			
Raw milk	n = 1		1 L Schott Duran glass bottles
Farm 5[4] (n = 11)			
Milking cups swabs	n = 10		Whirl-Pak Speci-Sponge (Whirl-Pak)
Raw milk	n = 1		1 L Schott Duran glass bottles

[1] Farm with a hybrid system (alternating housing/grazing).
[2] "Old" set of samples from Farm 1, collected from a pen with 3-month-old woodchips bedding materials.
[3] "New" set of samples from Farm 1, collected from a pen with 3-week-old woodchips bedding materials.
[4] Farm with a pasture-based system (year-round grazing).

3.1.1.1 Farm 1 – Hybrid farming

Samples from Farm 1 were collected in July 2018. Farm 1 housed about 3,000 dairy ewes and was located in the North Island of New Zealand. It was operated under a hybrid management scheme, meaning the flock alternated periods indoors and on-pasture, depending on the time of the year as well as on meteorological conditions (approximate time spent housed throughout the year: 25 %,

including in winter and throughout harsh weather). At the time of sampling, pregnant, nursing ewes and new-borns were housed in wall-less barns. Their living area consisted of woodchips-bedded pens further divided in smaller pens. To investigate potential farm-level variations of the local microbiota, two large pens were sampled. The first pen contained bedding materials that were set to be fully replaced after three months of use ("old" sample set) while the second had bedding materials that had been replaced three weeks before sampling ("new" sample set). As milking had not started at the time of bedding change, milking cup swabs were collected six weeks after the environmental sampling took place. According to historical data from the New Zealand National Climate Database (CliFlo, https://cliflo.niwa.co.nz/), the area experienced around 80 mm of rain and temperatures that ranged from -4.9 to 18.6°C during the 30 days preceding environmental sampling. The feeding regimen of the ewes consisted of grass silage that was manufactured on farm, a mineral rumen supplement aimed at improving rumen function and a mix consisting of various grains (e.g., corn, sorghum) that was fed during practice milking (in order to acclimate the ewes to the milking routine) and later on during milking. Composite samples collected from Farm 1 included sets of "old" and "new" samples for fence swabs, bedding materials, faeces, trough water and silage as well as a unique set of milking cups swabs samples. Raw milk samples could not be obtained for Farm 1.

3.1.1.2 Farm 2 – Pasture-based farming

Samples from Farm 2 were collected in September 2018. Farm 2 housed about 800 dairy ewes and was located in the North Island of New Zealand. Farm 2 was operating under a pasture-based system which meant that the ewes were grazing on pasture throughout the year. At the time of sampling, the 800 dairy ewes were divided in two flocks that were grazing on different paddocks. Composite environmental samples were collected for each flock on the paddock it had been grazing for the day and pooled together. Milking cups and raw milk samples were collected 6 weeks after the environmental sampling took place to align with the availability of farmers and ensure consistency with the sampling on Farm 1. According to historical data from the New Zealand National Climate Database (CliFlo, https://cliflo.niwa.co.nz/), the area experienced about 110 mm of rain and temperatures that ranged from 0.4 to 22°C during the 30 days preceding environmental sampling. The feeding regimen consisted of ryegrass and clover grazing as well as a mix of various grains that was fed during milking. Samples collected from Farm 2 included pasture (perennial ryegrass), soil, faeces, trough water, milking cups swabs and raw milk.

3.1.1.3 Farm 3 – Hybrid farming

Samples from Farm 3 were collected in September 2018. Farm 3 housed about 900 dairy ewes and was located in the North Island of New Zealand. Farm 3 operated under a hybrid system; ewes were housed in pens covered by wall-less barns and alternated periods housed or grazing depending on the time of the year (approximate time spent housed throughout the year: 45%). Three types of bedding materials were being trialled at the time: woodchips, miscanthus (also called elephant grass, a straw-like material) and a mixture of woodchips and miscanthus. Feeding regimen consisted of perennial ryegrass and clover grazing, grass silage and a mix of grains fed during milking. Raw milk was collected from Farm 3.

3.1.1.4 Farm 4 – Hybrid farming

Samples from Farm 4 were collected in September 2018. Farm 4 housed about 2,000 dairy ewes and was located in the North Island of New Zealand. Farm 4 operated under a hybrid system; ewes were housed in pens covered by wall-less barns and alternated periods indoors or grazing depending on the time of the year (approximate time spent housed throughout the year: 10%). Bedding materials consisted of woodchips and feeding regimen included perennial ryegrass, clover and lucerne grazing as well as silage and a mix of grains offered to the animals during milking. Raw milk was collected from Farm 4.

3.1.1.5 Farm 5 – Pasture-based farming

Samples from Farm 5 were collected in September 2018. Farm 5 housed about 3,000 dairy ewes and was located in the North Island of New Zealand. Farm 5 was operating under a pasture-based system which meant that the ewes were grazing on pasture throughout the year. Feeding regimen consisted of perennial ryegrass, clover and lucerne grazing as well as a mix of grains offered to the animals during milking. Samples collected from Farm 5 included raw milk and milking cups swabs.

3.1.1.6 Sheep dairy products

Sheep dairy products (n = 2) consisted of two types of sheep milk powder-based goods. They were collected from the manufacturer, kept sealed and away from light, and processed after their expiration date. The analysis of food samples was performed post-expiry in adherence to the manufacturer's

guidelines; dairy product A (DPA) was analysed five months after expiry and dairy product B (DPB) was analysed 11 months after expiry.

3.1.2 Sample collection

3.1.2.1 Farm 1 sampling

Two sets of environmental samples were collected from Farm 1. The first set was collected from a pen with 3-month-old woodchip bedding and are referred as the "old" samples (n = 34). The second set of samples was collected from an adjacent pen with 3-week-old woodchip bedding and are referred as the "new" samples (n = 42). To obtain samples representative of the pens, bedding materials [bedding old (BO), n = 8 and bedding new, n = 12 (BN)] and fresh faeces [faeces old (FO), n = 8 and faeces new (FN), n = 12]) were collected from each corner and central parts of each smaller pen. There were more samples of bedding and faeces collected for the "new" set as the pen surface available for the ewes was larger than for the "old" set. Silage samples [silage old (SO), n = 10 and silage new (SN), n = 10] were collected from the sides of the pen, during feeding of the animals. Bedding materials and silage samples were collected in Whirl-Pak sampling bags (Whirl-Pak) which were filled ¾, corresponding to about 50 g of silage and 100 g of bedding material per sample replicate. Fresh faeces were collected in half-filled stool containers, corresponding to about 20 g of faeces per sample replicate. Trough water samples [water old (WO), n = 4 and water new (WN), n = 4] were collected by taking 500 ml from four water troughs in each pen and pooling them in 1 L Schott Duran glass bottles. Fence swabs [infrastructure old (IO), n = 4 and infrastructure new (IN), n = 4] were collected with Ez-Reach sponges (World Bioproducts) pre-moistened with 20 ml of Butterfield's diluent (85 mg/L KH_2PO_4 in distilled H_2O) by thoroughly swabbing the inner side of one section of the fence, after feeding of the animal, at a fence location that had been visibly in contact with animals (i.e., fleece or skin contact, bites). Milking cup swabs (n = 10) were collected post-milking before any wash of the clusters by randomly selecting and thoroughly swabbing the inside liners of 10 milking cups using Whirl-Pak Speci-Sponges (Whirl-pak) pre-moistened with 15 ml of Butterfield's diluent. Samples were kept chilled during transportation and, upon arrival at the laboratory, frozen at -20°C until processed.

3.1.2.2 Farm 2 sampling

Environmental samples (n = 40) were collected from two paddocks where the animals were grazing. They consisted of grass (n = 12), soil (n = 12), faeces (n = 12) and trough water (n = 4). To obtain samples representative of the paddocks; faeces, soil and grass were collected from each corner and central parts of the paddocks. Soil (10-20 cm depth) and top grass samples were collected in Whirl-Pak sampling bags (Whirl-Pak) which were filled ¾, corresponding to about 50 g of grass and 100 g of soil per replicate. Fresh faeces were collected in half-filled stool sampling cups, corresponding to about 20 g of faeces per sample replicate. Trough water samples were collected by taking 500 ml from each of the two water troughs present in each paddock and pooling them in 1 L Schott Duran glass bottles. Milking cup swabs (n = 10) were collected post-milking before any wash of the clusters by randomly selecting and thoroughly swabbing the entire internal surface area of 10 milking cups using Whirl-Pak Speci-Sponges (Whirl-pak) pre-moistened with 15 ml of Butterfield's diluent. Raw milk (1 L) was collected aseptically from the vat after milking. Samples were kept chilled during transportation and, upon arrival at the laboratory, frozen at -20°C until processed.

3.1.2.3 Sampling of Farms 3, 4 and 5

In Farms 3, 4 and 5, raw milk (1 L) was collected aseptically from the vat after milking. Milking cups swabs (n = 10) were collected from Farm 5 post-milking before any wash of the clusters by randomly selecting and thoroughly swabbing the entire internal surface area of 10 milking cups using Whirl-Pak Speci-Sponge (Whirl-Pak) pre-moistened with 15 ml of Butterfield's diluent. Samples were kept chilled during transportation and, upon arrival at the laboratory, frozen at -20°C until processing.

3.1.3 Sample processing

Samples processed by this methodology included all environmental samples (pasture, soil, silage, faeces, fence swabs, trough water, bedding materials), milking cups and raw milk as well as dairy products. Table 3.2 recapitulates the specific conditions of incubation used for the isolation of bacteria across the different samples.

Samples were processed using a methodology adapted from Gupta and Brightwell (2017). Frozen samples were thawed overnight at 4°C. Biological replicates were pooled per farm and per sample type and thoroughly mixed to make a composite sample before aliquoting the required weight or volume for analysis.

Approximately twenty grams of solid samples (faeces, bedding materials, silage, soil, grass) were weighted, transferred to filtered Bagpage stomacher bags (Interscience) and mixed with 100ml of Butterfield's diluent. The mixture was homogenised using a Stomacher 400 (Seward) at high speed (or medium when sharp objects were present) for 120s. For each trough water samples, the total volume (2 L) was pooled, thoroughly mixed and 500ml was aliquoted in 50ml Falcon tubes. For milking cups swabs and fence swabs, the volume of Butterfield's diluent contained in the sponges was adjusted to 20ml and added to Falcon tubes. Swabs were further flushed with 10 ml of Butterfield's diluent to optimise bacteria recovery. For raw milk samples, 100 ml was aliquoted in Falcon tubes. For dairy products, 1 L of Butterfield's diluent was added to 100g of product, solubilised by vortexing and aliquoted in Falcon tubes.

These suspensions were centrifuged at 4°C for 1 hour at a speed of 3466 × g. Pellets were resuspended in volumes of Butterfield's diluent depending on sample texture and ranged from 5 to 25 ml. The resulting suspensions were serially diluted 10-fold, plated in duplicates on Cetrimide-Fucidin-Cephalosporin agar (CFC, Oxoid) and incubated aerobically for 48 h at 25°C to isolate and enumerate *Pseudomonas* species, according to the manufacturer's recommendations. Aerobic plate count at 35°C (APC_{35}) was performed for raw milk samples by serially diluting and plating the suspension on Columbia sheep blood agar (SBA, Fort Richard Laboratories) and incubating the plates under atmospheric conditions at 35°C for 24 h. APC_{35} and APC at 65°C (APC_{65}) were performed for dairy products by serially diluting and plating the suspension on SBA followed by incubation under atmospheric conditions at their respective temperature for 24 and 48 h, respectively.

The remaining volumes of bacterial suspensions were individually transferred to sterile Falcon tubes and heated at 80°C (± 1°C) for 10 min in a water bath to kill vegetative cells and harvest spores. After cooling at room temperature, spore suspensions were serially diluted in Butterfield's diluent and plated in duplicates on SBA, or milk agar IDF (International Dairy Foundation standards, MA, Fort Richard Laboratories). Following heat treatment, samples were plated and incubated aerobically as follow: SBA at 35°C for 24-48h, SBA and MA at 65°C, as well as 60°C for the milking cups swab samples for 24-48h and SBA at 7°C for 10 days to select mesophilic spore-formers, thermophilic spore-formers and psychrotrophic spore-formers, respectively. Obligate anaerobic spore-formers were isolated by using a modified enrichment technique described by Gupta and Brightwell (2017). One ml of heat-treated sample suspension was added to cooked meat glucose broth (CMG, Fort Richard Laboratories) supplemented with hemin (0.1%), vitamin K1 (1%) and yeast extract (0.0005%). This suspension was incubated at 35°C for 48 h in an anaerobic chamber (Don Whitley Scientific) with an atmosphere of 5% CO_2, 10% H_2 and 85% N_2 for 48 h. Enriched samples were centrifuged at 4°C for 1 h at 3,466 × g, pellets were resuspended in 5 ml of Butterfield's diluent and

serially diluted before plating on egg yolk agar made of Shahidi-Ferguson-Perfringens (SFP) agar base [Becton-Dickinson (Shahidi and Ferguson, 1971)] supplemented with 5% egg yolk (Fort Richard Laboratories,) and 30,000 units of Polymyxin B (Fort Richard Laboratories) per litre of media. Plates were left to dry, layered with SFP agar, and incubated at 35°C for 24 h under anaerobic conditions.

Following growth, all individual colonies from a selected plate obtained from each spore isolation method (20 to 100 colonies per plate and with the greater colony diversity) were propagated on SBA under their respective growth conditions until pure cultures were obtained. For fence swabs only, isolates with different plate morphologies were selected and propagated for each incubation condition. Spore-forming psychrotrophs were propagated at 20°C to shorten growth time. Pure cultures were preserved at -80°C in a cryobank by inoculating exponentially growing cells in a mix of 25% glycerol (Sigma-Aldrich) and tryptic soy broth (for aerobic isolates, Becton-Dickinson) or fluid thioglycolate (for anaerobic isolates, Fort Richards Laboratories). Due to the poor viability of some thermophilic cells stored at -80°C in glycerol, pure cultures of thermophilic isolates were also preserved in MAST CRYOBANK tubes (Mast Group) or in dried autoclaved surface soil collected in the vicinity of the Hopkirk Research Institute (Massey University, Palmerston North, New Zealand) and stored at -80°C.

Table 3.2. Culture conditions used for bacterial isolation

Target bacteria	Pre-treatment	Incubation conditions	F1	F2	F3	F4	F5	DA	DB
Mesophilic spores	80 ± 1°C 10 min	35°C SBA Atmospheric conditions 24-48 h	✕	✕	✕	✕	✕	✕	✕
Thermophilic spores		60 or 65°C SBA and MA Atmospheric conditions 48 h	✕	✕	✕	✕	✕	✕	✕
Psychrotrophic spores		7°C SBA and MA Atmospheric conditions 10 days	✕	✕	✕	✕	✕		✕
Clostridia		CMG 35°C 48h AnO_2 EYA 35°C 24 h AnO_2	✕	✕	✕	✕	✕	✕	✕
Mesophiles	None	35°C SBA Atmospheric conditions 24 h		✕‡	✕‡	✕‡	✕‡	✕	✕
Thermophiles		65°C SBA Atmospheric conditions 48h						✕	✕
Psychrotrophs		7°C SBA Atmospheric conditions 10 days	✕*						
Pseudomonas spp.		20°C CFC Atmospheric conditions 48h	✕	✕	✕	✕	✕		

F1-F5: Farm 1 to 5; DA: Dairy product A; DB: Dairy product B; SBA: Columbia sheep blood agar; MA: Milk agar; CMG: Cooked meat glucose broth; EYA: Egg yolk agar; CFC: Cetrimide Fucidin Cephalosporin agar; AnO_2. Crosses indicate that a given culture condition was employed across samples collected from a given location.

‡Incubation condition used for raw milk samples only; *Incubation condition used for fence swabs only

3.1.4 Enterobacterial Repetitive Intergenic Consensus PCR (ERIC-PCR) genotyping

ERIC-PCR was performed using the ERIC2 primer as described by Versalovic et al. (1991) and the protocol defined by Weijtens et al. (1999) with minor modifications. Each 25 µl of reaction mixture contained AmpliTaq Gold 360 Master Mix (1X, Thermo Fisher Scientific), bovine serum albumin (BSA, 0.02% w/v, Thermo Fisher Scientific) and the ERIC2 primer (1µM) with sequence 5′- AAG TAA GTG ACT GGG GTG AGC-3′ (Invitrogen, Thermo Fisher Scientific) in PCR grade H_2O. Template DNA consisted of a small amount of fresh bacterial colony added in the reaction mixture or of a 5 µl boil lysate preparation obtained by boiling exponentially growing cells for 10 min in phosphate buffer followed by rapid cooling on ice for at least 10min. The PCR was carried out in a Mastercycler® pro S (Eppendorf) with the following program: an initial step of denaturation for 5 min at 94°C followed by 40 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 25°C and elongation for 4 min at 72°C followed by a final elongation step of 10 min at 72°C after which the reactions mixtures were kept at 4°C. Amplified products were separated on a 1.5% UltraPure agarose (Invitrogen) gel, stained with ethidium bromide (0.5 µg/ml, Bio-Rad). Gels were visualised using the Image Lab software version 3.0 (Bio-Rad) and a Gel Doc XR+ (Bio-Rad). Bacterial isolates were assigned to ERIC groups based on their fingerprinting pattern and isolates with a unique intrasample ERIC pattern were further characterised. Whenever no ERIC fingerprinting could be obtained after multiple attempts (degraded DNA, no amplification), the isolates were included in the selection for further characterisation.

3.1.5 16S rRNA gene amplification and sequencing

The 16S rRNA gene sequence of representative isolates was amplified based on the methodology described by Boddinghaus et al. (1990) with minor modifications. Each 50µl of reaction mixture contained AmpliTaq Gold 360 Master Mix (1X), BSA (0.02% w/v), the primers pA with sequence 5′-AGA GTT TGA TCC TGG CTC AG-3′ and pH with sequence 5′- AAG GAG GTG ATC CAG CCG CA-3′, [1µM each, Invitrogen (Lane, 1991)] in PCR grade H_2O. A small amount of fresh bacterial colony was added as template DNA, directly in the reaction mixture, alternatively, genomic DNA was extracted using the high pure PCR template preparation kit according to the manufacturer recommendations (Roche Diagnostics). The PCR was carried out in a Mastercycler pro S (Eppendorf) running the following program. An initial denaturation step at 93°C for 3 min followed by 35 cycles

of denaturation-annealing-elongation at 92°C for 1 min, 55°C for 1 min and 72°C for 2 min, respectively, followed by a final elongation step at 72°C for 3 min and further held at 4°C. PCR products, approximately 1.5 kbp, were loaded on a 0.8% UltraPure agarose (Invitrogen) gel, stained with ethidium bromide (0.5 µg/ml, Bio-Rad). Gels were visualised using the Image Lab software version 3.0 (Bio-Rad) and a Gel Doc XR+ (Bio-Rad). PCR products were purified using the QIAquick PCR Purification Kit as per the manufacturer instructions (QIAGEN). The DNA concentration of purified PCR products was measured using a NanoDrop 1000 (Thermo Fisher Scientific) before adjusting the DNA concentration for one-way Sanger sequencing using the primers pA or pH and performed by Macrogen Inc. (South Korea, Seoul). Sequences were trimmed and arranged using Geneious Prime 2019.1.1 (https://www.geneious.com) and compared to type strains 16S rRNA gene sequences of cultured isolates from the Ribosomal Database Project [RDP, release 11, update 5, taxonomy 18, accessed 22/10/2020 (Cole et al., 2014)]. The RDP repository contains 16S rRNA sequences available from the International Nucleotide Sequence Database Collaboration [INSDC (Nakamura et al., 2013)]. Names of the organisms associated with the sequences were obtained as the most recently published synonym from the List of Prokaryotic names with Standing in the Nomenclature (LPSN, https://lpsn.dsmz.de/). As 16S rRNA gene fragments sizing 800-1200 bp were sequenced, isolates were assigned to the most closely related type strain species if their partial 16S rRNA sequence matched the type strain with a seqscore > 0.970. Isolates with lower similarity scores were assigned to the closest genus, with the name of the next most closely related species also mentioned [e.g., *Paenibacillus* sp. (tundrae)].

3.1.6 Qualitative spoilage assessment of the isolates

Isolates that were selected based on morphotypes or ERIC types were screened for proteolytic and lipolytic activities using plate-based assays. To assess protease activity, bacteria were plated on reinforced milk agar plates (peptone 5 g/L, yeast extract 6 g/L, NaCl 5 g/L, agar 15 g/L, skim milk powder 30 g/L) and incubated at their respective growth temperature, for 48-96 h. Protease activity was recorded positive when a clear zone was observed, caused by the hydrolysis of casein around the bacterial colony, and was assigned an intensity ranging from "+" (weak, faint casein hydrolysis), "++" (medium, casein hydrolysis only under the colony), and "+++" (strong, diffused casein hydrolysis). Lipase activity was assessed following the methodology described by Kouker and Jaeger (1987) with modifications. The base media tryptone glucose yeast agar (v = 745 ml), contained tryptone (6.7 g/L), yeast extract (6.7 g/L), K_2HPO_4 (1.3 g/L) and agar (13.4 g/L) and was sterilised by autoclaving. Glucose (1 g in 25 ml of mqH_2O), rhodamine B (2 ml of a 0.1% solution in mqH_2O;

Sigma Aldrich) and low acidity olive oil (33 ml, Sigma Aldrich) were added to the warm media after filter sterilization. Gum Arabic (10 g in 200 ml mqH_2O, Sigma Aldrich) was added after pressure-cooker sterilization at 15 psi for 10 min. The mixture was shaken vigorously by hand to solubilise olive oil in the aqueous phase prior to pouring the plates. Following growth of the isolates for 48 to 96 h, lipolytic activity was visualised under ultraviolet (UV) light. Negative control (water) and positive controls consisting of 20 µl of pure lipase from *Candida rugosa* (≥ 700 units/mg, Sigma Aldrich) diluted to 0.5, 0.25 and 0.125 mg/ml were used to assess the lipolytic activity of the isolates. To assess the natural fluorescence of some *Pseudomonas* which may yield false positives, they were visualised under UV light after incubation on both rhodamine B agar (test plate) and reinforced milk agar (negative control plate). *Pseudomonas* isolates with comparable fluorescence on both agar types were considered negative for lipase production.

3.1.7 Statistical analysis

For the study of bacterial diversity described in Chapters 4 & 5, analysis was carried out in the R environment (R Core Team, 2022) using the packages vegan for statistical analysis (Oksanen et al., 2020), and ggplot2 for data visualisation (Wickham, 2016). Shannon, Simpson and inverse Simpson diversity indexes were calculated using in-built functions of the vegan package by taking into consideration the bacterial species present at relative abundance > 1 % in a given sample. Bacteria which were present at abundance < 1 % in a given sample had been arbitrarily isolated on a morphological basis from lower dilution plates and therefore were not included in the analysis. For analysis of bacterial diversity between the samples, Bray-Curtis distances (a statistical measure used to assess the similarity between two datasets) and nonmetric multidimensional scaling (NMDS) were applied using the vegan package. The relative abundance of the different bacterial species was used to create similarity ranks (resemblance matrix) using the Bray-Curtis similarity. The matrix was then used for NMDS ordination.

3.1.8 *Clostridium perfringens* toxinotyping

Toxinotyping of the *Clostridium perfringens* strains isolated from Farm 1 was carried out using the Multiscreen enzyme-linked immunosorbent assay (ELISA) kit for detection of *C. perfringens* and α-toxin, β-toxin and ε-toxin (Bio-X Diagnostics) as per the manufacturer recommendations. To obtain a fresh suspension containing both the toxins and the bacteria, *C. perfringens* isolates (n = 31) were

cultured in TSB for 6 hours under anaerobic conditions. The ELISA test consisted of incubating wells containing immobilised monoclonal and/or polyclonal antibodies against α-toxin, β-toxin and ε-toxin of *C. perfringens*, as well as against a structural protein of this bacterium, with a fresh liquid culture of *C. perfringens*. Following incubation and wash steps, a peroxidase-labelled conjugate was added and bound to the immobilised antigen, if present. After further wash, the chromogen (tetramethylbenzidine) was added and in the presence of the conjugate, catalyses the transformation of tetramethylbenzidine, to tetramethylbenzidine diamine which can be visualised by photometry after the reaction has been stopped with phosphoric acid. For each test well, a corresponding negative control containing non-specific immobilised antibodies was used. Positive controls consisting of pure solutions of the target antigens (i.e., toxins or *C. perfringens* surface protein) were simultaneously assessed during each ELISA. To determine the positivity of the test, the optical density of the negative control was subtracted to the optical density of the sample and expressed as a percentage of the positive control. Threshold values were provided by the manufacturer to assess the results obtained for each sample.

3.2 Source attribution

3.2.1 ERIC-PCR genotyping

ERIC-PCR was performed using the ERIC2 primer as described by Versalovic et al. (1991) and the protocol defined by Weijtens et al. (1999) with minor modifications. Each 25µl of reaction mixture contained AmpliTaq Gold 360 Master Mix (1X, Thermo Fisher Scientific), Bovine Serum Albumin (BSA, 0.02% w/v, Thermo Fisher Scientific) and the ERIC2 primer (1 µM) with sequence 5′- AAG TAA GTG ACT GGG GTG AGC-3′ (Invitrogen) in PCR grade H_2O. Template DNA consisted of a cell lysate preparation obtained by boiling exponentially growing cells for 10 min in 1X Chelex resin (Bio-Rad) in PCR grade H_2O followed by rapid cooling on ice for at least 10min. The PCR was carried out in a Mastercycler® pro S (Eppendorf) with the following program: An initial step of denaturation for 5 min at 94°C followed by 40 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 25°C and elongation for 4 min at 72°C followed by a final elongation step of 10 min at 72°C after which the reactions mixtures were kept at 4°C. Amplified products were separated on a 1.5% UltraPure agarose (Invitrogen) gel, stained with ethidium bromide (0.5 µg/ml, Bio-Rad). Gels were visualised using the Image Lab software version 3.0 (Bio-Rad) and a Gel Doc XR+ (Bio-Rad). In each gel run, several wells were loaded with the 1 Kb+ DNA ladder to allow for normalisation and

comparison of the fingerprints across different gels. ERIC fingerprinting experiments were repeated at least twice to assess reproducibility of the fingerprints. For each isolate, DNA fragments that were not consistently showing on the agarose gel were excluded from the analysis.

3.2.2 Amplified rDNA (ribosomal DNA) Restriction Analysis (ARDRA) genotyping

ARDRA was performed by amplifying part of the 16S rRNA gene, the 16S-23S internal transcribed spacer and part of the 23S rRNA gene, resulting in a DNA fragment of about 4.5 kbp which was then digested by restrictions enzymes. The methodology of Pourshafie et al. (2005) was used with minor modifications. Each 50 µl of reaction mixture contained AmpliTaq Gold 360 Master Mix, BSA (0.02% w/v), and the primers with sequence 5'-AGAGTTTGATCMTGGCTCAG-3' (forward primer, 5'end of the 16S rRNA gene) and $ARDRA_R$ 5'-CGACATCGAGGTGCCAAA-3' (reverse primer, 5'end of the 23S rRNA gene), [1 µM each, Invitrogen (Lane, 1991)] in PCR grade H_2O. DNA template consisted of 5 µl of a boil lysate preparation or of genomic DNA obtained by extraction using the high pure PCR template preparation kit according to the manufacturer recommendations (Roche Diagnostics). The PCR was carried out in a Mastercycler pro S (Eppendorf) running the following program. An initial denaturation step at 94°C for 5 min followed by 35 cycles of denaturation-annealing-elongation at 94°C for 45 s, 60°C for 45 s and 72°C for 5 min, respectively, followed by a final elongation step at 72°C for 7 min and further held at 4°C. PCR products, approximately 4.5 kbp, were loaded on a 0.8% UltraPure agarose (Invitrogen) gel, stained with ethidium bromide (0.5 µg/ml, Bio-Rad). Gels were visualised using the Image Lab software version 3.0 and a Gel Doc XR+ (Bio-Rad). PCR products were purified using the QIAquick PCR Purification Kit as per the manufacturer instructions (QIAGEN). DNA fragments were digested overnight at 35°C in the following mixture (v = 40 µl): 4 µl of enzyme Tango buffer (10X, Thermo Fisher Scientific), 5µl of PCR grade H_2O, 1µl of restriction enzyme (10 units/µl) and 30µl of purified PCR product. The restrictions enzymes used were *HhaI* (Thermo Fisher Scientific) and *AluI* (Thermo Fisher Scientific,), and their restrictions sites are mentioned in Table 3.3. Digested DNA fragments were visualised on a 3% UltraPure agarose gel (Invitrogen) stained with ethidium bromide (0.5 µg/ml, Bio-Rad) and visualised as mentioned above. ARDRA fingerprinting experiments were repeated at least twice to assess reproducibility.

Table 3.3. Restriction enzymes used for ARDRA genotyping

Restriction enzyme	Restriction site[1]	Enzyme buffer
HhaI	5' G C G↓ C 3' 3' C↑ G C G 5'	Buffer Tango
AluI	5' A G↓ C T 3' 3' T C↑ G A 5'	

[1] Restriction sites are represented by arrows

3.2.3 Fingerprinting analysis

Analysis of fingerprinting data from ERIC or ARDRA genotyping was performed using the software PyElph with default settings (Pavel and Vasile, 2012). Briefly, gel images were cropped, adjusted and DNA migration lanes were detected, DNA fragments were annotated and matched with corresponding fragments of same molecular weight present on other lanes to produce DNA fragments similarity matrices. These matrices were used to compute the similarity matrix using Dice coefficient (Dice, 1945) which expresses the similarity level between two DNA patterns. Phylogenetic trees were built using the similarity matrix and the unweighted pair group method with arithmetic mean (UPGMA) sorting algorithm included in the built-in software phylogenetic tree generation tool of PyElph. The similarity matrices were extracted and the highest similarity value per sample was used to build radar charts and the corresponding heatmaps in the R environment using the ggplot2 package (Wickham, 2016).

3.2.4 Genomic DNA extraction for whole-genome sequencing

Whole-genome sequencing was performed on *Thermoactinomyces* spp. isolated from Farm 1 and Farm 2. Genomic DNA was prepared using the phenol-chloroform extraction method. Briefly,

Thermoactinomyces spp. isolates were grown in tryptic soy broth with shaking at 60°C for 16 to 40h. Cells were harvested by centrifugation in a tabletop centrifuge (13,000 rpm for 10 min) and frozen at -80°C. Prior to extraction, cells were rapidly thawed at 35°C and frozen at -80°C for two to four cycles in order to damage the cells and facilitate membrane permeabilization. Subsequently, cells were washed three times with TE buffer (Thermo Fisher Scientific), resuspended in 200 µl of genomic buffer (0.3423 g/ml of sucrose in TE buffer) and 50 µl of lysozyme (50 mg/ml; Sigma Aldrich) and incubated at 35°C for 90 min. Following incubation, 15 µl of RNase A (10 mg/ml; Thermo Fisher Scientific) and 100 µl of 20% N-lauroylsarcosine sodium salt in PCR grade H_2O were added, further incubated at 37°C for 30 min and 4 µl of Proteinase K (20 mg/ml, Ambion) was added before incubating the mixture again at 37°C for 30 min. The volume of the solution was adjusted to 600 µl with TE buffer and genomic DNA was extracted with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1, Sigma-Aldrich) three times. Following the last extraction step, 50 µl of 3M sodium acetate and three volumes of ice-cold absolute ethanol were added and the solution was incubated at -80°C for 1-16 hours. Precipitated DNA was washed with ice-cold 70% ethanol and air dried for at least 30 min. Finally, DNA was eluted in PCR grade H_2O overnight at 4°C. Quality checks were performed by spectrophotometry with a NanoDrop 1000 (Thermo Fisher Scientific), by fluorometry using a Qubit 2.0 fluorometer (Thermo Fisher Scientific) and checked by electrophoresis on a 0.8% UltraPure agarose (Invitrogen) gel, stained with ethidium bromide (0.5 µg/ml, Bio-Rad). Gels were visualised using the Image Lab software version 3.0 and a Gel Doc XR+ (Bio-Rad).

3.2.5 Genome sequencing & assembly

Genomic DNA extracted from the selected bacteria was sequenced using Illumina MiSeq version 3 sequencing platform at Massey Genome Services, New Zealand. DNA library preparation was carried out with the Celero PCR workflow with enzymatic fragmentation DNA-seq library preparation kit (NuGEN). The quality and quantification assessments of DNA libraries were carried out as a quality control check before sequencing. The resulting reads were quality trimmed, filtered, and *de novo* assembled with the A5-miseq assembler version 20160825 under the default settings (Coil et al., 2015). The genome completeness was assessed using BUSCO version 1.22 (Simão et al., 2015).

The genomes were assembled using unicycler version 0.4.8 (Wick et al., 2017) and protein coding genes were annotated using prodigal version 2.6.3 (Hyatt et al., 2010). Following this, repeat regions and non-coding RNAs were annotated using prokka version 1.14.6 (Seemann, 2014). The proteins were then aligned to the NCBI non-redundant database (downloaded 27/01/2022) using the "blastp"

function of diamond version 2.0.6 (Buchfink et al., 2015) and further annotated using InterProScan version 5.55-88.0 (Jones et al., 2014). The diamond alignments were imported into OmicsBox version 1.4 (Gotz et al., 2008) where they were annotated and assigned GO terms, which were then merged with the InterProScan results. Within OmicsBox, the annotations were further added to using eggNOG-Mapper 2.1.0 with EggNOG 5.0.2 (Huerta-Cepas et al., 2019). All programs were run using the default parameters except for the diamond aligner where XML output was specified to make it compatible with OmicsBox.

3.2.6 16S rRNA- and genome-based phylogeny of *Thermoactinomyces* spp. isolates

The genome sequence data were uploaded to the Type (Strain) Genome Server (TYGS, https://tygs.dsmz.de), for a whole genome-based taxonomic analysis (Meier-Kolthoff and Göker, 2019, Meier-Kolthoff et al., 2022). Information on nomenclature, synonymy and associated taxonomic literature was provided by TYGS's sister database, the List of Prokaryotic names with Standing in Nomenclature [LPSN, available at https://lpsn.dsmz.de (Meier-Kolthoff et al., 2022)]. The TYGS analysis was subdivided into the following steps.

3.2.6.1 16S rRNA gene-based phylogeny

The 16S rRNA gene sequences were extracted from the genomes using RNAmmer (Lagesen et al., 2007) and aligned as part of the TYGS analysis pipeline.

3.2.6.2 Pairwise comparison of genome sequences

For the phylogenomic inference, all pairwise comparisons among the set of genomes were conducted using the Genome BLAST Distance Phylogeny approach (GBDP) and accurate intergenomic distances inferred under the algorithm 'trimming' and distance formula d5 (Meier-Kolthoff et al., 2013), 100 distance replicates were calculated each. Digital DNA-DNA hybridization (dDDH) values and confidence intervals were calculated using the recommended settings of the Genome-To-Genome Distance Calculator [GGDC 3.0 (Meier-Kolthoff et al., 2013, Meier-Kolthoff et al., 2022)].

3.2.6.3 Phylogenetic inference

The resulting intergenomic distances were used to infer a balanced minimum evolution tree with branch support via FASTME 2.1.6.1 including superconvergent patch recovery (SPR) postprocessing (Lefort et al., 2015). Branch support was inferred from 100 pseudobootstrap replicates each. The trees were rooted at the midpoint (Farris, 1972) and visualised with PhyD3 (Kreft et al., 2017).

3.2.7 Multilocus sequence analysis typing (MLST)- and multiprotein sequence analysis typing (MPST)-based phylogeny of *Thermoactinomyces* spp. isolates

3.2.7.1 Ribosomal multilocus sequence analysis typing (rMLST)

The rMLST analysis was performed using the Bacterial Isolate Genome Sequence Database (BIGSdb) software hosted on the pubMLST website [https://pubmlst.org (Jolley and Maiden, 2010, Jolley et al., 2018)]. After the genomes of interest were uploaded to a scratch database, the genome comparator tool was used to run an rMLST analysis consisting of a BLAST search for the set of ribosomal proteins making up the 30S (loci BACT000001 to BACT000021, *rps* gene family) and 50S ribosomal subunits (loci BACT000030 to BACT000065, *rpl* and *rpm* genes families) followed by their concatenation and alignment. The construction of phylogenetic trees was done using the software Geneious version 2019.1 (Biomatters. https://www.geneious.com) with the Tamura-Nei genetic distance model, neighbour-joining clustering method and the similarity matrices generated after alignment of the concatenated gene sequences for each isolate.

3.2.7.2 Multiprotein sequence analysis typing (MPST)

For the MPST analysis, a set of gene sequences coding for putative proteins (proteases and lipases) were selected from the InterProScan analysis, and their amino acids sequences were imported in Geneious version 2019.1 (Biomatters. https://www.geneious.com) where they were concatenated and aligned. Phylogenetic trees were created using the Jukes-Cantor genetic distance model, neighbour-joining clustering method and the similarity matrices generated after alignment of the concatenated protein sequences in each isolate.

Chapter 4 - Ecological survey of the dairy spoilage microbiota of a barn-based sheep dairy farm and potential sources of milk contamination in the housing environment

4.1 Introduction

This research chapter presents an ecological investigation into the microbial communities found in the barn environment of a sheep dairy farm where animals are housed, with a specific focus on dairy spoilage bacteria. The investigation begins with the isolation of bacteria from environmental samples associated with the barn and milking environment of a sheep dairy farm. A culturomics approach is employed, utilising five selective culture conditions to isolate and enumerate bacteria according to their growth conditions. Bacterial isolates are subsequently screened for their ability to produce exolipases and exoproteases, aiming to further investigate bacteria with potential dairy spoilage capabilities. Bacteria exhibiting spoilage potential then undergo putative identification through a polyphasic approach, which includes DNA fingerprinting and partial 16S rRNA gene sequencing. This approach yields a detailed ecological overview of the culturable bacteria found in the barn environment of a sheep dairy farm. The primary aim of this research is to evaluate the prevalence of spoilage bacteria on sheep dairy farms and ascertain the species present in the barn environment that may be passed on to raw milk, and potentially impact its quality. Additionally, this article contributes empirical evidence to enhance our comprehension of microbial communities on sheep dairy farms and their spatial distribution. The outcomes of this study are instrumental in pinpointing microbial hotspots and potential sources of raw milk contamination on sheep dairy farms.

Alongside post-processing contamination (Alles et al., 2018) and in-product or in-plant bacterial growth (Scott et al., 2007), raw milk is one of the principal sources of dairy products contamination (Elmoslemany et al., 2010). Spoilage bacteria are ubiquitous in the farm environment and may contaminate raw milk upon milking. On bovine dairy farms, these bacteria have been isolated from feed (te Giffel et al., 2002, Scheldeman et al., 2005), soil (Garbeva et al., 2003, Vissers et al., 2007b), water (Brillard et al., 2015), faeces and farm slurry (Coblentz et al., 2014, Gupta and Brightwell, 2017), bedding materials (Murphy et al., 2019) as well as the milking equipment (Weber et al., 2019). As a result, the factors associated with bacterial contamination of bovine raw milk are diverse and include bedding and housing facilities (Murphy et al., 2019), feeding regime (Vissers et al., 2007c), cow cleanliness, as well as milking hygiene and the milking equipment (Masiello et al., 2017, Martin et al., 2019). However, the ecology of spoilage bacteria in sheep dairy farm environments has not been described and the factors and sources implicated in sheep milk contamination remain unclear.

The diversity of raw milk contaminants can differ across geographical locations as a result of variations in the composition of the native microbiota present in the farm environment but also due to the use of different farm practices across locations (Skeie et al., 2019). On sheep dairy farms, farming practices can differ between locations as a result of local farm factors such as climate, livestock breed, cultural background and financial or technological capabilities (Pulina et al., 2018). These farm practices define the living conditions of dairy livestock (e.g., indoors or outdoors), their diet (e.g., grazing or processed feed) and the conditions of milking (e.g., manual vs automatic milking) which have implications on the cycling of bacteria on-farm as well as on the bacterial species contaminating raw milk. For example, Wu et al. (2007) demonstrated that cows transiting from grazing to feedlotting led to an increase of their faecal mesophilic spore counts while also altering the predominant species of spore-formers present in faeces. Coorevits et al. (2008) highlighted key differences in the spore-forming microbiota of bovine raw milk produced on organic (fully outdoor, pasture grazing) and conventional (partially housed, total mixed ration as feed) dairy farms. Organic and conventional dairy farms are farming systems that refer to a broad set of farm practices associated with extensive and intensive farming, respectively. Across these farming systems, different components, albeit with similar function (e.g., soil/pasture versus bedding, pasture versus silage) are implicated in the cycling of bacteria and the contamination of raw milk, which is reflected by variations in the microbiota of raw milk (Coorevits et al., 2008). Housing of dairy livestock has become more common, particularly goats and sheep which may require protection from weather events and during pregnancy, but also to aid management in bovine dairy farms. The New Zealand sheep dairy industry has been trialling two farming systems: hybrid farming (housing and silage

feeding around winter or grazing for the remainder of the year) and pasture-based farming (year-round grazing). However, the impact of these farm practices on the microbiology of the farm environment and on the diversity of the contaminants entering ewe raw milk are unknown. Worldwide, there is a lack of data pertaining to the microbiota of sheep dairy farms. The presence and diversity of anaerobic spore-formers has been reported in ewe raw milk and cheeses (Arias et al., 2013, Turchi et al., 2016), but other spore-formers and psychotrophic non-spore-forming spoilage bacteria have been largely overlooked. The diversification of sheep dairy products means that a wider range of processing treatments may be applied to ewe milk, thus selecting for various spore-forming bacteria in the end-products. In addition, the volume of the dairy sheep production is much lower than that of dairy cows (by a factor 20 to 50 depending on breeds) which implies that sheep raw milk may be stored refrigerated on-farm for longer periods before collection by processers, thereby leaving time for psychrotrophic bacteria to proliferate in the bulk tank.

A better understanding of the ecology of spoilage bacteria present on sheep dairy farms may assist in the identification of key spoilage organisms, on-farm reservoirs of these bacteria and provide an assessment of their potential transmission to raw milk.

In this chapter, using a culturomics approach, I carried out an in-depth characterisation of the spoilage bacteria present in the barn environment of a sheep dairy farm during housing of the animals. The goals of this study were to (1) identify bacteria previously associated with bovine dairy spoilage or exhibiting spoilage potential and uncover potential reservoirs of these organisms on sheep dairy farms; (2) investigate possible farm-level variations of the microbiota present in the housing environment of dairy sheep; and (3) carry out a first assessment of the potential sources of raw milk contamination. To this end, farm sources present in two pens with bedding materials of different age (3-week-old vs 3-month-old) were sampled. *Pseudomonas* species as well as anaerobic mesophilic, aerobic mesophilic, thermophilic and psychrotrophic spore-formers were isolated from farm samples that included silage, faeces, bedding materials, fence swabs, trough water and milking cups swabs. Bacteria were qualitatively screened for spoilage potential using plate-based assays and spoilage-associated isolates were identified using a polyphasic approach.

4.2 Results

4.2.1 Bacterial counts

In order to enumerate spoilage-associated bacteria and recover microbial isolates for characterisation and identification, a culturomics approach was used for the culturing of farm samples (see section 3.1.3). After a 10-min heat treatment at 80°C followed by discriminative incubation conditions, aerobic mesophilic, thermophilic, psychrotrophic and anaerobic mesophilic spores could be recovered from every sample. The spores of *Clostridium* species were not enumerated as their isolation was carried out after enrichment. High concentrations of aerobic spores were recorded in fence swabs, bedding materials, silage, faeces, trough water and milking cups swabs (Figure 4.1). A more detailed overview of the bacterial count data is available in the appendix (Appendix 4.1). Low spore counts were recorded in corn (< 2.00 $\log_{10}$ cfu/g, Appendix 4.1) and the rumen supplement (< 2.50 $\log_{10}$ cfu/g), therefore these feed types were not included for the remainder of the study. There were no differences in the spore counts between the sets of "old" and "new" samples.

Mesophilic spore counts (MSC) were higher than thermophilic and psychrotrophic spores counts across all sample types and sample sets (Figure 4.1). Mean MSC ranged from a high of 6.92 $\log_{10}$ cfu/g in faeces to a low of 4.52 $\log_{10}$ cfu/swab in milking cups. In the environmental samples (i.e., silage, faeces, bedding, trough water and fence swabs), mean MSC were > 5.5 $\log_{10}$ cfu.

Thermophilic spore counts (TSC) were recorded after incubation on Columbian sheep blood agar (SBA) but also on milk agar (MA) when possible (Figure 4.1 & Appendix 4.1). TSC on SBA were highest in the faeces samples (mean 6.58 $\log_{10}$ cfu/g) and lowest in milking cups (3.22 $\log_{10}$ cfu/swab, Figure 4.1). Due to extensive swarming and spreading on plate, TSC on MA could not be obtained for the fence swabs, the trough water samples and milking cups after incubation at 65°C. When $TSC_{65°C}$ could be recorded on MA, they were consistently higher on SBA than on MA (bedding, faeces and silage). In milking cups, $TSC_{60°C}$ were similar on SBA and MA but $TSC_{60°C}$ on SBA were about 1 $\log_{10}$ higher (4.12 $\log_{10}$ cfu/swab) than $TSC_{65°C}$ on SBA (3.22 $\log_{10}$ cfu/swab). For the $TSC_{65°C}$ of the milking cups, extensive swarming on one of the SBA replicates prevented the obtention of a second replicate value for this condition. Apart from the fence swabs (4.16 $\log_{10}$ cfu/swab), mean TSC of the environmental samples were > 5 $\log_{10}$ cfu.

Psychrotrophic spores were the least abundant spores across most sample types (Figure 4.1). Psychrotrophic spore counts (**PSC**) were highest in bedding materials (mean 6 $\log_{10}$ cfu/g) and lowest in milking cups (3.61 $\log_{10}$ cfu/swab). Mean PSC were > 4 $\log_{10}$ cfu in the environmental samples.

Psychrotrophic vegetative cell counts were obtained for the fence swabs samples by direct plating (i.e., no heat-treatment) and incubation at 7°C for 10 days on SBA (mean 7.42 ± 0.35 $\log_{10}$ cfu/swab, Figure 4.1, see section 3.1.3). For the remaining samples, focus was shifted to *Pseudomonas* which were enumerated using direct plating on CFC agar. *Pseudomonas* were recovered from each sample types with the exception of silage (detection limit > 1 $\log_{10}$ cfu/g, Figure 4.1). Colony counts on CFC agar (CC) were consistently higher in the "new" set of samples (range 6.98 to 7.74 $\log_{10}$ cfu) than in the "old" set (range 5.72 to 6.04 $\log_{10}$ cfu). Notably, CC were highest in bedding "new" (BN, 7.74 $\log_{10}$ cfu/g) and lowest in bedding old (BO, 5.72 $\log_{10}$ cfu/g) while high CC were also recorded in milking cups (7.44 $\log_{10}$ cfu/swab).

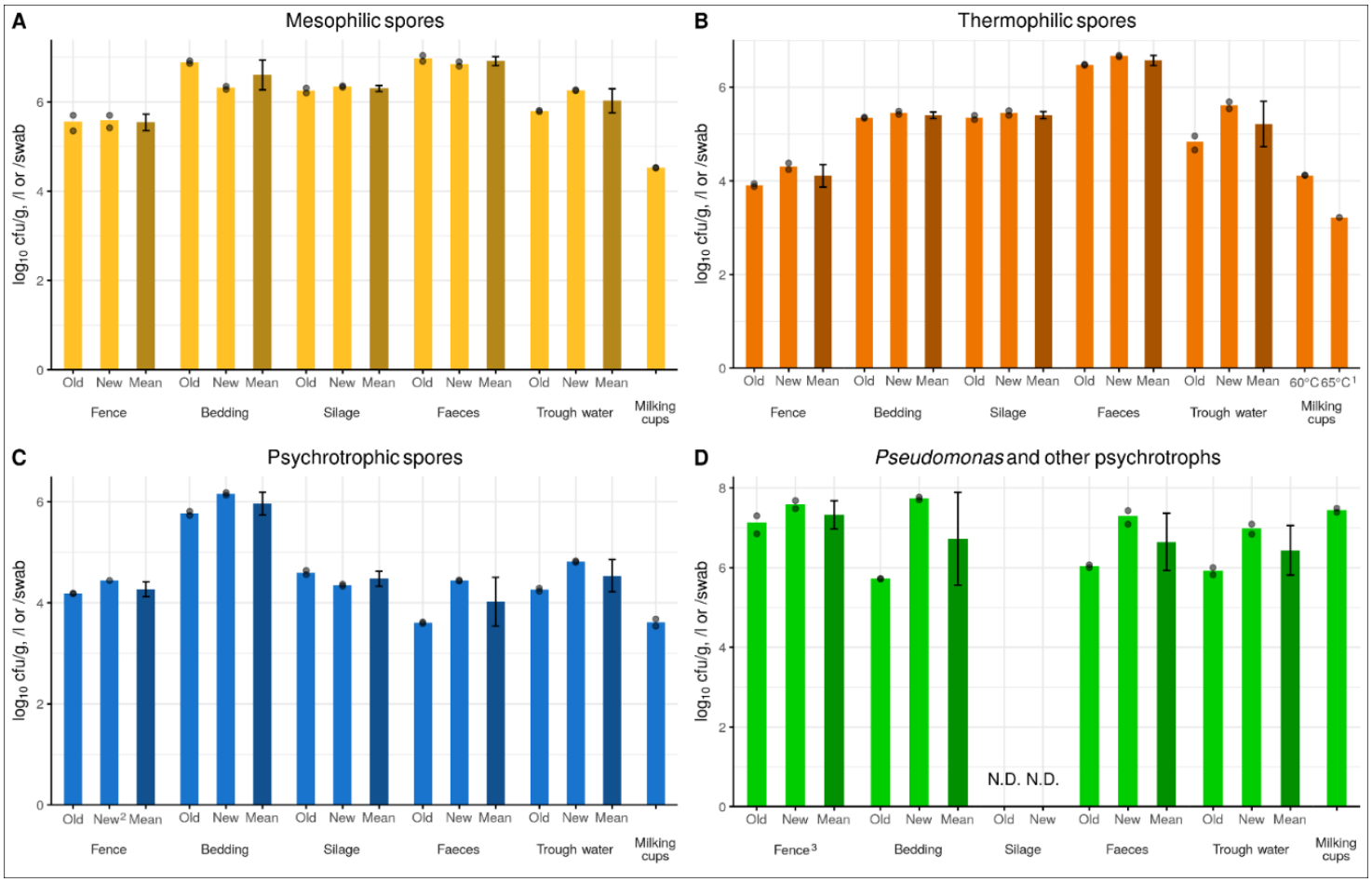


Figure 4.1. Bacterial counts in sources associated with the barn environment of a sheep dairy farm

Bar charts represent, unless stated otherwise, the average of two plating replicates on Columbia sheep blood agar (SBA) which are visible as black dots. Statistical testing could not be performed as the values were the average of two plating replicates. The terms "old" and "new" refer to the sample set for which the samples were collected ("old" = pen with 3-month-old bedding and "new" = pen with 3-week-old bedding).
A = Mesophilic spore counts. B = Thermophilic spore counts. C = Psychrotrophic spore counts. D = Psychrotrophic vegetative cells counts (fence samples) and CFC counts.
[1]Due to extensive swarming and spreading, only one plate replicate could be obtained for this condition.
[2]Due to dripping, only one plate replicate could be obtained for this condition.
[3]Psychrotrophic cell count: incubation at 7°C for 10 days on SBA

4.2.2 Prevalence and diversity of spoilage bacteria in the barn environment

Following enumeration across the 5 different culture conditions, bacterial colonies present on the isolation plates were subcultured to obtain pure cultures that could be characterised in more depths. A total of 1,753 spore-formers and 383 psychrotrophic bacteria were isolated from the samples collected in this study and a polyphasic approach was used for their identification. Aerobic bacteria were subjected to ERIC-PCR (see section 3.1.4) and isolates that displayed a unique intra-sample

ERIC signature (n = 1,215) were screened for spoilage potential (see section 3.1.6). ERIC-PCR on the clostridia isolates yielded faint to no bands, therefore, one to four isolates per plate morphotype (n = 243) were selected and screened for spoilage potential. In total, 68% (n = 1,459) of the collected isolates were screened for lipase and protease activity. Of these, 76% had some form of spoilage potential, 54% were positive for protease production, 47% were positive for lipase production and 25% were positive for concomitant lipase and protease production.

Sample-wise, representative isolates of ERIC types (aerobic isolates) or morphotypes (anaerobic isolates), which displayed spoilage potential, were identified by partial 16S rRNA gene sequencing (see section 3.1.5) and assigned to a species based on type strain sequences in the RDP repository. Isolates in a given ERIC-type or morphotype were assigned the same identification as that of their representative, leading to the identification of 86% (n = 1,844) of the isolates. For the remaining 292 isolates, identification was not attempted as they fell outside the scope of my study (i.e., lacked spoilage potential and did not cluster with any identified representative isolate). Bacteria with a 16S rRNA gene sequence similarity lower than 97% to type strains were assigned to the most closely related genus (e.g., *Bacillus* sp.) and grouped together if they shared at least 99% similarity in their 16S rRNA gene sequence, resulting in 49 clusters that could not be confidently assigned to type strains species (Appendix 4.2).

4.2.2.1 Prevalence, diversity and spoilage potential of aerobic spore-formers

The diversity of aerobic spore-formers spanned 33 genera and 92 species. Overall, the predominant genera were *Thermoactinomyces* (23% of the total relative abundance), *Bacillus sensu stricto* (*Bacillus s.s.,* 20%), *Psychrobacillus* (11%) and *Solibacillus* (9%). Among the screened aerobic spore-formers (n = 972), 76% were positive for spoilage potential with 55% being proteolytic, 45% being lipolytic and 24% being both lipolytic and proteolytic.

4.2.2.1.1 Aerobic mesophilic spore-formers

In this study, 494 aerobic mesophilic spore-formers were isolated, and a putative identification status was obtained for 436 (88%). A remarkable diversity of mesophilic spore-formers was observed, spanning 19 genera and 44 species (Figure 4.2, Table 4.1), including 13 clusters that could not be confidently assigned to a type strain species (Appendix 4.2). Despite this high diversity, 80% of the mesophilic species had a sample-wise abundance lower than 5%. Overall, the predominant genera were *Bacillus s.s.* (51%), followed by *Oceanobacillus* (11%) and *Alkalihalobacillus* (10%).

Bacillus licheniformis, *B. pumilus s.l.* and *Alkalihalobacillus clausii* were ubiquitous and the most abundant bacteria, representing 32%, 16% and 10% of the total abundance, respectively (Figure 4.2). *B. licheniformis* was highly abundant in most samples with the exception of faeces "old" (FO), faeces "new" (FN) and BO. Conversely, *B. pumilus s.l.* were highly abundant in FO and FN while the abundance of *A. clausii* was more homogeneously distributed throughout the samples. *Virgibacillus promii* was detected at low relative abundance in at least one sample of each sample type apart from milking cups, while *B. subtilis* and *Lederbergia galactosidilyticus* were detected at low relative abundance in at least one sample of each sample type except for bedding. *Oceanobacillus* spp. were particularly abundant in BO, FO, water "old" (WO) and water "new" (WN). *Oceanobacillus caeni* was the predominant species in BO and was also abundant in WO and WN while *Oceanobacillus sojae* was particularly abundant in FO and was also detected in WO and WN. Other genera of mesophilic spore-formers also detected included *Bhargaveae*, *Cytobacillus*, *Lysinibacillus*, *Nialla*, *Ornithinibacillus*, *Priestia*, *Pseudobacillus*, *Psychrobacillus*, *Robertmurraya*, *Siminovitchia*, *Solibacillus* and *Sporosarcina*.

Using the bacterial relative abundance data, and tools from the vegan package (see section 3.1.7), a descriptive community analysis was performed. A high diversity of mesophilic spore-formers was observed throughout the samples (Shannon diversity = H' > 1.60, Appendix 4.3). According to the Shannon, Simpson and inverse Simpson diversity indexes, the trough water samples (WN and WO) harboured the greatest mesophilic spore diversity. Bray-Curtis (BC) dissimilarity metrics and NMDS analysis further suggested that the mesophilic spore diversity of the trough water samples was different to that of other sample types (Appendix 4.4). On the NMDS plot (stress = 0.095), faeces and silage clustered together with milking cups (Appendix 4.5). Although bedding samples grouped apart from this cluster, BC metrics revealed that the pair BN/silage "old" (SO) was most similar across the samples (BC = 0.48, Appendix 4.4), followed by the pairs FO/FN (BC = 0.50), SO/FN (BC = 0.53) and SO/milking cups (BC = 0.56).

In general, most mesophilic spore-formers isolated from milking cups were also found in the environmental samples (Figure 4.2). These included *A. clausii*, *B. pumilus s.l.* and *B. licheniformis* (all ubiquitously detected), *B. subtilis* [isolated from infrastructure "old" (IO) and "new" (IN), SO, FN, FO, WN and WO), *Led. galactosidilyticus* (IO, infrastructure "new" (IN), SO, FN, FO and WO), *O. caeni* (IO, BO, FO, WN and WO) and *Solibacillus silvestris* (WN and WO). However, *Alkalihalobacillus rhizosphaerae*, *Lederbergia ruris* and *Oceanobacillus oncorhynchi* were only isolated from milking cups.

Out of the 379 mesophilic spore-forming isolates screened for spoilage potential, 77% demonstrated either lipase or protease activity. Variations in spoilage potential were observed at the genus level

(e.g., *Paenibacillus* spp.) but also amongst a given species (e.g., *A. clausii*). A wide range of species of mesophilic spore-formers was screened for spoilage potential (n = 44). However, in 70% of them, less than five isolates were screened for lipase and protease activity, and only one isolate was screened in half of these species of mesophilic spore-formers (Figure 4.3). Proteolytic bacteria accounted for 66% of the isolates and included species such as *B. licheniformis*, *B. pumilus s.l.*, *A. clausii*, *B. subtilis*, *V. promii*, *Caldibacillus hisashii*, *Paenibacillus lautus* and *Bhargavaea ginsengi*. Lipolytic bacteria were less common, representing 49% of the mesophilic isolates, and mainly included *B. pumilus s.l.*, *A. clausii*, *Oceanobacillus sojae*, *B. subtilis*, *Cald. hisashii*, *Robertmurraya siralis*, *Paenibacillus woosongensis* and *P. lautus*. Concomitant lipase and protease activity was observed in 37% of the isolates, mainly *B. pumilus s.l.*, *B. subtilis*, *Cald. hisashii*, and *P. lautus*. At the genus level, strong spoilage potential, often species-specific, was noted for *Bacillus s.s*, *Alkalihalobacillus*, *Paenibacillus* and *Lysinibacillus* whereas, apart from *O. sojae*, *Oceanobacillus* species exhibited weak spoilage potential.

Table 4.1. Bacteria isolated from the fence swab samples

"Old" fence swabs (IO)	"New" fence swabs (IN)
Mesophilic spore-formers	
Alkalihalobacillus clausii	*Alkalihalobacillus clausii*
Bacillus licheniformis	*Bacillus licheniformis*
Bacillus pumilus s.l.	*Bacillus pumilus* s.l.
Bacillus subtilis	*Bacillus subtilis*
Lederbergia galactosidilyticus	*Lederbergia galactosidilyticus*
Lysinibacillus sp. (*pakistanensis*)	*Oceanobacillus sojae*
Lysinibacillus xylanilyticus	*Oceanobacillus* sp. (*polygoni*)
Oceanobacillus caeni	*Ornithinibacillus* sp. (*bavariensis*)
Paenibacillus woosongensis	*Paenibacillus* sp. (*lentus*)
Priesta megaterium s.l.	*Paenibacillus woosongensis*
	Siminovitchia sp. (*fordii*)
	Solibacillus silvestris
	Virgibacillus proomii
Thermophilic spore-formers	
Brevibacillus aydinogluensis	*Anoxybacillus rupiensis*
Caldibacillus kokeshiiformis	*Bacillus smithii*
Geobacillus lituanicus	*Parageobacillus caldoxylosilyticus*
Geobacillus thermodenitrificans	*Saccharomonospora viridis*
Parageobacillus caldoxylosilyticus	*Thermoactinomyces vulgaris*
Thermoactinomyces vulgaris	*Ureibacillus suwonensis*
Ureibacillus suwonensis	*Ureibacillus terrenus*
Psychrotrophic spore-formers	

Paenibacillus odorifer	*Neobacillus* sp. (*ginsengisoli*)
Psychrobacillus spp.	*Paenibacillus odorifer*
Solibacillus spp.	*Peribacillus psychrosaccharolyticus*
	Psychrobacillus spp.
	Solibacillus spp.
Psychrotrophic bacteria	
Acinetobacter lwoffii	*Acinetobacter lwoffii*
Arthrobacter arilaitensis	*Acinetobacter* sp. (*lwoffii*)
Bacillus mycoides s.l.	*Arthrobacter arilaitensis*
Microbacterium hydrocarbonoxydans	*Arthrobacter bergerei*
Pseudomonas extremorientalis	*Bacillus mycoides* s.l.
Pseudomonas fragi	*Brachybacterium* sp. (*tyrofermentans*)
Pseudomonas poae	*Carnobacterium* sp. (*maltaromaticum*)
Pseudomonas yamanorum	*Jeotgalicoccus* sp. (*psychrophilus*)
Psychrobacter faecalis	*Paeniglutamicibacter antarcticus*
Staphylococcus equorum	*Pseudomonas poae*
Stenotrophomonas rhizophila	*Pseudomonas yamanorum*
	Psychrobacter maritimus
	Staphylococcus equorum

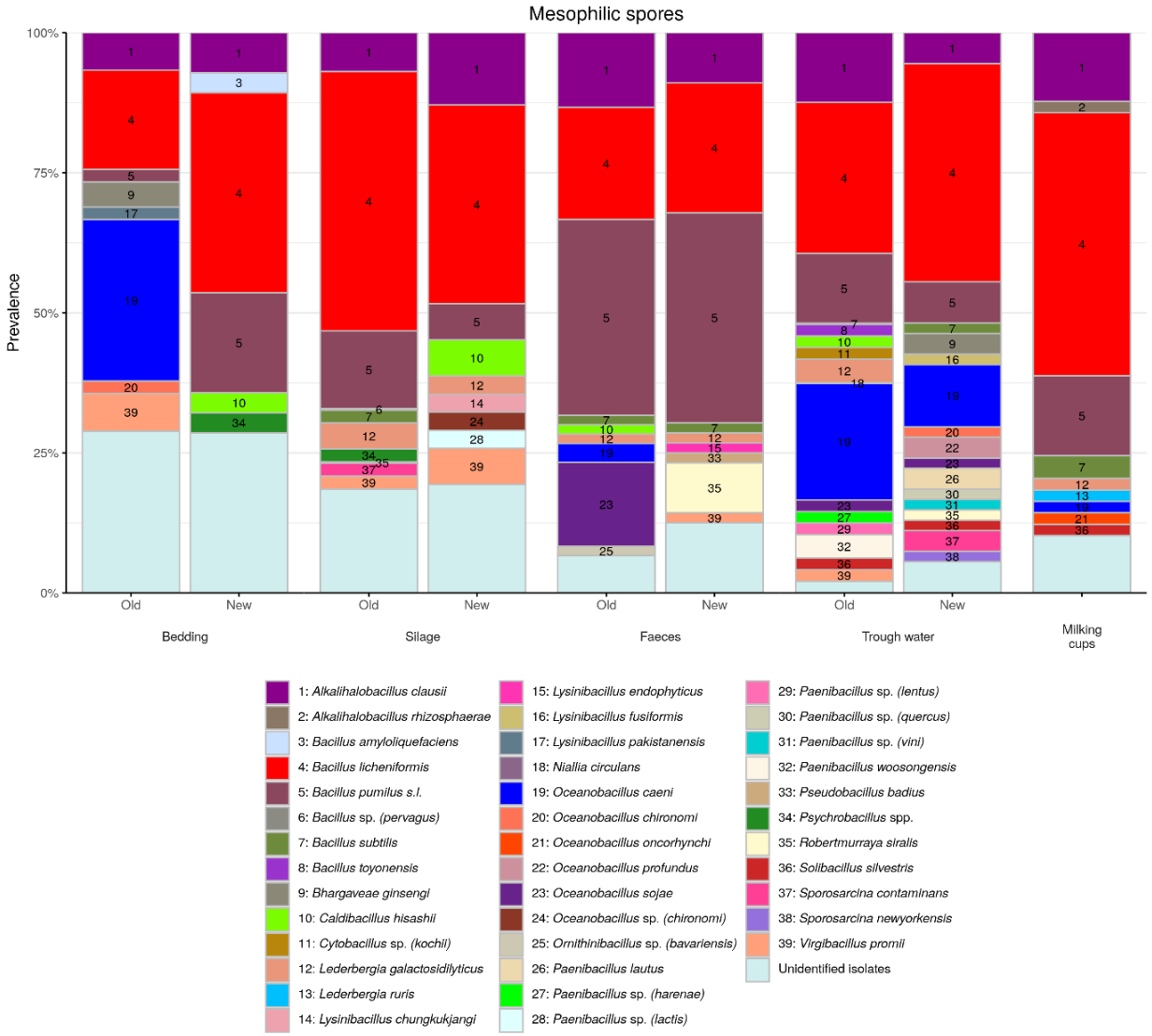


Figure 4.2. Diversity of mesophilic spores in the barn environment of a dairy sheep farm.

Coloured boxplots with a number represent the relative prevalence of a bacteria in a given sample. The terms "old" and "new" refer to the sample set for which the samples were collected ("old" = pen with 3-month-old bedding while "new" = pen with 3-week-old bedding). The proportion of unidentified isolates is visible as pale turquoise rectangles on the bottom of each bar graph. Isolates referred to as "sp." Could not be confidently attributed to a type strain species based on their partial 16S rRNA gene sequence, further information on these isolates is available in Appendix 4.2.

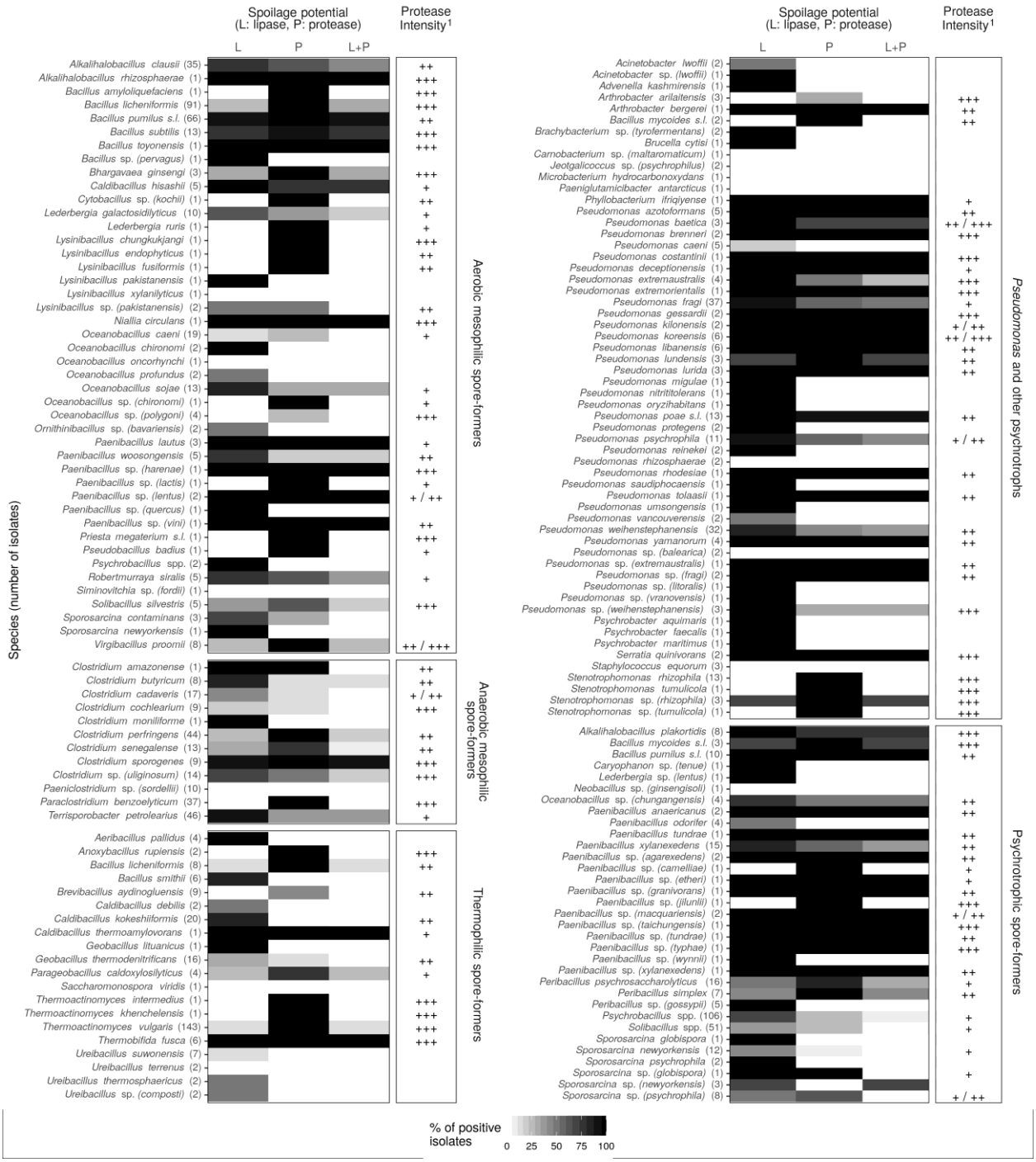


Figure 4.3. Spoilage potential of the spore-formers isolated from the barn environment of a dairy sheep farm

For a given species or cluster, the proportion of isolates positive for lipase and/or protease activity is visible in shades of grey (the more isolates showing the corresponding enzymatic activity, the darker the shade). Enzymatic screening was performed for each isolate with a sample-wise unique Enterobacterial repetitive intergenic consensus (ERIC) gel pattern using qualitative plate-based assays. The numbers in the brackets next to each species or cluster shows the number of isolates screened for spoilage potential for that species.

[1]The intensity of the protease activity was ranked as weak (+, weak casein hydrolysis), medium (++, casein hydrolysis only directly under the colony) or strong (+++, diffused casein hydrolysis around the colony). The protease intensity displayed for each species or cluster corresponds to the most common protease intensity for that species.

4.2.2.1.2 Aerobic thermophilic spore-formers

The diversity of thermophilic spore-forming bacteria spanned 11 genera and 20 species (Figure 4.4), including two clusters that could not be confidently identified (Appendix 4.2). Overall, the predominant genera consisted of *Thermoactinomyces* (67%), *Caldibacillus* (13%), *Geobacillus* (5%) and *Bacillus s.s.* (5%).

Across all samples, *Thermoactinomyces vulgaris*, *Caldibacillus kokeshiiformis*, *Thermobifida fusca*, *B. licheniformis* and *Geobacillus thermodenitrificans* accounted for more than 90% of the total abundance, with *T. vulgaris* alone accounting for 67% of it (Figure 4.4). *Thermoactinomyces vulgaris* was predominant across the samples after incubation on SBA but its relative abundance was lower on MA at both 60°C (milking cups) and 65°C (SN). *Caldibacillus kokeshiiformis* was the second most common thermophilic species and was highly abundant in WO and milking cups after incubation at 65°C. *Cald. kokeshiiformis* was also detected in IO, silage (SO and SN) and faeces (FO and FN). *Geobacillus s.l.* (i.e., *Geobacillus* and *Parageobacillus*) were only detected in the fence samples (IO and IN) and silage (SN, Figure 4.4 & Table 4.1). *Geobacillus thermodenitrificans* was highly abundant in SN after incubation on MA at 65°C but was otherwise only detected on SBA in IO. Thermotolerant *B. licheniformis* were predominant in milking cups at 60°C but they were not detected from any sample following incubation at 65°C. *Thermobifida fusca* was only isolated from the faeces samples (FO and FN). Other genera of thermophilic spore-formers included *Aeribacillus*, *Anoxybacillus*, *Brevibacillus*, *Sacchamonospora* and *Ureibacillus*.

The overall diversity of thermophilic spore-formers was low, and lower after incubation on SBA (H' < 0.90) than on MA (H' > 1.40, Appendix 4.3). Aberrant similarities between BO, BN and WN (BC = 0, Appendix 4.4) as well as their clustering on the NDMS plot (stress = 0.059, Appendix 4.5) was caused by the fact that only one species (*T. vulgaris*) was taken into account for the NMDS and Bray-Curtis analyses, being the only one present at abundance > 1% in these samples.

Based on the results of the BC dissimilarity metrics and NMDS analysis, the bacterial diversity of the milking cups at 60°C on MA and 65°C on SBA was different to that of other samples (Appendix 4.4 & 4.5). WO was somewhat similar to milking cups incubated at 65°C on SBA (BC = 0.63). *Thermoactinomyces kenchelensis*, *B. licheniformis*, *Ureibacillus thermosphaericus* and *Caldibacillus thermoamylovorans* were only isolated from milking cups following incubation at 60°C on MA (Figure 4.4). Other bacteria isolated from milking cups at 60°C and 65°C were also detected in other samples, namely *T. vulgaris* (ubiquitous), *Cald. kokeshiiformis* (IO, SN, SO, FN, FO and WO), *U. suwonensis* (IO, IN, SN and WN) and *Aeribacillus pallidus* (SN, SO and FN).

A total of 265 thermophilic isolates were screened for spoilage potential and 70% were positive for lipase or protease activity. Proteolytic thermophiles represented 65% of the isolates and included *T. vulgaris* (Figure 4.3), *B. licheniformis*, *Thermobifida fusca*, *Parageobacillus caldoxylosilyticus*, *Anoxybacillus rupiensis*, *Thermoactinomyces* spp. and *Cald. thermoamylovorans*. Lipolysis was less common, being detected in 23% of the isolates, including species such as *Cald. kokeshiiformis*, *Bacillus smithii*, *Thermobifida fusca*, *Aer. pallidus*, *Geobacillus lituanicus* and *Cald. thermoamylovorans*. Concomitant lipase and protease activity was recorded in 12% of the isolates, mainly in those identified as *Thermobifida fusca* and *Cald. thermoamylovorans* but also rarely for *T. vulgaris*. At the genus level, *Geobacillus s.l.* demonstrated moderate spoilage potential, which was often species-specific, while *Ureibacillus* spp. showed weak spoilage potential, only rarely producing lipases.

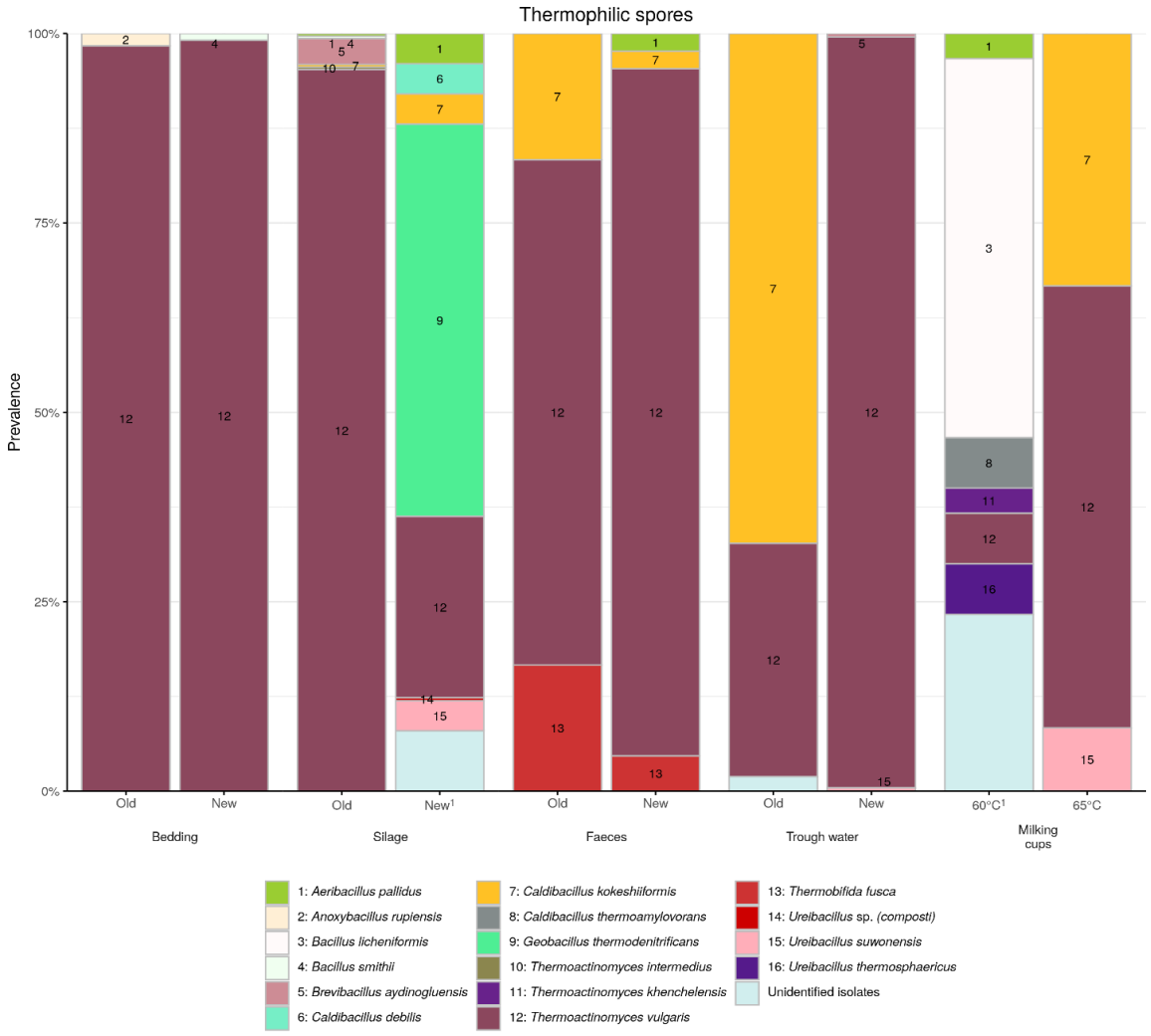


Figure 4.4. Diversity of thermophilic spores in the farm environment of a dairy sheep farm.

Coloured rectangles with a number represent the relative prevalence of a bacteria in a given sample after incubation at 65°C on SBA. The terms "old" and "new" refer to the sample set for which the samples were collected ("old" = pen with 3-month-old bedding while "new" = pen with 3-week-old bedding). The proportion of unidentified isolates is visible as pale turquoise rectangles on the bottom of each bar graph. The isolate referred to as "sp." could not be confidently attributed to a type strain species based on their partial 16S rRNA gene sequence, further information on this isolates is available in Appendix 4.2.

[1] Thermophilic diversity on MA as extensive spreading and swarming prevented bacterial isolation from SBA

4.2.2.1.3 Aerobic psychrotrophic spore-formers

The diversity of psychrotrophic spore-formers spanned 11 genera and 33 species (Figure 4.5), including 18 clusters for which reliable identification could not be obtained (Appendix 4.2). The predominant genera of psychrotrophic spore-formers were *Psychrobacillus* (31%), *Solibacillus* (27%), *Bacillus sensu lato* (11%, *Bacillus s.l.* : *Bacillus* spp. and recently reassigned former *Bacillus* members such as *Peribacillus* and *Alkalihalobacillus*) and *Paenibacillus* (9%).

Psychrobacillus and *Solibacillus* spp. were ubiquitous and the most abundant psychrotrophic spore-formers detected across the samples (Figure 4.5). Although some *Psychrobacillus* and *Solibacillus* isolates could be identified to the species level based on their 16S rRNA gene sequence (i.e., *Psy. psychrodurans*, *Psy. psychrotolerans*, *Psy. insolitus*, *Psy. lasiicapitis* and *So. silvestris*, and *So. isronensis*), for most isolates, the 16S gene PCR produced multiple DNA fragments of same size (~ 1.5 kb) that shared a 140 bp homology which matched part of the 16S rRNA gene sequence of *Psychrobacillus* or *Solibacillus* type strain species. Psychrotrophic *Bacillus s.l.* and *Paenibacillus* spp. were also ubiquitously found across the samples, but at lower relative abundance. *Bacillus s.l.* were most abundant in FO, BO, SN and trough water (WO and WN). *Bacillus pumilus s.l.* was particularly abundant in FO, bedding materials (BO and BN) and milking cups. Other *Bacillus s.l.* species included *B. mycoides s.l.*, *A. plakortidis*, *Peribacillus psychrosaccharolyticus* and *Peri. Simplex*. *Paenibacillus* were most abundant (> 10%) in silage (SO and SN), FO and WN. *Paenibacillus xylanexedens* was the most abundant *Paenibacillus*, particularly in silage and faeces. Other *Paenibacillus* species were detected at a low sample-wise relative abundance in at most two samples and included 11 clusters of isolates most closely related to *Paenibacillus* species for which definitive identification could not be obtained (Appendix 4.2). Other genera of psychrotrophic spore-formers included *Caryophanon*, *Lederbergia*, *Neobacillus*, *Oceanobacillus* and *Sporosarcina*.

The psychrotrophic spore diversity of the different samples was heterogeneous; BN and FN were the least diverse (H' < 1.50) while silage (SO and SN) and FO were the most diverse (H' > 1.90, Appendix 4.3). The NMDS plot (stress = 0.149, Appendix 4.5) revealed clustering of BO and FO, BN and FN, WO and WN as well as of SO and SN. Bray-Curtis metrics further suggested a link between the trough water samples (BC = 0.60, Appendix 4.4) but also highlighted the pair SN/WO as most similar (BC = 0.45). Although milking cups somehow clustered with BO on the NMDS analysis plot, relatively high Bray-Curtis metrics were associated with milking cups (BC > 0.67).

Apart from three species, *Paenibacillus* sp. (*tundrae*), *Paenibacillus* sp. (*wynnii*) and *Sporosarcina newyorkensis*, the psychrotrophic spore-formers isolated from milking cups were also recovered from other samples (Figure 4.5). Although highly abundant in milking cups (27%), *S. newyorkensis* was

not recovered from any other sample. Psychrotrophic spore-formers isolated from milking cups and the environmental samples included *Psychrobacillus* spp. (ubiquitously detected), *Solibacillus* spp. (ubiquitous except FO), *Bacillus pumilus s.l.* (isolated from BN, BO and FO), *Oceanobacillus* sp. (*chungangensis*) [BO, SO and FO], *P. xylanexedens* (SN, SO, FO, WN and WO), *Peribacillus psychrosaccharolyticus* (IN, BN, BO, SN, FN, WN and WO), *Peri. simplex* (BO) and *Sporosarcina* sp. (*psychrophila*) [SO, FN, FO and WN].

There was a total of 328 (74%) psychrotrophic spore-formers that were screened for spoilage potential, and 71% of those were positive for either lipase or protease activity. Proteolytic bacteria represented 34% of the isolates and included species such as *Peri. psychrosaccharolyticus* (Figure 4.3), *B. pumilus s.l.*, *A. plakortidis* and *Peri. simplex*. Lipolytic activity was more common, being detected in 56% of the isolates, such as *Psychrobacillus* spp., *P. xylanexedens*, *B. pumilus s.l.*, *A. plakortidis*, *Peribacillus* sp. (*gossypii*) and *Oceanobacillus* sp. (*chungangensis*). Concomitant lipase and protease activity was detected in 18% of the isolates, predominantly for *B. pumilus s.l.* and *A. plakortidis*. At the genus level, *Paenibacillus* and *Bacillus s.l.* were strong enzyme producers, producing both lipases and proteases. *Solibacillus* spp. showed weak spoilage potential while *Sporosarcina* spp. had moderate spoilage potential, producing mostly lipases.

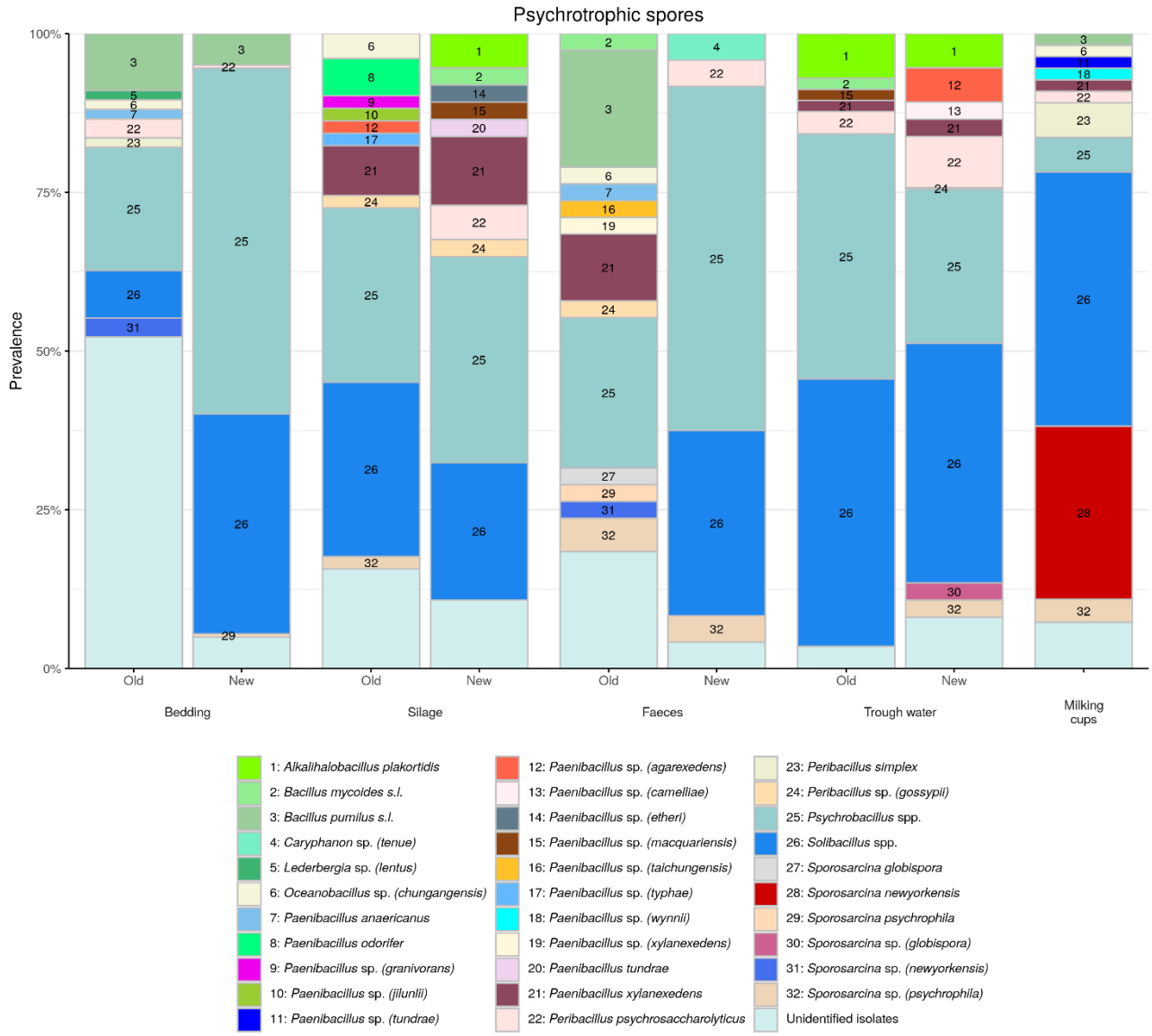


Figure 4.5. Diversity of psychrotrophic spores in the farm environment of a dairy sheep farm.

Coloured rectangles with a number represent the relative prevalence of a bacteria in a given sample. The terms "old" and "new" refer to the sample set for which the samples were collected ("old" = pen with 3-month-old bedding while "new" = pen with 3-week-old bedding). The proportion of unidentified isolates is visible as pale turquoise rectangles on the bottom of each bar graph. Isolates referred to as "sp." Could not be confidently attributed to a type strain species based on their partial 16S rRNA gene sequence, further information on these isolates is available in Appendix 4.2.

4.2.2.2 Diversity and spoilage potential of anaerobic spore-formers

Following cooked meat glucose broth (CMG) enrichment and plating on egg yolk agar, 436 anaerobic spore-formers were recovered. Intrasample morphotype representatives were assigned to type strains species based on their 16S rRNA gene sequence and isolates with comparable plate morphologies

were assigned to the same species. These isolates spanned 12 species, including two clusters that could not be confidently assigned to type strains species (Figure 4.6 & Appendix 4.2). The most commonly isolated clostridia were *Terrisporobacter petrolearius*, *Clostridium perfringens*, *Paraclostridium benzoelyticum* and *C. cadaveris*. Silage harboured the most diverse clostridial population (SN, 7 different species, Figure 4.6) while trough water showed the least diverse (WO, 3 species). Clostridial diversity was similar between SN and SO (5 shared species) as well as between FN and FO (4 shared species) but differed between BN and BO as well as between WN and WO (1 shared species). Clostridia isolated from milking cups included *T. petrolearius* (ubiquitously detected), *Paracl. benzoelyticum* (detected in IO, BN, SO, SN, FO, FN), *C. perfringens* (BN, SO, FN and WO) and *C. moniliforme* (not detected elsewhere).

A total of 243 clostridia were screened for spoilage potential and 73% were positive for either protease or lipase activity. Proteolytic bacteria accounted for 52% of the isolates and included *C. perfringens*, *Paracl. benzoelyticum*, *C. senegalense*, *C. sporogenes* and *C. amazonense* (Figure 4.3). Lipolytic clostridia represented 39% of the isolates and mainly included *T. petrolearius*, *C. sporogenes*, *Clostridium* sp. (*uliginosum*), *C. butyricum*, *C. moniliforme* and *C. amazonense*. Concomitant lipase and protease activity was observed in 9% of the isolates, mainly *C. sporogenes* and *C. amazonense*.

Based on the results of amplified rDNA restriction analysis (ARDRA), representative isolates of *C. perfringens* isolated from NB (n = 4), OS (n = 7), OW (n = 4) and NP (n = 3) as well as all *C. perfringens* isolated from milking cups (n = 12) were screened for the production of *C. perfringens*-specific surface antigens, α-toxin, β-toxin and ε-toxin using an ELISA (Appendix 4.6). All isolates were confirmed as *C. perfringens* (presence of *C. perfringens* specific surface antigens) and the majority (28/30) showed α-toxin production. Two isolates from silage (OS) did not produce α-toxin. No production of β-toxin or ε-toxin was recorded among the isolates.

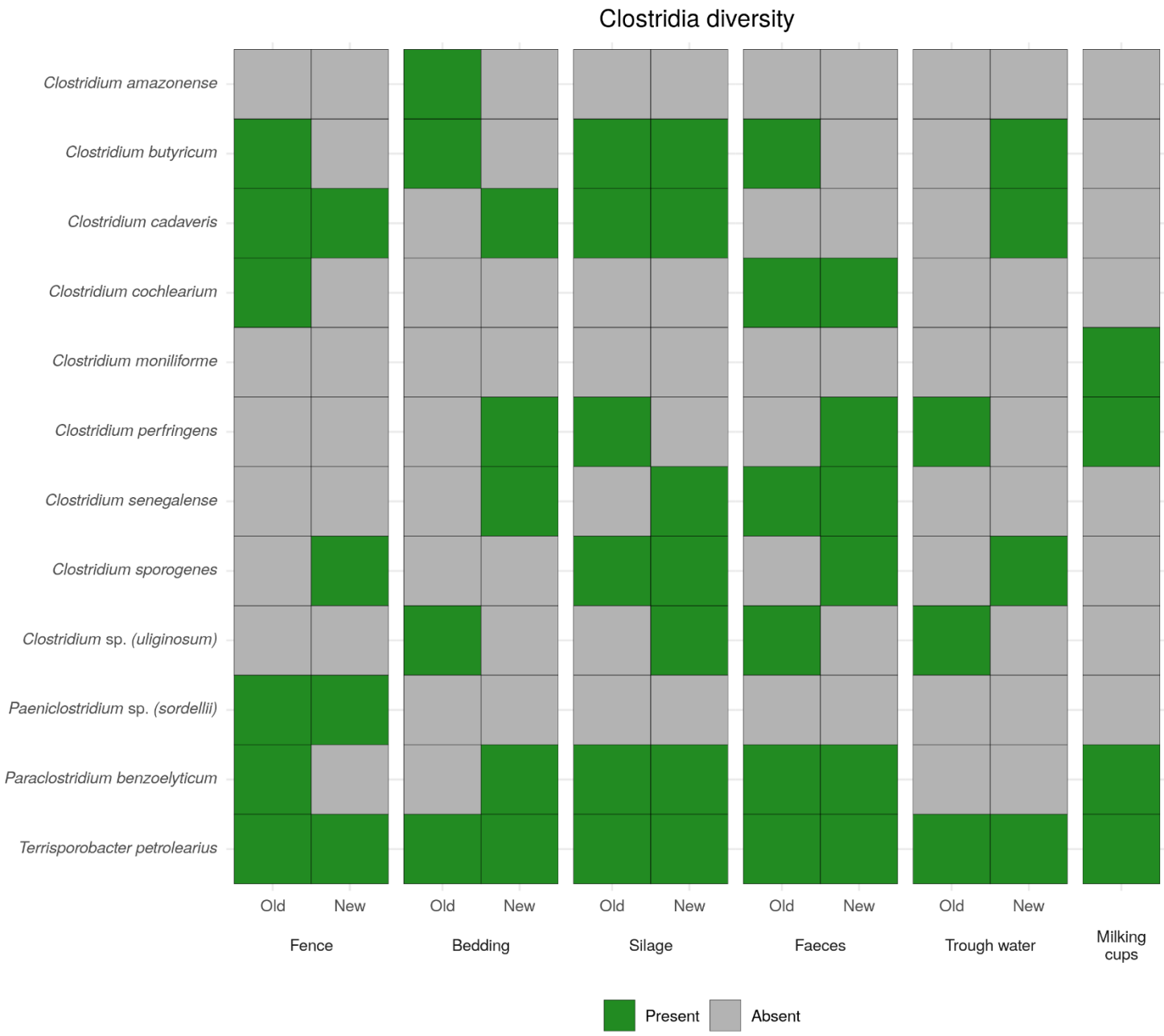


Figure 4.6. Clostridial diversity of the farm samples collected at a dairy sheep farm following cooked meat glucose starch broth (CMG) enrichment.

Representatives of the isolates retrieved following anaerobic CMG enrichment were identified by partial 16S rRNA gene sequencing and compared with the sequence of the RDP depository. The detection of at least one isolate of a specific clostridial species in a given sample is pictured as green squares while grey squares indicate the absence of detection of the organism. The terms "old" and "new" refer to the sample set for which the samples were collected ("old" = pen with 3-month-old bedding while "new" = pen with 3-week-old bedding

4.2.2.3 Prevalence and spoilage potential of *Pseudomonas* and other psychrotrophs

Psychrotrophic bacteria from the fence swabs were isolated after incubation for 10 days at 7°C on SBA and included 12 genera: *Acinetobacter*, *Arthrobacter*, *Bacillus*, *Brachybacterium*, *Carnobacterium*, *Jeotgalicoccus*, *Microbacterium*, *Paeniglutamibacter*, *Pseudomonas*, *Psychrobacter*, *Staphylococcus* and *Stenotrophomonas* (Table 4.1). To increase the recovery rate of *Pseudomonas*, the remaining samples (bedding, faeces, trough water, silage and milking cups) were instead incubated on CFC agar. No isolates could be recovered from the silage samples (SO and SN) following incubation on CFC agar.

The bacterial diversity on CFC spanned six genera, namely *Pseudomonas*, *Stenotrophomonas*, *Serratia*, *Advenella*, *Brucella* and *Phyllobacterium* (Figure 4.7). Across the samples, the predominant species recovered from CFC agar were *Pseudomonas fragi*, *Ps. weihenstephanensis*, *Stenotrophomonas rhizophila*, *Ps. libanensis* and *Ps. caeni*. Although *Ps. fragi* and *Ps. weihenstephanensis* were ubiquitously detected at high abundance from the environmental samples, they were not isolated from milking cups. Apart from *Ps. fragi* and *Ps. weihenstephanensis*, the predominant bacteria differed across sample types. *Pseudomonas caeni* and *Pseudomonas* sp. (*balearica*) were highly abundant in bedding materials, *Stenotrophomonas rhizophila* was highly abundant in faeces while the bacteria most abundant in trough water were *Ps. poae*, *Ps. psychrophila* and *Pseudomonas* sp. (*fragi*).

Although a high species diversity was observed across the samples ($1.70 < H' < 2.30$, Appendix 4.3) with the exception of FO ($H' = 0.85$), the diversity of the different samples was heterogeneous, as seen by the relatively high Bray-Curtis metrics ($BC > 0.64$, Appendix 4.4). The NMDS plot analysis underlines this heterogeneity and highlights outlier samples, namely FO, FN and milking cups (Appendix 4.5). Nonetheless, both the NMDS analysis and Bray-Curtis metrics suggested some degree of similarity between the bacterial diversity of WO, WN and BN ($0.64 < BC < 0.71$).

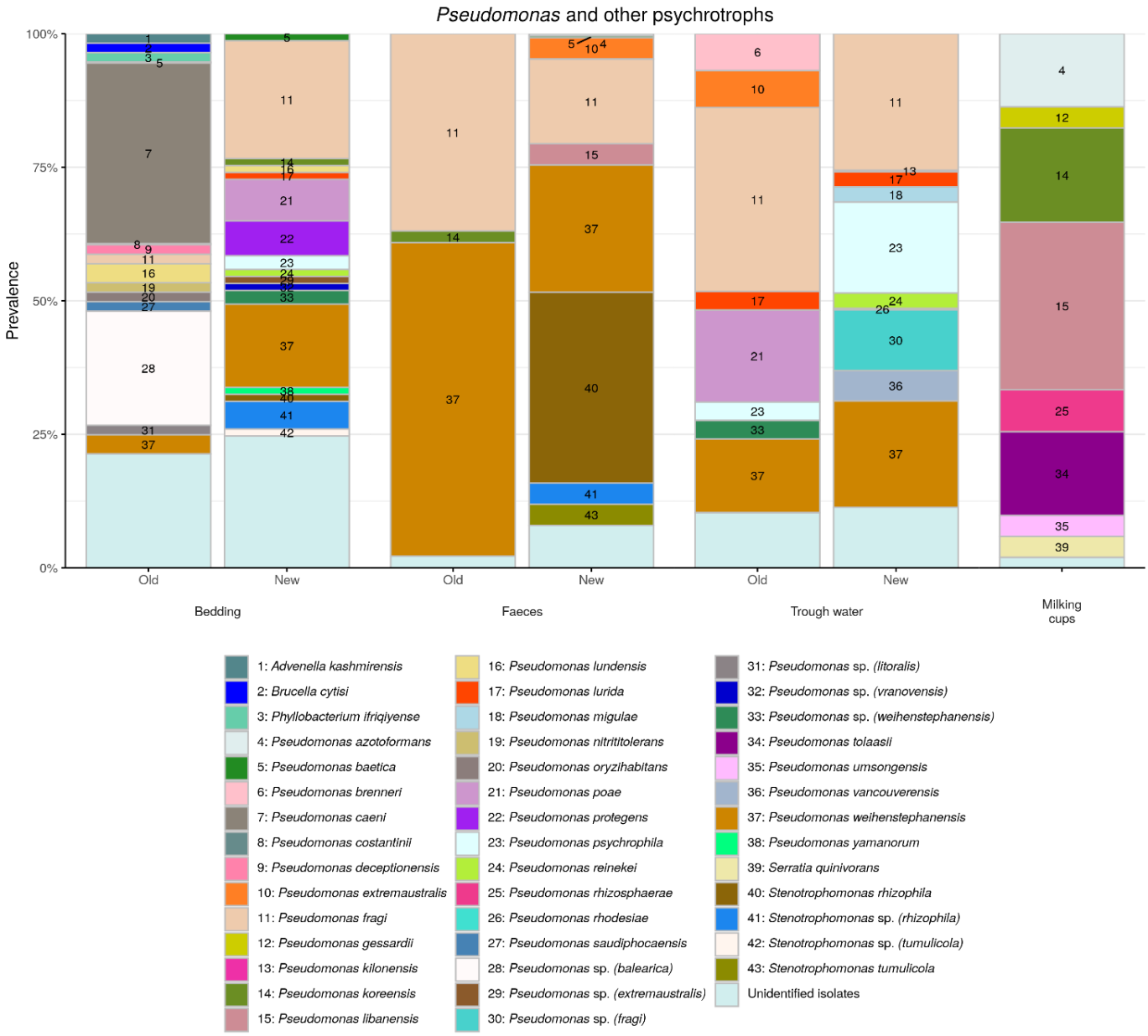


Figure 4.7. Diversity of *Pseudomonas* and other psychrotrophic species in the farm environment of a dairy sheep farm.

Coloured rectangles with a number represent the relative prevalence of a bacteria in a given sample. The terms "old" and "new" refer to the sample set for which the samples were collected ("old" = pen with 3-month-old bedding while "new" = pen with 3-week-old bedding). The proportion of unidentified isolates is visible as pale turquoise rectangles on the bottom of each bar graph. Isolates referred to as "sp." Could not be confidently attributed to a type strain species based on their partial 16S rRNA gene sequence, further information on these isolates is available in Appendix 4.2.

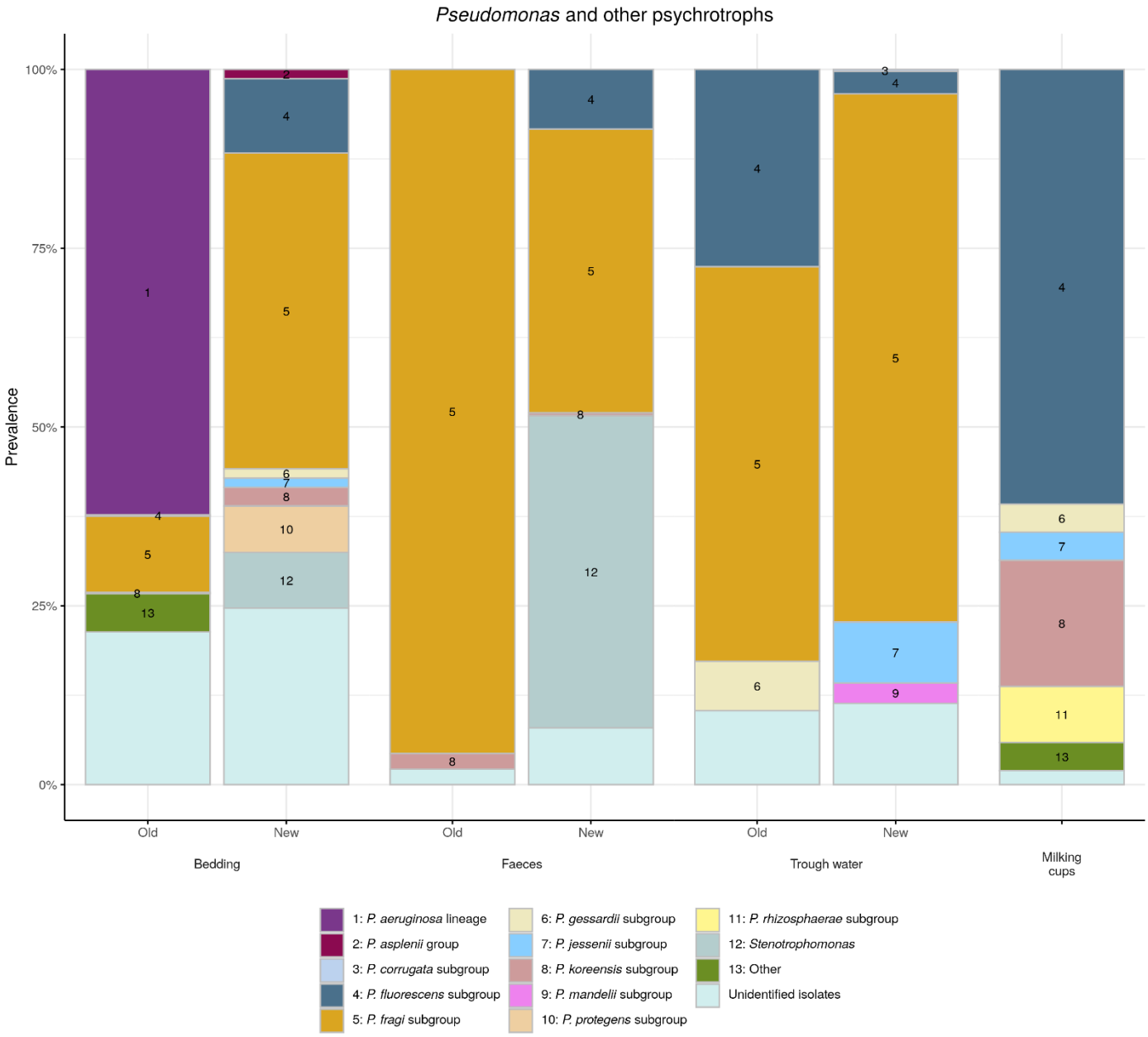


Figure 4.8. Diversity of *Pseudomonas*, sorted by phylogenetic groups, and other psychrotrophic bacteria in the farm environment of a dairy sheep farm.

Coloured rectangles with a number represent the relative prevalence of a bacteria in a given sample. The terms "old" and "new" refer to the sample set for which the samples were collected ("old" = pen with 3-month-old bedding while "new" = pen with 3-week-old bedding). No isolates were recovered after incubation of the silage samples on CFC agar. The proportion of unidentified isolates is visible as pale turquoise rectangles on the bottom of each bar graph. Isolates referred to as "sp." Could not be confidently attributed to a type strain species based on their partial 16S rRNA gene sequence, further information on these isolates is available in Appendix 4.2. Grouping of the *Pseudomonas* species was done according to the phylogeny described by Garrido-Sanz et al. (2016).

The species Diversity of milking cups was markedly different to that of the environmental samples, (BC > 0.94, Appendix 4.4). *Pseudomonas gessardii*, *Ps. rhizosphaerae*, *Ps. tolaasii*, *Ps. umsongensis* and *Serratia quinivorans* were only isolated from milking cups. Other species isolated from both milking cups and the farm environment included *Ps. azotoformans* (detected in FN), *Ps. koreensis* (BN and FO) and *Ps. libanensis* (FN).

To enable a broader perspective of their ecology, *Pseudomonas* species were assigned to taxonomic groups previously described by multilocus sequence analysis [(Garrido-Sanz et al., 2016), Appendix 4.7]. Members of the *Ps. fluorescens* lineage, and especially those of the *Ps. fluorescens* complex, were predominant across the samples (Figure 4.8). Bacteria belonging to the *Ps. aeruginosa* lineage (i.e., *Ps. caeni*, *Ps. oryzihabitans*, *Ps. nitrititolerans*, *Ps. saudiphocaensis*, *Pseudomonas* sp. (*litoralis*) and *Pseudomonas* sp. (*balearica*)], were only detected in BO in which they were highly abundant. Members of the *Ps. fragi* subgroup (*Ps. deceptionensis*, *Ps. fragi*, *Ps. lundensis*, *Ps. psychrophila*, and *Ps. weihenstephanensis*) were predominant in the environmental samples but were not detected in milking cups. In contrast, members of the *Ps. fluorescens* subgroup (*Ps. azotoformans*, *Ps. constantinii*, *Ps. extremaustralis*, *Ps. lurida*, *Ps. poae*, *Ps. rhodesiae* and *Ps. tolaasii*) were predominant in milking cups but not in the environmental samples. Other members of the *Pseudomonas fluorescens* lineage were detected at low total abundance and included members of the *Ps. asplenii* and *Ps. rhizosphaerae* groups as well as of the *Ps. koreensis*, *Ps. jessenii*, *Ps. gessardii*, *Ps. corrugata*, *Ps. mandelii* and *Ps. protegens* subgroups.

Spoilage potential was assessed for 244 isolates and was present in 78% of those. Proteolytic activity was recorded in 51% of the isolates, mainly in bacteria identified as belonging to the *Ps. fluorescens*, *Ps. corrugata*, *Ps. gessardii* and *Ps. koreensis* subgroups as well as *Stenotrophomonas* species (Figure 4.3, Appendix 4.7). Proteolytic species included *Stenotrophomonas rhizophila*, *Ps. poae*, *Ps. koreensis*, *Ps. libanensis* and *Ps. azotoformans*. Lipase activity, which was recorded in 67% of the isolates, was particularly common for members of the *Ps. fluorescens*, *Ps. fragi*, *Ps. protegens*, *Ps. mandelii*, *Ps. corrugata*, *Ps. gessardii*, *Ps. jessenii* and *Ps. koreensis* subgroups as well as the *Ps. asplenii* group. Lipolytic species included *Ps. fragi*, *Ps. weihenstephanensis*, *Ps. poae*, *Ps. psychrophila*, *Ps. koreensis*, *Ps. libanensis* and *Ps. azotoformans*. There was in total 39% of the isolates exhibiting concomitant lipase and protease activity. These bacteria belonged to the *Ps. fluorescens*, *Ps. corrugata*, *Ps. gessardii* and *Ps. koreensis* subgroups, and included species such as *Ps. poae*, *Ps. koreensis*, *Ps. libanensis* and *Ps. azotoformans*. Bacteria members of the *Ps. aeruginosa* lineage had weak spoilage potential, exclusively producing lipases in some instances.

4.3 Discussion

4.3.1 Approach

With a strong focus on bovine dairy farms, previous studies have identified correlations between the bacterial load of raw milk and that of several farm sources (i.e. soil, bedding materials, silage), as well as specific farm practices [i.e. feeding scheme, bedding management (Arias et al., 2013, Miller et al., 2015a, Martin et al., 2019, Murphy et al., 2019)]. On cow dairy farms, bacteria associated with spoilage or with spoilage potential are prevalent, and their primary sources include soil, plant matter and commensal communities associated with livestock (Siala et al., 2000, Hong et al., 2009, Keshri et al., 2019). However, despite the apparent involvement of the farm environment in the contamination of raw milk, few studies have taken a holistic farm approach to the issue of dairy quality and considered both a variety of samples and the use of different isolation conditions to survey the prevalence of spoilage bacteria on dairy farms (Scheldeman et al., 2005). Sheep dairy farms share many traits with their bovine counterparts, including environmental (e.g., pastures, soils) and functional or management (feeding scheme, housing, milking) features. Therefore, it could be expected that they harbour similar microbial populations, both in numbers and diversity.

To ensure that the observed bacterial diversity was representative of the populations prevalent in the general environment, the present study relied on a thorough sampling plan. The analysis of composite samples, which consisted of a mix of discrete samples collected at different locations in the pens, permitted a more global overview of the farm microbiota. Composite samples are considered to reflect more accurately the microbiological composition of large samples in which microorganisms may be heterogeneously distributed (Murphy et al., 2021). Further, the analysis of two sample sets, originating from pens with bedding materials of distinct ages, provided two complementary perspectives of the populations present at the farm and highlighted farm-level variations of the microbiota. Although the predominant bacteria and spore counts were similar between the “old” and “new” sets, some bacteria were more abundant in a specific sample set (e.g., *Pseudomonas* spp. or *Oceanobacillus* spp. in the “new” or “old” sample sets, respectively). These findings stress the importance of a well-designed sampling plan and on the advantage of analysing composite samples to capture the bacterial diversity of dairy farms more accurately.

In my study, a culturomics approach was used to survey the prevalence and diversity of spoilage bacteria associated with the barn environment of a sheep dairy farm. The term culturomics, which was coined by Lagier et al. (2012), consists in the high-throughput, culture-dependent microbiological analysis of samples. By using a set of isolation conditions (i.e., different pre-treatments, incubation

temperatures, growth media composition) the aim of this approach is to accommodate the complex growth requirements of the microbiota present in a sample. The resulting isolates can then be identified using high-throughput methods such as MALDI-TOF or the sequencing of housekeeping genes for which well-documented and curated databases exist (e.g., 16S rRNA gene, *rpoB*). Compared to culture-independent methods such as metagenomics, a culture-dependent approach yields functional isolates that can then be characterised in more depth, both phenotypically and genetically. Culturomics have been successfully used for the isolation of fastidious or so-called unculturable members of the gut microbiota, but also to study those less abundant and generally concealed by other predominant organisms (Lagier et al., 2018). In their study, Lagier et al. (2012) screened three human faecal samples using 212 different culture conditions and isolated 32,500 microorganisms spanning 117 genera and 340 species of bacteria of which 174 had never been described previously in the human gut. My approach was similar, albeit more modest: five discriminative culture conditions were used to isolate bacteria present on a sheep dairy farm, yielding 2,136 isolates spanning 52 genera and 159 species.

Culture conditions used as part of the culturomics approach aimed to select for those bacteria able to initiate spoilage in various dairy products, by proliferating at different stages of the dairy continuum. Aerobic spore-formers were isolated by using a combination of a heat treatment and discriminative incubation temperatures (7, 35 and 65°C) similar to previous work, although thermophilic spore-formers are often isolated at 55°C (Buehner et al., 2014, Miller et al., 2015b, Murphy et al., 2019). Clostridia were isolated after CMG enrichment, which successfully prevented the growth of facultative anaerobes such as *Bacillus* and *Paenibacillus* species (Gupta and Brightwell, 2017). *Pseudomonas* were isolated with minimal interference from other genera by using a cocktail of antibiotics to which they are inherently resistant (Mead and Adams, 1977). These conditions of isolation were complementary; few bacteria were redundantly detected across the different conditions. *Bacillus* was the only genus recovered after mesophilic, thermophilic and psychrotrophic incubation. Similar observations were reported by Miller et al. (2015b) in bovine raw milk and dairy powders but, in addition to mesophilic and psychrotrophic isolates, these authors also recovered thermophilic *Paenibacillus* spp. after incubation at 55°C.

The isolation of dairy-associated thermophilic spore-formers is routinely carried out at 55°C but temperatures ranging 50 – 70°C are common throughout the powder manufacturing process, during preheating and evaporation (Burgess et al., 2010). In my study, incubation of the environmental samples at 55 and 60°C on both MA and SBA revealed highly abundant populations of thermotolerant *B. licheniformis* which spread on plates and prevented reliable enumeration and isolation (Murphy et al., 2021). At 65°C, the growth of *B. licheniformis* was inhibited but incidental swarming was still

observed for bacteria identified as *Brevibacillus aydinogluensis* and *Bacillus smithii*. Spreading and swarming are common features of aerobic spore-forming bacteria isolated from cow raw milk (Miller et al., 2015a), milk powders (Murphy et al., 2021) as well as bedding materials (Murphy et al., 2019), and may hinder the accurate detection of these contaminants in the farm environment and dairy products. Although MA yielded lower TSC than SBA, it limited the occurrence of swarming and spreading phenotypes. In addition, more diverse thermophilic populations were recovered with MA, but the relative abundance of *T. vulgaris* decreased as compared to SBA. These findings suggest that a single method of isolation is insufficient to accurately depict the thermophilic microbiota of farm samples, considering some bacteria were only isolated from one type of agar media.

Several growth media, including brain heart infusion agar, milk agar, plate count agar, and sheep blood agar in the present study, are routinely used for the isolation and enumeration of spore-forming bacteria from dairy products and the farm environment. Recent studies have shown that the type of media used for bacterial isolation may influence spore counts as well as the observed plate diversity (Kent et al., 2016, Murphy et al., 2021). In this study, the use of SBA for the isolation of aerobic spore-formers yielded a large diversity of mesophilic and psychrotrophic spore-forming isolates. Although thermophilic spores were less diverse on SBA than on MA, spore counts were consistently higher on SBA. This suggests that the combination of several growth media may be required to accurately describe the bacterial diversity of farm samples, particularly among thermophilic spore-formers. Murphy et al. (2021) showed that a single set of testing methods was insufficient to capture variations in the microbial load of dairy powders. The same authors recommended a set of methods, namely the simultaneous use of several heat treatments (80°C/12 min, 100°C/30 min and 106°C/30 min), spread plating on plate count milk agar and incubation at 32°C and 55°C, as intended to optimally assess variations in spore count across bovine dairy powders. In addition to the growth media used, the temperature of the heat-treatment used to select for spores, the method of sampling, as well as the training of laboratory staff are factors that can significantly affect observed spore counts in bovine milk and dairy powders (Kent et al., 2016, Murphy et al., 2021). Although SBA is rarely used to isolates aerobic spore-formers (Gupta and Brightwell, 2017) and has not yet been thoroughly evaluated for its accuracy, the recovery of high spore counts and a broad diversity of bacteria across three aerobic spore isolation conditions (psychrotrophs, mesophiles and thermophiles) suggest that this media is suitable for the isolation and propagation of spore-formers associated with the dairy farm environment. Future work may consider evaluating the accuracy of SBA as compared to other widely used media (e.g., milk agar, brain heart infusion agar) to study the spore-forming bacterial diversity of dairy products and farm samples.

4.3.2 High levels of spores are found in the sheep dairy farm barn environment

Elevated levels of bacterial spores in the dairy farm environment represent a potential risk to milk quality. High spore concentrations may be found in several farm sources (e.g., bedding, feed, soil) and they have been associated to higher spore counts in bovine bulk tank raw milk (Vissers et al., 2007b, Vissers et al., 2007d, Martin et al., 2019, Murphy et al., 2019). Farming systems and practices impact the dynamics of the cycling of bacteria in the farm environment as they define which components are involved in this process but also the extent of their contribution. In the farm considered in this study, dairy ewes were housed in sheltered pens with woodchips bedding and fed a combination of silage, corn, and a rumen supplement. As the animals did not have access to pastures at the time of sampling, these feeds represented the main exogenous source of spores into the barn environment. Some of the bacterial spores present in feed and trough water would have been ingested by the animals and shed in faeces, further contaminating bedding materials and the udders of the animals, before ultimately gaining access to raw milk upon milking. Swab samples of the pen fences were also analysed as dairy ewes were found to frequently rub against or bite fences and fence posts, suggesting they may represent potential reservoirs or vectors of bacteria.

This study highlights the presence of high spore counts in a variety of sources associated with the barn environment of a sheep dairy farm, which underpins their potential transfer to raw milk. High concentrations of aerobic spores capable of growing at several temperatures (i.e., mesophiles, thermophiles and psychrotrophs) have been previously reported on bovine dairy farms (Martin et al., 2019) but their occurrence on ovine dairy farms remained largely unassessed until now. Therefore, the results of this study suggest that similar spore loads are found on bovine and ovine dairy farms and provide baseline spore counts for several sources associated with the sheep dairy farm barn environment. The recorded spore counts were on the higher end of the ranges previously reported in bovine dairy farms, particularly for (reported range for MSC; TSC; PSC) silage (1.27 – 6.22; 0.66 – 5.59; 0 – 2.6 $\log_{10}$ cfu/g) and wood-based bedding materials [3.13 – 6.4; 1.97 – 5.75; 0.95 – 4.83 $\log_{10}$ cfu/g (Buehner et al., 2014, Martin et al., 2019, Murphy et al., 2019)]. The potential to achieve spore counts as low as < 2 $\log_{10}$ cfu/g in key farm sources such as silage and bedding materials emphasise the potential to limit contamination of raw milk on farm by controlling the microbial load present in the farm environment.

High concentrations of bacterial spores on dairy farms may result from two processes: the persistence of spore-formers as endospores or their proliferation as vegetative cells and subsequent sporulation. The cycling of bacteria in the farm environment produces a flow of bacterial spores which has the potential to increase their concentration in specific farm sources. Because of their resilience, these spores persist in the environment and thereby increase in concentration, provided that the number of spores entering a system is higher than those dying or being removed from it (Nilsson and Renberg, 1990). This process may explain the high concentrations of bacteria present in environments that would not be expected to support their growth (e.g., thermophilic spore-formers in surface bedding or trough water, obligate anaerobic spore-formers in aerobic environments). Previous studies have shown that many spore-formers are capable of proliferating in several components of the farm, such as during ensiling (Vissers et al., 2007a, Borreani et al., 2013), in soils and presumably on pastures, including upon establishing symbiotic relationships with plant species (Escobar Diaz et al., 2021), in bedding materials (Magnusson et al., 2007b, Godden et al., 2008) and in the GIT of livestock (Hong et al., 2009). Recent research has unravelled the potential for bacteria with growth requirements not expected to be met in the farm environment (e.g., thermophilic or anaerobic spore-formers), to be actively proliferating in some sources, leading to their dispersal and persistence in other components of the farm. Some species of thermophilic spore-formers can grow at temperatures as low as 30°C, which implies that they may proliferate in self-heating farm sources such as ensiled forage, composted plant matter, the gastrointestinal tract (GIT) of animals or during warm weather (Cross, 1968, Pahlow et al., 2003, Kikue et al., 2019, Giambra et al., 2021). Anoxic environments that may support the growth of anaerobic spore-formers include soil, sections of the GIT of mammals and effluent ponds (Julien et al., 2008, Gupta and Brightwell, 2017, Shabana et al., 2021). Although endospores are a quiescent bacterial state, counts of spores and vegetative cells are often correlated as these physiological states are dependent upon one another (Magnusson et al., 2007b). Preventing the on-farm proliferation of spore-forming bacteria in key reservoirs such as bedding or silage has been a successful strategy to improve the microbiological quality of raw milk (Vissers et al., 2007b, Vissers et al., 2007d, Murphy et al., 2019). However, additional research is required to confirm and identify farm sources in which the growth of spore-formers may be effectively controlled, such as soils and pastures, feed, drinking water or even in the GIT of dairy livestock. Controlling the proliferation or build-up of spoilage bacteria on-farm may be achieved through farm interventions (e.g., chemical or physical removal of spore-formers, use of probiotic strains), or through the adjustment of existing management practices (e.g., improved feed manufacturing and storage, hygiene, or pasture management schemes) and will be key to limit the contamination of raw milk on-farm.

In the present study, faeces and silage were identified as key sources of mesophilic and thermophilic spores and displayed the highest MSC and TSC across the samples. On cow dairy farms, Martin et al. (2019) recorded the highest concentrations of mesophilic and thermophilic spores in manure, although these values were lower by a 1 $\log_{10}$ factor as compared to the ones reported here. The consumption of feed containing high spore counts, such as silage or feed concentrate, may contribute to increase the spore load present in the faeces of dairy livestock due to a concentration effect (Wu et al., 2007, Martin et al., 2019). Silage with high spore counts has lower nutritive value, which decreases milk yield, and its consumption by dairy cows was shown to increase the concentration of spores in their faeces (Arias et al., 2013, Borreani et al., 2019). The silage analysed in this study had high aerobic spore counts, suggesting these bacteria may have proliferated at some stage during ensiling. Mesophilic, and perhaps thermophilic spore-formers may also be capable of proliferating in some sections of the animals GIT where temperatures neighbour 37°C (Brod et al., 1982, Lee et al., 2019). Indeed, several species of thermophilic spore-formers have demonstrated growth at temperatures as low as 30 to 40°C (Yasawong et al., 2011, Kikue et al., 2019). To better understand the transmission dynamics of thermophilic spores on dairy farms, further research is required to identify primary sources that may act as incubators of these bacteria and contribute to their dissemination in the farm environment. As these organisms are particularly resilient and challenging to remove from milk, a reduction in the number of thermophilic spores present raw milk will be key to fulfil the stringent spore thresholds set for the manufacturing of high-quality dairy powdered goods such as infant formula.

In this study, aerobic spore counts were notably high in bedding materials. Throughout use, the deposit of faeces and urine on bedding materials provides a source of bacteria and nutrients that may promote bacterial growth (Magnusson et al., 2007b, Godden et al., 2008). The high MSC and TSC recorded here in faeces (6.92 ± 0.10 and 6.58 ± 0.11 $\log_{10}$ cfu/g, respectively) may contribute to explain the corresponding high spore counts in bedding materials (6.69 ± 0.34; 5.41 ± 0.07 $\log_{10}$ cfu/g, respectively). However, faeces PSC were much lower than in bedding materials (4.20 ± 0.48 and 6.00 ± 0.23 $\log_{10}$ cfu/g, respectively), which suggest the involvement of other processes and contamination sources. This may indicate that (1) high counts of psychrotrophic spores were present in unused bedding and persisted; (2) psychrotrophic spore-formers proliferated in the bedding; (3) there were additional sources of bedding contamination; or a combination of those. Martin et al. (2019) reported similar PSC in the faeces of dairy cows (4.23 ± 0.66 $\log_{10}$ cfu/g) but significantly lower PSC in sawdust bedding (2.39 ± 1.93 $\log_{10}$ cfu/g). Magnusson et al. (2007b) showed that *B. cereus* was actively growing in the bedding of dairy cows and that their incidence was modulated by factors such

as frequency of faeces deposit, and intrinsic bedding characteristics such as pH and bedding type. On cow dairy farms, several other factors were found to impact the growth of bacteria in bedding, including water activity, nutrient content, and initial bedding microbiota (Godden et al., 2008, Murphy et al., 2019). The contribution of these factors to the contamination of bedding materials used on sheep dairy farms will require future assessment. In my study, the most abundant psychrotrophic spore-formers isolated from bedding materials were *Solibacillus* and *Psychrobacillus* species as well as *B. pumilus sensu lato*. Analysis of unused bedding samples would be required to assess the initial presence of these bacteria as well as to evaluate their potential growth over time. High mesophilic and thermophilic spore counts have been reported in a variety of unused bedding materials[up to 6 $\log_{10}$ cfu/g (Murphy et al., 2019)], demonstrating the potential for high initial spore counts, but there is a lack of data pertaining to the PSC of unused bedding materials (Martin et al., 2019).

The detection of similar mesophilic, thermophilic and psychrotrophic spore counts in 3-week-old or 3-month-old woodchips bedding material suggests that bedding replacement may not affect spore counts in the medium to long term. Magnusson et al. (2007b) showed that complete sawdust bedding replacement caused a 2 $\log_{10}$ cfu/g reduction of both vegetative cells and spores of *B. cereus*; however, these populations were restored to their initial levels (5 to 6 $\log_{10}$ cfu/g) after four to six weeks of use. Thus, strategies that aim at mitigating spore counts in bedding materials should aim to achieve long-lasting effects. This may be attained by maintaining adequate bedding and housing cleanliness, including through frequent topping of the bedding area with fresh bedding materials, and by selecting materials with low initial bacterial counts or with inhibitory properties towards spoilage bacteria (Rowbotham and Ruegg, 2015, Murphy et al., 2019).

4.3.3 Ecology of aerobic spore-formers in the barn environment

There is growing evidence suggesting that the majority of the spore-forming contaminants found in the bovine dairy processing environment and dairy products originate from the dairy farm environment, with raw milk being the vector of this transmission. This phenomenon is clearly observed when considering mesophilic spore-formers; species such as *B. licheniformis*, *B. cereus s.l.*, *B. subtilis* and *B. pumilus s.l.* are frequently isolated at high abundance from the bovine dairy farm environment, in raw milk and in end-products (Scheldeman et al., 2005, Miller et al., 2015b). To a lesser extent, a similar trend has been reported for psychrotrophic spores (Huck et al., 2008). However, the populations of thermophilic spores present in cow raw milk, and therefore potentially on bovine dairy farms, differ to those found in dairy powders (Miller et al., 2015b). Throughout the

dairy continuum, the diversity of spore-forming bacteria present in milk shifts, from farm to fork, as the stringency of storage and processing applies selective pressure on the microbiota of raw milk (Huck et al., 2008, Miller et al., 2015b, McHugh et al., 2020). Bacteria with greater fitness or survivability in a set of defined environmental conditions will be more likely to proliferate or persist in these environments and ultimately contaminate end products (Postollec et al., 2012). Early identification of these contaminants in the farm environment can help evaluate the presence of potential microbial threats to dairy quality and their origin on-farm.

The results of my study suggest that the spores of bacteria associated with dairy spoilage are prevalent and abundant on sheep dairy farms, which echoes what has been reported on cow dairy farms. My study provides a detailed overview of the abundance of these bacteria and also identifies yet unreported bacterial species that may impact the quality of sheep dairy products. Across the spore-forming bacteria recovered from this study, spoilage potential was noticeably common (55% positive for protease and 45% positive for lipase) which is not surprising considering many spore-formers are saprotrophs and scavengers involved in the recycling of organic matter (Voronina et al., 2011). Lucking et al. (2013) screened 126 mesophilic and thermophilic spore-forming bacteria associated with the dairy processing environment or product spoilage and found that 50% and 28% showed proteolytic or lipolytic activity, respectively. However, in my study, spoilage potential was dependent on the bacterial group considered. Protease activity was generally most common for thermophiles and aerobic or anaerobic mesophiles while lipolysis was more commonly observed in aerobic mesophiles and psychrotrophs. Nonetheless, high variations in enzyme production across isolates belonging to the same genus or species (e.g., *Psychrobacillus* and *Solibacillus* spp., *L. galactosidilyticus*) suggests that spoilage potential can be heterogeneous within the same taxa.

The genus *Bacillus* constitutes one of the most versatile bacterial taxa and is the predominant genus of dairy-associated spore-formers. The heterogenous growth capabilities found across members of this genus explains their ubiquity alongside the dairy continuum but also in the general environment (Hong et al., 2009). With the recent advances in genome sequencing, phylogenetic tools and increasing availability of data, the phylogeny of the *Bacillus* genus has been re-evaluated and many bacteria have been reassigned to novel genera, including but not limited to *Alkalihalobacillus*, *Caldibacillus*, *Lederbergia* and *Peribacillus* (Gupta et al., 2020a, Patel and Gupta, 2020). This updated taxonomy was used in my study and may explain the low observed proportions of *Bacillus s.s.* that were recorded (23% of the overall aerobic isolates, 51% of the aerobic mesophilic isolates). On cow dairy farms, in raw milk and dairy products, *Bacillus s.s.* were found to represent 80% of the psychrotrophic and mesophilic spore-forming microbiota (Huck et al., 2008, Lucking et al., 2013,

Masiello et al., 2017). In my study, *Bacillus* species were highly prevalent and detected across the three aerobic spore detection methods, but most particularly as mesophiles. The prevalent and predominant mesophilic spore-formers isolated from the samples considered in my study consisted of bacteria previously implicated in bovine dairy spoilage. In particular, *B. licheniformis, B. pumilus s.l., A. clausii* and *B. subtilis* were ubiquitous and/or highly abundant but also demonstrated strong spoilage potential, in line with previous findings (De Jonghe et al., 2010, Lucking et al., 2013). *Bacillus licheniformis*, *A. clausii*, *B. pumilus s.l.* and *B. subtilis* constitute dairy-ubiquitous spore-formers that are consistently isolated at high abundance from cow dairy farms, raw milk, the processing environment and end-products (Buehner et al., 2014, Zain et al., 2015, Martinez et al., 2017). Some strains of these organisms may be psychrotolerant (e.g., *B. cereus s.l.*), thermotolerant (e.g., *B. subtilis*), or both (e.g., *B. licheniformis*), which may add to their adaptability and contribute to their ubiquity. Several *Bacillus s.l.* are also associated with the gut microbiota of mammals. Bacteria including but not limited to *B. subtilis, B. licheniformis, B. pumilus s.l., B. cereus s.l* and *A. clausii* have been evaluated or commercialised as probiotics in both human and dairy livestock (Fakhry et al., 2008, Haldar and Gandhi, 2016, Lopetuso et al., 2016). Their successful use as probiotics suggests they may colonise the GIT of mammals and live out their full life cycle, which could contribute to explain their high prevalence and abundance in livestock faeces and at the farm level. Amongst these, *B. cereus s.s.* is a bacteria involved in both dairy quality and safety (Webb et al., 2019). Although *B. cereus s.s.* has been isolated from silage, bedding and animal faeces on bovine dairy farms (Magnusson et al., 2007a), it was not isolated from this farm environment. Silage, pastures and particularly soil constitute the principal reservoirs of this bacterium, and the grazing period has been associated with higher abundance of *B. cereus* in cow faeces and raw milk (Wu et al., 2007, Coorevits et al., 2008). My results suggest that housing may be associated with a lower incidence of *B. cereus s.s.* in the environment of dairy ewes, and perhaps also in raw milk. This substantiates the findings of Coorevits et al. (2008) who showed that *B. cereus* was more prevalent in organic farms than in conventional dairy farms, the latter housing the animals for longer periods of time, thereby also decreasing exposure to pastures. However, this will need to be confirmed by surveying the prevalence of *B. cereus s.s.* on additional sheep dairy farms, including farms operating under a pasture-based system.

Spoilage bacteria are widely distributed on dairy farms and as a result, their entry in raw milk is highly likely. However, by reducing the prevalence and abundance of these bacteria on dairy farms, there is potential to limit the numbers of bacteria gaining access to milk. Until recently, strategies to control the bacterial populations in different environments mainly consisted in their eradication. Yet, research

on environmental probiotics has demonstrated that fighting microorganisms with bacteria could be more sustainable, biologically stable on the long term and sometimes more efficient than chemical or antimicrobial-based disinfection (Caselli et al., 2016, Berings et al., 2017). Therefore, there is potential to limit the proliferation of spoilage bacteria and reduce their prevalence on dairy farms by introducing microorganisms that may compete with these bacteria for nutrients and the colonisation of ecological niches. For example, lactic acid bacteria can be added to forage prior to ensiling to ensure proper and rapid acidification of the silage, thereby preventing the growth of deleterious bacteria such as bacilli and clostridia (Muck et al., 2018, Keshri et al., 2019). In my study, the predominance of bacteria not associated with spoilage in specific sources could provide potential candidates for a similar approach. Mesophilic isolates identified as *Oceanobacillus caeni* demonstrated weak spoilage potential, but they were highly abundant in trough water and most particularly in older bedding materials. Although their detection in trough water may have resulted from cross-contamination of the water troughs by bedding materials and faeces, their high abundance in bedding suggests these bacteria proliferated and may have been able to outcompete *Bacillus* species such as *B. licheniformis* and *B. pumilus s.l.*, which were less abundant in this instance. Driehuis et al. (2014) isolated *O. caeni* after stringent heat treatment (30 min 106°C) and 37°C incubation of compost bedding collected from bovine dairy farms, demonstrating their presence in different types of bedding. Some species of *Oceanobacillus* are capable of disrupting biofilm production and quorum sensing, two biological processes facilitating environmental colonisation (Kavita et al., 2014, Seghal Kiran et al., 2014), which suggests they could have an antagonistic effect on other bacteria in bedding materials. Further characterisation of the bacteria isolated from bedding materials, for example through genome mining, phenotyping microarrays and *in situ* competition assays, may help identify potential candidates to be used as environmental probiotics. Livestock and poultry bedding inoculants are readily available in the market and supposedly have an action on the abundance of pathogens (e.g., *Salmonella* and mastitis-causing bacteria) or may improve animal health and comfort by diminishing bedding ammonia content and other unpleasant smells (Pedroso et al., 2013, Lallemand Animal Nutrition, 2021). These prebiotics and probiotics consist of a combination of lactic acid bacteria, *Bacillus* species, lignocellulosic enzymes, and nutrients. *Bacillus pumilus*, *B. subtilis* and *B. amyloliquefaciens* are the *Bacillus* species most commonly used for this purpose, however, they are also spoilage bacteria, and it is yet unknown if their use as environmental probiotics has the potential to increase their occurrence on dairy farms and in raw milk.

In the present study, the predominant psychrotrophic spore-formers detected in the farm environment consisted of *Solibacillus* and *Psychrobacillus* which demonstrated weak spoilage potential. Huck et

al. (2008) identified *Bacillus* and *Paenibacillus* species as the predominant psychrotrophic spore-formers present on cow dairy farms. However, this study was conducted before *Psychrobacillus* and *Solibacillus* were split from the *Bacillus* genus (Krishnamurthi et al., 2009, Krishnamurthi et al., 2010), implying that, if present, these bacteria may have been misidentified as *Bacillus* species. *Solibacillus* and *Psychrobacillus* spp. are only incidentally isolated from cow dairy products (Kent et al., 2016, Da Silva Duarte et al., 2020) as they are quickly outcompeted by *Bacillus* and *Paenibacillus* spp. in refrigerated milk (Masiello et al., 2017, Buehler et al., 2018). Ivy et al. (2012) isolated *Solibacillus* and *Psychrobacillus* species at low abundance among psychrotrophic spore-formers present in bovine raw and pasteurised milk and as well as from dairy farm environments. In my study, their spoilage potential was weak to moderate, which corroborates previous assessments (Ivy et al., 2012, Buehler et al., 2018) and suggests that these genera are likely not associated with milk quality.

Isolates of *Psychrobacillus*, *Solibacillus Sporosarcina, Bacillus* and *Paenibacillus* could be detected across multiple isolation methods, mainly as mesophiles and psychrotrophs. Notably, *Paenibacillus* were found at moderate abundance amongst psychrotrophs (9%) and mesophiles (2%). In the absence of post-pasteurisation contamination, spoilage of pasteurised milk is often attributed to *Paenibacillus* species that persist in milk or colonise the processing plants (Doll et al., 2017). Although they are not the predominant genus in raw and freshly pasteurised milk, *Paenibacillus* can account for two-third of the spore-forming microbiota after refrigerated milk storage and they may produce hydrolytic enzymes capable of causing spoilage in fluid milk (Huck et al., 2008, Ribeiro Júnior et al., 2016). My results support the hypothesis that *Paenibacillus* species are not the predominant genus of psychrotrophic spore-formers present at the early stages of the dairy continuum (i.e., in the farm environment and in fresh raw milk). Instead, after contaminating raw milk at low concentrations from the farm environment, the spores of *Paenibacillus* can survive pasteurisation and proliferate in refrigerated pasteurised milk with higher fitness than *Bacillus* spp. and other thermoduric psychrotrophs. Doll et al. (2017) isolated *P. xylanexedens*, *P. tundrae* and *P. odorifer* from bovine bulk tank milk but only isolated the latter two in pasteurised milk, with *P. odorifer* being predominant. In my study, silage and trough water were the main reservoirs of *Paenibacillus* species and *P. xylanexedens*, *P. tundrae* and *P. odorifer* were all isolated from silage, with *P. xylanexedens* being also isolated from faeces and trough water. Silage has been documented as an important source of *Paenibacillus* which are facultative anaerobes and may proliferate during ensiling (Borreani et al., 2013, Borreani et al., 2019). Future research is needed to assess if *Paenibacillus* species, but also other spore-forming bacteria are capable of growth in water. The presence of these bacteria in trough water may also be a consequence of cross-contamination by other farm sources, predominantly feed

and bedding materials but also the animals and their faeces (LeJeune et al., 2001, Ayscue et al., 2009). In my study, there were 15 clusters of *Paenibacillus* isolates that could not be confidently identified and which may represent novel *Paenibacillus* species. The isolation of a large set of putative novel species of *Paenibacillus* has also been reported in bovine dairy products (Ivy et al., 2012, Doll et al., 2017), which emphasises our current incomplete understanding of this genus. To resolve the phylogeny of these putatively novel bacteria, whole-genome sequencing, phenotyping assays and additional phylogenetic analysis (e.g., sequencing of housekeeping genes, WGS) will need to be carried out.

Thermoactinomyces vulgaris was the most prevalent and abundant thermophilic bacteria isolated in this study. *Thermoactinomyces vulgaris* has been isolated from bovine raw milk and dairy powders in the USA (Buehner et al., 2014, VanderKelen et al., 2016) and was also found to be highly prevalent in cow dairy powders from China (Yuan et al., 2012, Sadiq et al., 2016a). I recorded strong proteolytic activity for all the *T. vulgaris* isolates and for the thermophilic actinomycetes in general (i.e. *Thermoactinomyces* and *Thermobifida*) while lipase activity was rare. These results are in line with previous reports (Elwan et al., 1978, Falkowski and Janda, 2003) and suggests that these bacteria have the potential to produce hydrolytic enzymes during the manufacturing of dairy powders, which could impact their quality. Spores of *T. vulgaris* are highly heat-resistant and can survive treatments as high as 120°C for 30 min, which underpins their potential transfer from raw milk to dairy powders (Sadiq et al., 2016b). Sources of *Thermoactinomyces vulgaris* on dairy farms include soil, bedding, animal feed, manure and compost (Cross, 1968, Kotimaa et al., 1991, Li et al., 2019, Giambra et al., 2021). Specifically, *Thermoactinomyces* spores originate from manure (Cross, 1968) and are more generally associated with agricultural activity (Unsworth et al., 1977, Nilsson and Renberg, 1990). When large amounts of *Thermoactinomyces* spores are aerosolised from hay, dust or other agricultural substrates, inhalation by farm staff may trigger hypersensitivity pneumonitis and cause farmer's lung disease, an immunologically mediated inflammatory disease of the lungs (Frank and Haselwandter, 1982, Brinkmann et al., 2014). Therefore, controlling the numbers of these spores in the farm environment could have benefits not only for dairy quality but also for the health of farm workers. Thermophilic actinomycetes and other thermophilic bacteria can proliferate on-farm in self-heating plant matter that is composted or naturally composting (Van Den Bogart et al., 1993). In addition, high numbers of thermophilic bacteria may be present in several feedstuffs, including silage (> 5 $\log_{10}$ cfu/g in my study), and will be ingested and shed by dairy livestock (Martin et al., 2019). Although optimal growth temperatures of thermophilic spore-formers range between 50 to 60°C, many are capable of growth at 30 to 35°C, including *T. vulgaris*, *Thermobifida fusca*, *Bacillus*

kokeshiiformis and *Aeribacillus pallidus*, while other genera like *Geobacillus* and *Ureibacillus* may also proliferate around 40°C (Yasawong et al., 2011, Kikue et al., 2019). During ensiling and the subsequent storage of silage, the stack may reach temperatures well above 30°C as a result of microbial metabolic activity taking place during fermentation and particularly throughout aerobic deterioration (Pahlow et al., 2003, Kung, 2011). Further, in healthy sheep, rumen temperatures are close to 37°C (Brod et al., 1982). These environments may provide somewhat suitable conditions for the proliferation of thermophilic spore-formers, which could contribute to the spore concentrating effect observed in the faeces of dairy livestock. In support of this hypothesis, the thermophilic spore-formers isolated from faeces were identified as those species able to grow at 30 to 37°C (Kikue et al., 2019), i.e., *T. vulgaris*, *Thermobifida fusca*, *Cald. kokeshiiformis* and *Aer. pallidus*.

In addition to the ubiquitous *B. licheniformis*, *Anoxybacillus* and *Geobacillus* species constitute the majority of the thermophilic contaminants present in bovine, ovine and caprine dairy powders (Rueckert et al., 2004, Sadiq et al., 2016a, Delaunay et al., 2021). *Geobacillus* have been previously isolated at low relative abundance from cattle raw milk and feed (Scheldeman et al., 2005, Miller et al., 2015b) but not *Anoxybacillus.* In this study, I isolated *Anoxybacillus rupiensis* and three species of *Geobacillus s.l.* (*G. thermodenitrificans*, *G. lituanicus* and *Parag. caldoxylosilyticus*) from silage, bedding and on fences. Notably, the silage analysed here contained close to 5 $\log_{10}$ cfu/g of *G. thermodenitrificans*. I recorded weak spoilage potential in *G. thermodenitrificans* but it was one of the most potent biofilm-forming bacteria present in cow milk in the study by Karaca et al. (2019), underlining its potential to colonise dairy powder manufacturing plants and contribute to biofouling during processing runs.

Miller et al. (2015b) showed that the thermophilic contaminants of dairy powders significantly differ from the populations isolated from raw milk. Based on my findings, it seems plausible that the main contaminants of dairy powders, obligate thermophilic spore-formers, are present at low abundance in the farm environment and contaminate raw milk at low levels. Using culture-based methods, thermotolerant spore-formers such as *B. licheniformis*, which are more abundant in the farm environment, are detected and mask the growth of obligate thermophiles such as *Geobacillus s.l.* and *Anoxybacillus* species. However, during the powder manufacturing process, their greater fitness at high temperatures could favour their proliferation over that of thermotolerant bacteria and further lead to the colonisation of the pipelines through biofilm formation, thereby leading to their predominance in finished powders products (Murphy et al., 1999). Once settled in a biofilm at the plant, thermophilic spore-formers may persist in the manufacturing environment and adapt independently to the communities present in the farm environment, which could explain the genomic

divergence between these populations (Burgess, 2016). Whereas the bacterial diversity present in raw milk and dairy powders differs, raw milk TSC often remain under strict requirements from manufacturers that aim to limit biofouling during processing and achieve low spore counts in finished products (Murphy et al., 2016, Murphy et al., 2019). Therefore, monitoring the prevalence of thermophilic spore-formers present in the farm environment and entering raw milk may help improve the quality of raw milk destined for the manufacturing of dairy powders. An identification of the farm sources involved in the proliferation of thermophilic spore-formers will provide further insight on ways to disrupt the transmission of these bacteria and reduce their prevalence in the farm environment.

Some species of spore-formers produce spores capable of surviving high heat treatments (i.e., > 100°C), such as those used during ultra-high temperature (UHT) processing. As many of these bacteria are found on dairy farms, they have the potential to be carried from raw milk to highly heat-treated dairy products (Scheldeman et al., 2005). The present study isolated *B. smithii*, *Cald. thermoamylovorans*, *U. thermosphaericus* and *Aer. pallidus* (thermophiles) as well as *B. licheniformis*, *V. promii*, *B. amyloliquefaciens*, *B. subtilis* (mesophiles) and *Peri. simplex* (psychrotrophs) from various sources of the farm environment. These bacteria have been previously isolated from dairy powders, raw milk, silage and fodder following high-heat treatments [e.g., 125°C for 30 min (Witthuhn et al., 2011, Sadiq et al., 2016b, Stoeckel et al., 2016)].

4.3.4 Ecology of *Clostridium* and related genera in the barn environment

In New Zealand, several hard and semi-hard sheep milk cheeses are manufactured, such as Pecorino and Manchego (Peterson and Prichard, 2015). Butyric acid bacteria (also called lactate fermenting clostridia) can negatively affect the quality of hard and semi-hard cheeses as a result of metabolic activity during ripening. Cheese defects such as late blowing and other organoleptic alterations are mainly the result of lactate fermentation (production of gas and butyric acid) as well as protein hydrolysis and fermentation (production of peptides and amines) by clostridia. Clostridial fermentation during cheese ripening may be prevented by the partial removal of clostridial spores present in milk using processes such as bactofugation and microfiltration or by the addition of compounds preventing spore germination [e.g., lysozyme, nitrate (Bergère et al., 1969, Ávila et al., 2014)]. However, the effectiveness of these interventions is limited, particularly when butyric acid bacteria spore counts are high in raw milk (Vissers et al., 2007b). A successful strategy to limit the incidence of late blowing defects in cheeses has been to monitor and control the amount of clostridial

spores contaminating raw milk at the farm level. A better understanding of the diversity and sources of clostridial spores on sheep dairy farms is required to initiate targeted actions to reduce their occurrence in milk. Until now, limited data was available on the prevalence of clostridial spores on NZ dairy farms. The results of my study suggest that clostridia are widely distributed in the NZ sheep dairy farm environment as they were isolated from each sample considered. Several clostridia previously implicated in cheese late blowing events were found to be prevalent, namely *C. sporogenes*, *C. butyricum* and *C. perfringens*, while two species frequently associated with cheese spoilage, *C. tyrobutyricum* and *C. beijerinckii*, were not detected from any sample.

The dairy farm environment harbours large concentrations of clostridial spores, including in soil and manure (around 4 $\log_{10}$ cfu/g) but also in feed, particularly silage [2 to 7 $\log_{10}$ cfu/g (Julien et al., 2008, Driehuis et al., 2016, Borreani et al., 2019)]. Silage has been identified as an important source of clostridial spores in raw milk because these bacteria can proliferate during ensiling when conditions are suitable (see section 2.5.1.2). The spores of butyric acid bacteria (BAB) present in silage are ingested by dairy livestock and may be shed in faeces, further contaminating the surface of the udders and entering raw milk during milking (Driehuis et al., 2016). Feeding silage to dairy livestock can increase the incidence of late blowing in manufactured cheese, therefore its use is often prohibited in sheep dairy farms dedicated to the manufacturing of protected designation of origin cheeses (Pomiès et al., 2014). The silage samples analysed in this study exhibited the greatest diversity of spoilage-associated clostridia and shared five common species, *C. butyricum*, *C. sporogenes*, *Paracl. benzoelyticum* (related to *Paracl. bifermentans*, formerly *C. bifermentans*) as well as *C. perfringens*, all of which have implications for the quality of cheese and silage (Reindl et al., 2014, Bermudez et al., 2016, Driehuis et al., 2018). The clostridia able to impact the quality of cheeses are also generally implicated in silage quality because similar metabolic processes, namely lactate fermentation and protein hydrolysis, are responsible for the spoilage of these products. The main clostridia involved in silage deterioration include *C. tyrobutyricum*, *C. beijerinckii*, *C. butyricum* and *C. perfringens* (Driehuis, 2013). The role of clostridia such as *C. sporogenes*, *Paracl. benzoelyticum*, *T. petrolearius* and *C. cadaveris* during ensiling remains unclear but these bacteria are frequently isolated from finished silage (Zheng et al., 2017, Li et al., 2020, Zheng et al., 2020). In the study of Li et al. (2020), the abundance of *C. cadaveris* was higher in clostridial silage while that of *T. petrolearius* was higher in silage of good quality. It is possible that some of these bacteria are contributing in some ways to the ensiling process or that their abundance is linked to the physicochemical properties of silage, and therefore to its quality. In the present study, a set of strongly proteolytic clostridia were isolated from silage, namely *Paracl. benzoelyticum*, *C. senegalense* and

Clostridium spp. (*uliginosum*). These bacteria may contribute to initiate clostridial fermentation during ensiling through proteolysis. Insights into the phenotype (e.g., pH tolerance) and genotype of these bacteria may provide further information on their molecular capabilities and potential involvement during ensiling. Additionally, their inoculation in silage and monitoring of the ensiling process, similarly to the study of Zheng et al. (2020) with *C. perfringens*, could further our understanding of the dynamics of these bacteria during ensiling.

Contrary to proteolysis, lipolysis during cheese ripening is desirable to a certain extent as it contributes to the development of unique cheese sensory attributes (Collins et al., 2003). Here, lipase activity was notable in *C. butyricum, C. sporogenes, T. petrolearius, C. cadaveris, Clostridium* sp. (*uliginosum)* and *C. perfringens*. Lipase activity has been previously reported for some clostridia including *C. butyricum* (Duan et al., 2017), *C. sporogenes* (Bacques et al., 1983) and *C. perfringens* in which it may contribute to pathogenicity (Blagoveshchenskii et al., 1972). Although lipolysis naturally occurs during the ripening of cheeses, clostridia-associated lipolysis has the potential to impact product quality in semi-hard cheeses in which this process is limited, such as in Cheddar or Gouda cheese (Bills and Day, 1964).

In addition to impact cheese quality, several clostridia are of medical importance due to the toxins they may produce. *Clostridium botulinum* is an infamous pathogen, but other species are also able to cause disease in humans and animals, including but not limited to *C. perfringens*, *C. tetani*, *C. difficile*, *C. novyi* and *C. butyricum* (Gill, 1982, Fenicia et al., 2002). Although not involved in the spoilage of dairy powders, the presence of clostridial spores in powdered milk is undesirable (Barash et al., 2010). *Clostridium perfringens* is of particular importance as this bacterium can cause disease in both humans (e.g., food poisoning) and livestock (e.g., enterotoxaemia). *Clostridium perfringens* can be divided into seven different toxinotypes, denoted A to G, each producing a unique combination of toxins (Rood et al., 2018). *Clostridium perfringens* was ubiquitously detected in my study and the isolates produced only α-toxins, suggesting the toxinotype of these isolates to be type A (Appendix 4.6). *Clostridium perfringens* type A, which is considered the most common toxinotype, are food-poisoning human pathogens associated with temperature-abused cooked meals (Petit et al., 1999). Recently, Rood et al. (2018) proposed two new *C. perfringens* toxinotypes: type F and type G. Type F, which carries both the α-toxin gene and the *cpe* gene (coding for the *Clostridium perfringens* enterotoxin) is responsible for human food-poisoning and non-foodborne *C. perfringens*-mediated diarrhoea (McClane et al., 2006). Type G toxinotypes produce α-toxin and the NetB toxin, and they have been shown to cause necrotic enteritis in chickens (Keyburn et al., 2008). The ELISA kit I used to evaluate the production of toxins did not allow for detection of the CPE and NetB toxins. As type

F and G, like type A, produce α-toxins, future studies will need to be carried out to confirm the toxinotype of these *C. perfringens* isolates. This can be achieved using PCR to survey the presence of the *cpe* and *netB* genes (Rood et al., 2018), which may be complemented by a functional study of these genes and associated toxins. *Clostridium perfringens* types B, C, D and E, but generally not types A, F or G are responsible for diseases such as enterotoxaemia, necrotic enteritis and dysentery in livestock, with sheep and lambs being particularly susceptible to these illnesses (Petit et al., 1999). My results suggest that the *C. perfringens* toxinotypes involved in sheep and lamb disease were not present in the sheep dairy farm environment. In New Zealand, sheep are generally vaccinated against *C. perfringens* types C and D as part of the pre-lamb vaccinations. The abundance of these *C. perfringens* toxinotypes in the farm environment may decrease if these bacteria are not able to colonise the GIT of dairy sheep, which, alongside faeces, could act as a reservoir of these bacteria.

In this study, CMG enrichment enabled the recovery of *Clostridium* and related species without interference from facultative anaerobes such as *Bacillus* and *Paenibacillus*. This selective growth is likely mediated by compounds with antimicrobial properties secreted by clostridia during CMG enrichment (Pahalagedara et al., 2020). It is plausible that these antimicrobials compounds may inhibit the growth of some clostridia or that the enrichment process favours the growth of specific species, which may introduce bias in the diversity observed. Furthermore, as my identification approach focused on isolates exhibiting lipase or protease activity, species that consistently lack these enzymes may have been overlooked. Due to the enrichment step, the abundance of the different clostridia could not be determined. However, *C. perfringens*, *Paracl. benzoelyticum* and *T. petrolearius* were by far the most frequently isolated clostridia, suggesting that the growth of these bacteria is favoured by CMG enrichment or that they were highly abundant in the samples.

4.3.5 Ecology of *Pseudomonas* in the barn environment

Another goal of this study was to survey the prevalence of spoilage-associated psychrotrophic bacteria present in the barn environment of a sheep dairy farm. The ecology of these bacteria has been extensively studied in milks from several animal species and locations, highlighting geographical and seasonal variations of the predominant species (Hantsis-Zacharov and Halpern, 2007, de Garnica et al., 2011, Hahne et al., 2019). However the prevalence and diversity of psychrotrophic bacteria on dairy farms has seldom been reported (Druce and Thomas, 1970). Following direct plating and psychrotrophic incubation (7°C for 10 days on SBA) of the fence samples, I recovered bacteria linked to psychrotrophic dairy spoilage (e.g., *Acinetobacter*, *Arthrobacter* and *Pseudomonas*), including

spore-formers (e.g., *Bacillus*), but also bacteria lacking spoilage potential (e.g., *Microbacterium*, *Paeniglutamicibacter*), or exhibiting spoilage activity but likely not associated with milk quality due to their poor fitness at milk refrigeration temperatures, as documented elsewhere [e.g., *Psychrobacter* (Bowman, 2006, Quigley et al., 2013)]. To focus on the main dairy spoilage-associated psychrotrophic bacteria, namely *Pseudomonas* species, CFC media was used for their isolation from the remaining samples. *Pseudomonas* and closely related genera such as *Stenotrophomonas* and *Burkholderia* can be selectively isolated with a cocktail of antibiotics (cetrimide, fusidic acid and cephaloridine) to which they are intrinsically resistant (Mead and Adams, 1977). In some environments, the presence of natural or acquired antibiotic resistance in other bacteria (e.g., *Serratia*, *Proteus*, *Klebsiella*) may hinder the recovery of *Pseudomonas* and related genera (Tryfinopoulou et al., 2001). In the present study, CFC agar successfully enabled the recovery of *Pseudomonas* spp. with minimal interference from other genera, which accounted for 1% of the relative abundance on CFC agar, suggesting that this media is suitable for the isolation of pseudomonads in samples collected from the dairy farm environment.

CFC agar has been extensively used to survey the prevalence of *Pseudomonas* spp. in dairy products and raw milk across a variety of animal species and locations (Leriche and Fayolle, 2012, de Moura Aguiar et al., 2019, Bruzaroski et al., 2020). Due to their ability to form biofilms, *Pseudomonas* spp. are also commonly isolated from livestock-associated drinking water systems and the milking equipment, but their ecology remains poorly understood in the broader dairy farm environment (Vidal et al., 2017, Maes et al., 2019). Therefore, the present work provides baseline *Pseudomonas* count data for several sources of the sheep dairy farm environment. Although high concentrations of *Pseudomonas* counts were recorded in bedding, trough water and animal faeces, they were not detected from silage. Pseudomonads represent one of the predominant bacteria on fresh herbage, but their abundance decreases during the early steps of ensiling as a result of harsh environmental conditions [e.g., low pH, O_2 depletion (Keshri et al., 2019, Zhang et al., 2020)]. However, in a recent study, Ogunade et al. (2018) detected *Pseudomonas* and *Stenotrophomonas* in lucerne silage, both before and after ensiling. The same authors showed that the abundance of these genera was negatively correlated with silage pH, ammonia-N concentrations as well as yeast and mould counts, all of which are indicators of poor silage quality (Ogunade et al., 2018). High silage pH is a sign of poor fermentation while high levels of ammonia-N can be indicative of protein breakdown typically caused by native plant proteases or proteolytic bacteria, mostly clostridia (Kung and Shaver, 2001). Being less resistant than spores, the combination of acidic and anoxic conditions in silage, together with

sample freezing, may have also damaged vegetative bacterial cells and prevented the recovery of culturable *Pseudomonas* from silage in my study.

Whereas spore counts were similar in the two pens (i.e., with either "old" and "new" bedding materials), *Pseudomonas* counts were consistently higher in the set of "new" samples. This difference was, on average, higher by a 1.2 $\log_{10}$ factor across the samples and was higher by a 2 $\log_{10}$ factor in bedding materials. This may suggest that *Pseudomonas* proliferated in fresher bedding materials which, as a result, may have resulted in a greater number of these bacteria being dispersed from bedding to the rest of the barn environment. Another possible explanation contributing to this phenomenon may include the presence of high concentrations of *Pseudomonas* in fresh, unused bedding. Patel et al. (2019) recorded initial counts of non-coliform Gram-negative bacteria (which include *Pseudomonas* and other genera) of 2.53 ± 2.18 $\log_{10}$ cfu/cm^3 in a set of unused bedding materials such as wood shavings, straw, and other organic materials. The same authors found initial counts of these bacteria to be highly variable across the different unused bedding samples (< 1.4 to 6.34 $\log_{10}$ cfu/cm^3), pointing to the importance of selecting bedding materials with low initial bacterial load (Patel et al., 2019). As the natural habitat of *Pseudomonas* includes plants and soil, it is plausible that they may be present and survive in fresh plant-based bedding materials, if they are not stringently processed before use. Bacterial levels in unused bedding are influenced by several external and intrinsic factors, including bacterial load in materials used to formulate bedding materials, contamination during on-farm processing and storage, and proliferation during bedding storage, all of which depend on intrinsic bedding factors such as nutrient availability, pH and water activity (Murphy et al., 2019). Fresh, unused bedding materials carry nutrients contained in plant matter (e.g., lignocellulosic compounds) and, together with animal defecation and bedding temperatures, could provide a suitable growth environment for *Pseudomonas* both during storage and use of bedding (Magnusson et al., 2007b, Godden et al., 2008). The presence of lignocellulosic enzymes in genomes of *Stenotrophomonas* and *Pseudomonas* further highlights their ability to release the sugars found in plant matter, which could then be utilised for growth (Prabhakaran et al., 2015, Chukwuma et al., 2021). Apart from my work, the ecology of *Pseudomonas* in bedding remain largely unassessed and additional research is required to survey their prevalence and abundance in a variety of organic (e.g., straw, wood-based, recycled manure) and inorganic (e.g., limestone, sand) bedding materials. In addition, the growth of *Pseudomonas* in bedding should be further investigated, particularly as my results suggest that the concentration of *Pseudomonas* may be affected by bedding replacement. By understanding how bedding management practices might affect the microbial dynamics of bedding

materials, farmers could plan or adapt their bedding management schemes accordingly and limit the abundance of *Pseudomonas* species on-farm throughout lactation and milking of the animals.

Despite noticeable differences in the *Pseudomonas* counts of the two sample sets, a core microbiota, consisting of members of the *Pseudomonas fragi* subgroup, in majority *Ps. fragi* and *Ps. weihenstephanensis,* was consistently detected at high abundance from both sample sets. *Pseudomonas fragi* has been identified as one of the predominant psychrotrophic bacteria in bovine raw milk and the dairy processing environment, but also in water and soil (Morrison and Hammer, 1941, Marchand et al., 2009, Meng et al., 2017). In the present study, the isolation of *Ps. fragi* from fences, bedding materials, faeces and trough water suggests that the farm environment is an important source of these bacteria. *Pseudomonas weihenstephanensis* was formally discovered by von Neubeck et al. (2016) and may have been previously overlooked as *Ps. fragi* or *Ps. fluorescens* with which it shares many phenotypic and genotypic traits (Marchand et al., 2009, von Neubeck et al., 2016). Nonetheless, *Ps. weihenstephanensis* has been isolated from bovine raw milk (von Neubeck et al., 2015), freshwater salmonid fish farms (Duman et al., 2021) and chicken meat (Lee et al., 2017a), which highlight its prevalence in food systems. Although *Pseudomonas weihenstephanensis* was ubiquitous in my study, its notably high abundance in faeces could suggest manure as a farm source of these bacteria. In the milking cups, members of the *Ps. fluorescens* subgroup were particularly abundant, namely *Ps. libanensis*, *Ps. tolaasi* and *Ps. azotoformans*, all of which exhibited strong spoilage potential, producing both lipases and proteases. In the study by Yuan et al. (2018b), *Ps. libanensis* and *Ps. azotoformans* were amongst the most potent producers of cold-active lipases and proteases. *Pseudomonas azotoformans* was also shown to produce thermostable proteases ($D_{90°C}$ = 69 min) and lipases [$D_{90°C}$ = 33 min (Yuan et al., 2018b)] and was identified as the second most abundant *Pseudomonas* in German bovine raw milk (Hahne et al., 2019). *Pseudomonas azotoformans* is also known to produce grey and blue pigments, causing organoleptic defects in bovine milk (Evanowski et al., 2017) while *Ps. libanensis* produces olfactory compounds during cheese ripening (Morales et al., 2005). Accounts of *Ps. tolaasii* are rare in the context of the dairy production (Ranieri and Boor, 2009, Van Tassell et al., 2012) and this bacterium is the causative agent of brown blotch disease in edible mushrooms (Soler-Rivas et al., 1999). *Pseudomonas fluorescens* was long believed to be the predominant species in refrigerated milk but it was not isolated in the present study. In the past, the frequent detection of this bacterium may be attributable to inaccurate identification methods which often relied on biochemical testing or partial 16S rRNA gene sequencing (Marchand et al., 2009). Partial 16S rRNA gene sequencing is often recognised as insufficient to correctly identify *Pseudomonas* species, in particular when short fragments are sequenced (~ 400 bp), due to the high

similarities in their 16S rRNA gene sequence (Gomila et al., 2015). Here, 16S rRNA gene fragments 800 to 1100 bp in size were sequenced, which, together with ERIC-PCR clustering and morphological features, enabled reliable identification of these bacteria. To ensure correct identification of *Pseudomonas* species, complementary approaches should be considered, including multilocus sequence typing, biochemical testing or MALDI-TOF mass spectrometry (De Jonghe et al., 2011, Carrascosa et al., 2021).

Stenotrophomonas spp. include both human opportunistic pathogens (*S. maltophila*) and plant growth promoting rhizobacteria (*S. rhizophila*). The strains of *Stenotrophomonas* isolated in the present study were all proteolytic but never lipolytic which is consistent with the findings of Wolf et al. (2002) but differs to that of De Jonghe et al. (2011) who found strong lipolytic potential in *Stenotrophomonas* isolated from bovine milk. Although *Stenotrophomonas rhizophila* can grow at 4°C, highlighting its ability to proliferate in refrigerate raw milk, multiple studies have reported that *Stenotrophomonas* are only detected during early storage of raw milk and are later outcompeted by fast-growing psychrotrophs (De Jonghe et al., 2011, Boubendir et al., 2016). Potential farm reservoirs of *Stenotrophomonas* in the sheep dairy farm barn environment were identified as faeces and bedding materials.

4.3.6 Potential sources of milk contamination during housing

Milking cups were used as a proxy to investigate the transmission of bacteria from the farm environment to raw milk. Cup liners showed no apparent tearing or marks and were washed regularly with a hot alkaline wash, meaning they would have contained minimal contamination prior to milking. This means that the bacteria recovered from milking cup swabs were, in majority, those transferred to raw milk during milking by either contact with the udders of the ewes or from external contamination such as dust or faeces. This sampling strategy circumvented the detection of bacteria that may form biofilms in the milking pipeline and contaminate raw milk as it flows to the bulk tank (Bava et al., 2017). High concentrations of spores and particularly *Pseudomonas* were recorded in the milking cups. Scheldeman et al. (2005) recorded MSC of 2.58 $\log_{10}$ cfu/swab in milking cup samples collected from bovine dairy farms and corresponding raw milk MSC of 6.74 $\log_{10}$ cfu/l. Weber et al. (2019) did not detect *Pseudomonas* from milking cups swabs collected on two German bovine dairy farms (detection limit > 0.4 $\log_{10}$ cfu/cm^2) while Capodifoglio et al. (2016) recorded post milking *Pseudomonas* counts on teat cups ranging between 2.56 to 3.17 $\log_{10}$ cfu/ml in Brazil. Conceivably, the sampling approach and equipment used (i.e., swab material, swabbing technique,

timing of the sampling with regards to milking) have the potential to impact the number of bacterial cells that are sampled and therefore affect laboratory counts. In addition, bacterial counts in milking cups are often reported with different units (log_{10} cfu/ml, /swab or /cm^2 in the above examples) which limits comparisons across studies.

Although raw milk was not collected in the present study, the high bacterial counts of the milking cups suggest the possible transfer of a large quantity of bacteria from the farm environment to raw milk. By quantifying the MSC of dirt present on the udder surface (e.g., manure, bedding materials) and in raw milk collected from dairy cows, Vissers et al. (2007c) estimated a quantity of dirt transferred to raw milk ranging between 3 to 300 mg/l. We can broadly assume that the transfer of dirt to raw milk follows similar principles on bovine and ovine dairy farms and that, in the farm considered here, the dirt was predominantly composed of faeces and bedding materials as the animals were kept indoors. Using the mean spore counts (MSC; TSC; PSC) I obtained for bedding materials (6.60; 4.41 and 5.96 log_{10} cfu/g, Appendix 4.1) and faeces (6.92; 6.60 and 4.00 log_{10} cfu/g) as well as the equation proposed by Vissers et al. (2007c), stating that the concentration of spores transmitted into raw milk by dirt is equal to the mass of dirt entering raw milk multiplied by the concentration of spores in dirt, expected spore counts in raw milk would range between 4.08 to 6.40, 2.89 to 6.08 and 1.48 to 5.44 log_{10} cfu/l for MSC, TSC and PSC, respectively. These estimates assume a transfer of dirt ranging from 3 to 300 mg per litre of raw milk, as calculated by Vissers et al. (2007c). The upper range of these spore counts is similar to thermoduric counts (which include spores and other thermoduric bacteria) reported in ovine raw milk [5.92 – 6.25 log_{10} cfu/l (de Garnica et al., 2013, Jiménez-Sobrino et al., 2018)] and well above spore counts reported in bovine raw milk [MSC = 3.50; TSC = 3.36; PSC = 2.76 log_{10} cfu/l (Martin et al., 2019)]. If we further assume that the transfer of *Pseudomonas* from dirt present on the udders to raw milk follows a similar transmission pattern to that of spores, the equation mentioned above produces estimated *Pseudomonas* counts ranging between 4.49 to 6.92 log_{10} cfu/l of raw milk. Bruzaroski et al. (2020) recorded *Pseudomonas* counts in ewe milk of 6.63 log_{10} cfu/l while those of bovine raw milk varied across different studies [6.56 log_{10} cfu/l (Leriche and Fayolle, 2012); 8.73 log_{10} cfu/l (de Moura Aguiar et al., 2019)] as a result of different farming and milking practices used across countries and locations. However, the dynamics of the transmission of bacteria to raw milk may vary between species capable of growing at different temperatures, and as a result of variations in the composition of their cell membrane [e.g. varying cell adhesion properties and polarity (Martin et al., 2019, Murphy et al., 2019)]. In the present study, *Pseudomonas* appeared to be transferred from the farm environment to milking cups at a greater rate than spores, which suggests that their transfer follows different dynamics. The transmission of

bacteria to raw milk is governed by a complex set of factors (e.g., related to the livestock species, general milking hygiene, farm practices), but my results emphasise the importance of achieving low spore concentrations in components of the farm that may be involved in the transfer of bacteria to raw milk. Ultimately, as the presence of bacteria on the udders of dairy livestock cannot be entirely avoided, best hygienic practices during milking will be key to limit the transfer of these contaminants to raw milk.

Extensive sampling of the farm environment and a culturomics approach uncovered a substantial set of spoilage bacteria dwelling in the sheep dairy farm environment. The transfer of these bacteria to milking cups, and potentially raw milk, was evaluated by comparing the bacteria isolated from the environmental samples and the milking cups. Limitations of this approach include that (1) due to timing between bedding change and start of milking, milking cups were collected six weeks after the collection of other sample types, (2) bacteria present at low abundance in the environment could not be detected but may still have been transferred to milking cups, and (3) the detection of a given bacteria in multiple samples does not necessarily indicate transmission across these samples. The microbiota of the dairy farm exhibits extensive genetic variability which requires strain-resolution genotyping for reliable source tracking (te Giffel et al., 2002, VanderKelen et al., 2016). Therefore, the results discussed here are merely a suggestion of candidate species which may be transmitted from the farm environment to milking cups as a result of milking, and which require further Investigation. Future work will include genotyping of these isolates using techniques such as multilocus sequence analysis of housekeeping genes or DNA fingerprinting.

Although ubiquitous, *Pseudomonas* generally thrive in soil (Li et al., 2013) and in association with plants (Ogunade et al., 2018), but few studies have highlighted the transfer of *Pseudomonas* across these sources to raw milk. Vacheyrou et al. (2011) assessed the transmission of environmental bacteria to raw milk and identified hay, the air of the milking parlour, dust and cow teat surface as potential sources of *Pseudomonas* contamination in bovine milk. Here, populations of *Pseudomonas* isolated from milking cups were different to the ones found in the environmental samples, although the milking cups isolates showed strong spoilage potential. However, three of the main bacteria isolated from milking cups and identified as potential dairy spoilers, namely *Ps. azotoformans*, *Ps. koreensis* and *Ps. libanensis*, were each detected from at least one faeces sample, albeit at low relative abundance. Differences in the *Pseudomonas* diversity recovered from milking and the rest of the environmental samples may be attributable to the six weeks gap between their sampling date. It is also possible that the components implicated in the contamination of milking cups by *Pseudomonas*

spp. include sources not assessed in this study, e.g., dairy workers, the animals and the milking environment (Bava et al., 2017, Evanowski et al., 2020).

The statistical analytical tools applied to the aerobic mesophilic, thermophilic and psychrotrophic bacterial diversity datasets suggest potential routes of milking cups contamination that differed based on the spore detection method considered. The analysis revealed an association between the mesophilic spore diversity of silage, faeces, bedding and milking cups, suggesting that mesophilic spore contamination may be driven by factors related to feed and animal hygiene. These findings are consistent with the conclusions of other studies that have identified bedding materials (Murphy et al., 2019), silage (te Giffel et al., 2002), and manure (Vissers et al., 2007d) as key sources of mesophilic spores contamination in raw milk. In previous work on bovine dairy farms, adequate animal hygiene was also found to be particularly important to achieve low mesophilic spore counts in raw milk, suggesting the transfer of bacterial spores present on livestock to raw milk during milking (Vissers et al., 2007d, Martin et al., 2019). Notably, my results suggest that bacteria such as *B. licheniformis* and *B. subtilis* could be originating from silage while others such as *B. pumilus s.l.* may stem from faeces. This is consistent with the findings of te Giffel et al. (2002) who identified *B. licheniformis*, *B. subtilis* and *Paenibacillus* spp. from grass silage and demonstrated the transfer of some of these bacteria to cow raw milk using RAPD-PCR.

Due to the markedly low bacterial diversity observed across thermophilic spore formers, the analytical methods used here were not robust enough to draw accurate conclusions with regards to their transmission to milking cups. However, *Thermoactinomyces vulgaris* isolated from milking cups were diverse (8 different ERIC profiles across 15 isolates) which suggests the involvement of different sources. The detection of *Aer. pallidus*, *Cald. kokeshiiformis* and *U. suwonensis* from silage, faeces and milking cups suggest that feed may act as a source of thermophilic spore contamination in milking cups, intermediately transmitting through the GIT of livestock, faeces, and their udders, to gain entry in raw milk. Scheldeman et al. (2005) isolated *Aer. pallidus* from green crops, fodder, the milking equipment and raw milk on bovine dairy farms, further suggesting that contamination by this bacterium is mediated by feed but also that it accumulates in the milking equipment despite thorough cleaning procedures (Scheldeman et al., 2005).

The psychrotrophic spore diversity of the milking cups was most similar to that of bedding materials and faeces. Like previous observations amongst mesophilic isolates, possible sources of *B. pumilus s.l.* in milking cups were bedding and faeces. Bedding contamination is largely driven by faecal contamination and the proliferation of bacteria in bedding (Godden et al., 2008, Murphy et al., 2019).

High concentrations of psychrotrophic spores in bedding materials may contribute to their rate of attachment to the udders of dairy ewes and increase the contribution of bedding materials to the contamination of the milking cups. *Sporosarcina newyorkensis* represented close to a third of the psychrotrophic spore diversity of the milking cups but it was not detected from the environmental samples. *Sporosarcina newyorkensis* has been isolated from bovine raw milk and human clinical samples, which suggests it may be associated with dairy workers (Coorevits et al., 2008, Wolfgang et al., 2012).

4.4 Conclusion

The dairy farm environment represents a crucial reservoir of bacteria that can be transmitted to raw milk via various farm components, including animal feed, drinking water, faeces, and living areas. While extensive studies have investigated the distribution of dairy spoilage bacteria on bovine dairy farms, sheep dairy farms remain undercharacterised in this regard. This study addresses this gap by conducting a comprehensive survey of bacterial populations in the barn environment of a sheep dairy farm during animal housing. Representing one of the most extensive culture-based microbiological surveys in a dairy farm setting, these results demonstrate the prevalence of spoilage bacteria across five culture conditions in various barn-associated samples. The identification of these spoilage bacteria using a polyphasic approach unveiled the ubiquitous presence of species historically linked to microbial communities found in cow dairy farms and products, underscoring noteworthy parallels in the microbiological aspects of ovine and bovine dairy production. Furthermore, the study identified several microbial hotspots in the farm environment, highlighting them as possible sources of milking cup contamination. The high concentrations of spores and *Pseudomonas* spp. detected across the samples considered in this study, in particular milking cups, underline their potential transfer to raw milk during milking. Several farm components had particularly high spore counts and were identified as potential reservoirs of these bacteria (e.g., thermophilic spores in faeces, psychrotrophic spores in bedding). Samples collected from the pen with fresher bedding materials had *Pseudomonas* counts consistently higher than the samples collected from the pen with older bedding. Intrinsic farm-level factors (e.g., pen exposure to weather) may be responsible for this effect but further research would be required to investigate any effects a change of bedding might have on the populations of *Pseudomonas* present in bedding materials. Preserving the quality of dairy requires producers to minimise the transfer of contaminants from the farm environment to raw milk. By controlling the growth of these bacteria in the environment and by breaking their cycling at the dairy farm, there is

potential for farmers to reduce the bacterial load present in the farm environment and thereby mitigate that of raw milk.

The bacterial count data was complemented by an analysis of the diversity and spoilage potential of these bacteria across the farm, which enabled the identification of potential reservoirs of spoilage bacteria. Among *Pseudomonas* as well as mesophilic and thermophilic spore-formers, predominant populations consisted of bacteria with strong spoilage potential. Some of these bacteria have been associated with dairy spoilage (e.g., *B. licheniformis*, *B. pumilus*, *Ps. fragi*, *Ps. weihenstephanensis*) while others constitute emerging contaminants whose involvement in dairy spoilage remains unclear (e.g., *T. vulgaris*, *Cald. kokeshiiformis*) and will require further assessment.

This study also identified bacteria not associated with spoilage being present at high abundance in specific farm sources (e.g., *Oceanobacillus* and members of the *Pseudomonas aeruginosa* lineage in woodchips bedding). It seems plausible that some of these bacteria have the potential to compete with spoilage-associated species and contribute to reduce their abundance in the environment by competing for nutrients. Further work may consider assessing the potential for some of these bacteria to be used as environmental probiotics, perhaps in combination with farm interventions, in an effort to limit the over-colonization of the farm environment by spoilage bacteria.

Faeces and silage were identified as key sources of milking cups contamination, both in terms of bacterial load and diversity, however with some involvement from other sources, bedding and trough water. The possible sources of milking cups and milk contamination that were identified in this study will require further testing to confirm the transfer of bacteria throughout different sources. To this end, the isolates obtained during this study have been preserved, which will enable future investigation of their phylogeny.

Overall, the isolation, characterisation, and spoilage assessment of more than 2,000 isolates distributed over five conditions of culture provide a unique overview of the farm microbiota. A similar approach undertaken on a larger set of sheep dairy farms, including farms with similar or different farming systems (e.g., pasture-based), would be required to further assess the observations of this study. Nonetheless, the data obtained here may be used by industrials and scientists as a tool enabling quick evaluation of the potential quality threat posed by bacteria isolated from dairy products. In addition, this study provides recommendations on potential research perspectives aiming to improve our understanding of the ecology of spoilage bacteria in the barn environment of sheep dairy farms. Such knowledge will be key to design and implement tailored interventions aiming at

limiting milk contamination and improve its microbiological quality on New Zealand sheep dairy farms.

Chapter 5 - Ecological survey of the dairy spoilage microbiota of a pasture-based sheep dairy farm and potential sources of milk contamination in the grazing environment

5.1 Introduction

In Chapter 4, an extensive survey of culturable bacterial communities within the barn environment of a sheep dairy farm uncovered significant concentrations of bacterial spores and *Pseudomonas* spp. in farm components such as silage, bedding materials, and faeces. My findings implicated these sources in the potential shedding of a substantial number of bacteria into the farm environment and possibly into raw milk. Furthermore, the identification of a core microbiota pervasive across the farm and in milking cups, dominated by bacterial species with strong spoilage potential, underscored the likelihood of high bacterial transmission within the barn environment and to milking cups. This, in turn, indicated the environmental origin of some bacterial contaminants in milking cups. Chapter 5 extends this investigation, employing the same approach and methodology as Chapter 4, to survey the culturable bacterial communities in the grazing environment of dairy ewes. This examination aims to uncover bacterial hotspots and identify potential sources or routes of raw milk contamination associated with the distinctive conditions of grazing, where the natural environment (e.g., pastures, soil) may represent an important source of raw milk contamination.

In the natural environment, dairy spoilage bacteria such as *Bacillus s.l.*, *Paenibacillus*, *Geobacillus s.l.*, *Clostridium s.l.* and *Pseudomonas* spp. can be found in soil, water, decaying organic matter, on plants and in animals where they fill a variety of ecological niches as saprotrophs, symbionts, or pathogens (Hong et al., 2009, Dignam, 2016, Kikue et al., 2019). Consequently, spoilage bacteria are ubiquitous on bovine dairy farms and several major sources of milk contamination have been identified, including components of the farm such as soil (Vissers et al., 2007d), bedding materials (Murphy et al., 2019), feed, and the faeces of dairy livestock (Vissers et al., 2007b, Arias et al., 2013). Because of the intrinsic relationship between the components of the farm, bacteria are transmitted and cycle in the environment, ultimately gaining access to the udders of the animals and to raw milk upon milking (Vissers et al., 2007c, Vissers et al., 2007d). After attachment of the milking clusters, dirt particles from farm sources (e.g., soil, bedding materials, faeces) that have adhered to the udders of dairy livestock can, with the bacteria they contain, flow into raw milk.

Across dairy farms, the function of the components involved in the cycling of bacteria remains similar (e.g., feed, lying area), but the choice of farming systems can affect the dynamics of this cycling by modifying the nature of these components (e.g., types of feed) or their degree of interaction with other sources of the environment (e.g., grazing/housing time). As a result, farming systems and farming practices can have an impact on the microbial load and diversity of both the farm environment and raw milk (Coorevits et al., 2008, Martin et al., 2019). A variety of farming systems are used in the farm dairy production, ranging from highly extensive, in which the use of available resources is maximised in order to minimise costs, to highly intensive, in which high implementation and running costs generate high yield (Pulina et al., 2018).

In Europe, sheep dairy farming has undergone intensification stemming from the shrinkage of farmland area available for the grazing of livestock but also to optimise the yield of the production (Keenleyside and Tucker, 2010, Pulina et al., 2018). Sheep are often continuously housed to ease management and provide shelter from weather and predators (Harrison, 1980, Turchi et al., 2016). On bovine dairy farms, the intensification of the dairy production (housing, use of processed feed) has been associated with a decrease in some facets of the microbiological quality of milk (Vissers et al., 2007b). Specifically, processed feeds such as silage have been identified as a key source of milk contaminants (te Giffel et al., 2002, Arias et al., 2013) while bedding materials can also provide suitable environmental conditions for the growth of bacteria (Godden et al., 2008, Murphy et al., 2019). In European sheep dairy flocks dedicated to the production of high-quality cheeses and products, grazing can be mandated while the use of wet, preserved forages is often limited or forbidden to achieve low bacterial counts in raw milk (Pomiès et al., 2014, Burtscher et al., 2020).

With about 30% of the country covered by grassland (StatsNZ, 2020), the New Zealand dairy production leverages high pasture availability to alleviate operational costs, relying on grazing as principal feed source and frequently keeping the animals exclusively outside. Unlike their bovine counterparts, dairy sheep and goats are more commonly housed, and experimenting around farming practices in New Zealand has led to the trial of several types of production systems on ovine dairy farms, ranging from fully pasture-based to hybrid systems in which animals are alternated between grazing and housing. Recently, several bovine dairy farms have been converted to sheep dairy farming as a mean of diversifying the farm production. The conversion of these pasture-based farms requires minimal financial input as most of the existing infrastructure can be used (e.g., pastures, walkways). Because sheep dairy farming remains in its early development in New Zealand, experimentation around farming systems is required to identify practices that best fit New Zealand natural resources, climate, and farming culture. However, these choices also have implications for the microbiological quality of raw milk.

In the case of sheep dairy farms, which have been understudied, the interplays between farming systems and the microbiota of the farm environment or raw milk remain unclear. Coorevits et al. (2008) highlighted variations between the microbial diversity of raw milk produced on organic and conventional cow dairy farms. The same study also highlighted a trend of higher spore counts in milk produced from farms with a production system considered more intensive (i.e., preserved feed, housing). Several other farm practices have been associated with increased bacterial contamination of both cow (Martin et al., 2019) and sheep raw milk (Arias et al., 2013) but there is a lack of holistic data pertaining to the implications of ovine farming systems on the microbiology of the farm. Pasture-based sheep dairy farms are likely to become more common in New Zealand due to their ease of implementation in the current landscape, however the microbiota of these farms, and thus the bacteria that may enter ewe milk, remain unknown. The microbiota of pasture-based dairy farms is made up, in majority, of the native populations present in the natural environment, i.e., in the soil and pastures of the different paddocks grazed by the animals. As compared to barned dairy animals, grazed ewes have therefore access to a wider living area, which also changes according to the grazing rotation, thereby suggesting that the animals may be exposed to a greater and more heterogeneous range of bacteria.

The aim of the present study was to characterise the microbiota of a pasture-based sheep dairy farm to survey and identify the predominant species of spoilage bacteria found in the grazing environment of dairy ewes. These results will allow a risk assessment of the potential microbial hotspots and sources of milk contamination that may be found in the pasture environment. The culturomics

approach applied during Chapter 4 was also used in this work, thus the results will provide a contrasting perspective of the microbiota found in different sheep dairy farm environments.

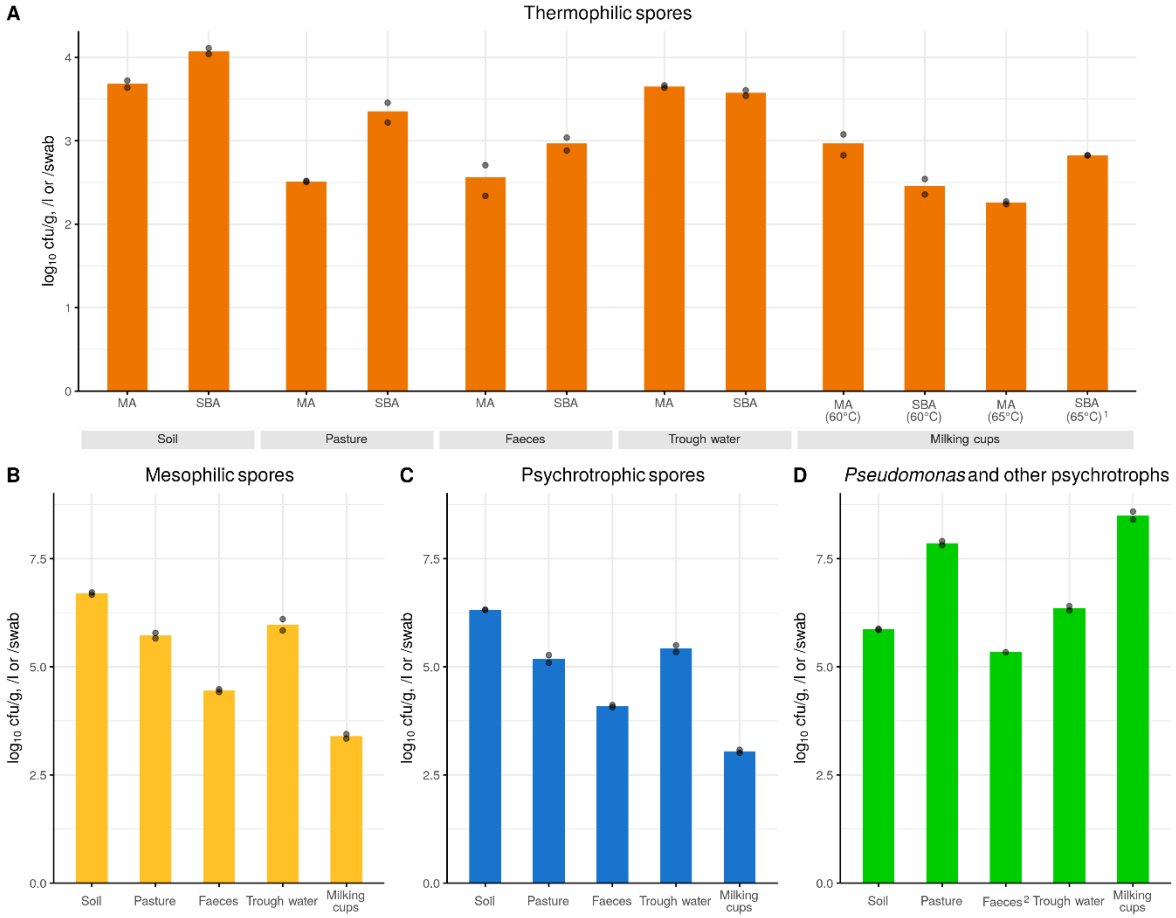
5.2 Results

5.2.1 Bacterial counts

To enumerate spoilage-associated bacteria and recover microbial isolates for characterisation and identification, a culturomics approach identical to that used in section 4.2 was used for the culturing of farm samples (see section 3.1.3). After a heat treatment of 80°C for 10 min and four different culture conditions, aerobic mesophilic, thermophilic and psychrotrophic as well as anaerobic spore formers were recovered from soil, faeces, pasture, trough water and milking cup swabs. Clostridial spores were not enumerated as they were enriched prior to their isolation. For aerobic spores, counts ranging 2.46 to 6.69 cfu $\log_{10}$ were recorded, with soil displaying the highest spore counts in each category (Figure 5.1). Mesophilic spore counts (MSC), thermophilic spore counts (TSC) and psychrotrophic spore counts (PSC) across the environmental samples followed a similar distribution; in decreasing order, soil had the highest counts, followed by trough water, pasture and faeces. MSC and PSC were similar across the samples (range 4.09 – 6.69 $\log_{10}$ cfu) while TSC were lower (range 2.52 – 4.07 $\log_{10}$ cfu). At 65°C, TSC could be recorded on both SBA and MA for all sample types with limited spreading and swarming from the isolates on the agar plates. In the environmental samples, the TSC at 65°C on SBA (2.96 – 4.07 $\log_{10}$ cfu) and on MA (2.51 – 3.68 $\log_{10}$ cfu) were similar. Milking cups spore counts were similar across the three aerobic spore detection methods (range 2.26 – 3.39 $\log_{10}$ cfu/swab, respectively TSC and MSC).

Cetrimide fucidine cephaloridine agar (CFC) was used to enumerate *Pseudomonas* and related genera from sources of the farm environment (section 3.1.3), with counts ranging between 5.34 $\log_{10}$ cfu/g (faeces) to 8.50 $\log_{10}$ cfu/swab (milking cups). Across the environmental samples, trough water, faeces and soil had similar colony counts on CFC agar (CC, 5.34 – 6.35 $\log_{10}$ cfu/g or /l) while pasture had markedly higher CC (7.86 $\log_{10}$ cfu/g). In the milking cups, CC were particularly high (8.50 $\log_{10}$ cfu/swab) and higher than in any other sample type.

Figure 5.1. Concentrations of bacterial spores and *Pseudomonas* and other psychrotrophs in sources associated with pasture environment of a sheep dairy farm



Bacterial counts are presented in log_{10} cfu/g for soil, pasture, and faeces, log_{10} cfu/l and log_{10} cfu/swab for milking cups. Bar charts represent, unless stated otherwise, the average of two plating replicates which are visible on the graphs as black dots.

[1]Due to extensive swarming and spreading, only one plate replicate could be obtained for this condition.

[2]Extensive fungal growth and spreading of the isolates on one of the isolation plates prevented the collection of a duplicate value for this count.

5.2.2 Prevalence and diversity of spoilage bacteria in the pasture-based sheep dairy farm environment

Following enumeration across the 5 different culture conditions, bacterial colonies present on the isolation plates were subcultured to obtain pure cultures that could be characterised in more depths. Some 1,210 spore-formers and 279 psychrotrophic bacteria were isolated from the samples analysed this study. Fingerprinting of the isolates using ERIC-PCR enabled their subtyping and clustering according to their ERIC profiles (see section 3.1.4), which aided the identification of these bacteria. Apart from some thermophilic (n = 4) and mesophilic (n = 12) isolates that could not be propagated after their original isolation, all isolates (n = 1,473) were screened for spoilage potential using plate-based assays (see section 3.1.6). Spoilage screening results revealed that 86% of the isolates exhibited

some form of spoilage potential: 68% were positive for protease activity, 50% for lipase activity and 32% for both protease and lipase activity. On a sample-wise basis, representative isolates which displayed spoilage potential were selected for 16S rRNA sequencing identification based on morphological features (anaerobic isolates) and ERIC profiles (aerobic isolates, see section 3.1.5). The identification status of representative bacteria was used to identify other isolates with similar ERIC types and plate morphology, leading to the identification of 90% of the isolates (n = 1,340). The remaining 149 isolates were not identified as they lacked spoilage potential and did not cluster with any representative bacteria. The bacteria that could not be confidently assigned to a type strain species using the Ribosomal Database Project [RDP, isolates with seqmatch score < 0.970 (Cole et al., 2014)] were instead assigned to the closest genus [e.g. *Pseudomonas* sp. (*fragi*)] and grouped together if they shared at least 99% similarity in their 16S rRNA gene sequence, resulting in 49 clusters of bacteria that could not be confidently identified to the species level (Appendix 5.1).

5.2.2.1 Prevalence, diversity and spoilage potential of spore-forming bacteria

5.2.2.1.1 Aerobic mesophilic spore-formers

Mesophilic spore-formers were particularly diverse and spanned 16 genera and 45 species (Figure 5.2) including 17 clusters that could not be confidently identified (Appendix 5.1). The bacterial diversity was particularly high in soil, trough water and milking cups (Shannon diversity = H' > 2.4) and to a lesser extent in faeces (H' = 1.794), but not in pasture (H' = 0.837, Appendix 5.2). The predominant genera of aerobic mesophilic spore-formers included *Bacillus s.s.* (35% of the total relative abundance), followed by *Priestia* (22%), *Paenibacillus* (8%) and *Solibacillus* (5%).

Across the samples, the diversity of mesophilic spore-forming bacteria was heterogeneous. High metrics of dissimilarity across all samples pairs (BC > 0.80, Appendix 5.3) and an ordination stress of 0 obtained for the NMDS analysis plot (Appendix 5.4) were explained by the fact that only 13 out of 45 species of bacteria were detected in two or more samples. *Bacillus cereus s.l. (*i.e., *B. cereus* and closely related species such as *B. mycoides*, *B thuringiensis*), *Priestia megaterium s.l.* (i.e., *Pr. megaterium* and *Pr. aryabhattai*) and *B. pumilus s.l.* (i.e., *B. pumilus* and closely related species such as *B. safensis*, *B. aerophilus* and *B. stratosphericus*) were the most abundant bacteria and the only species detected ubiquitously. There were four other bacterial species detected in three or more samples, namely *B. licheniformis* (detected in faeces, trough water and milking cups), *Psychrobacillus* spp. (soil, pasture, trough water and milking cups) as well as *Neobacillus* sp.

(*bataviensis*) and *Solibacillus* spp. (both in soil, trough water, and milking cups). Some bacteria were particularly abundant in specific environmental samples, namely *B. pumilus s.l.* in faeces, *Psychrobacillus* spp. in pasture, *Neobacillus*, *Solibacillus* and *Paenibacillus* spp. in water and *Lysinibacillus* spp. in soil. Other bacteria were detected at a low total relative abundance and included members of *Alkalihalobacillus*, *Brevibacillus*, *Cytobacillus*, *Fredinandcohnia*, *Mesobacillus*, *Ornithinibacillus*, *Peribacillus*, *Robertmurraya* and *Viridibacillus*.

A remarkable diversity of bacteria made up of 22 species was recovered from the milking cups, but only 12 of these bacteria were also isolated from the environmental samples. In addition to the ubiquitous *B. cereus s.l.*, *Pr. megaterium s.l.* and *B. pumilus s.l.*, bacteria simultaneously detected in milking cups and the environment included *Psychrobacillus* spp. (soil, pasture, trough water), *B. licheniformis* (faeces, trough water), *Solibacillus* spp. (soil and trough water), *A. clausii* (faeces), *L. macroides* (trough water), and *P. tundrae* (trough water). *Bacillus licheniformis* was the most abundant bacteria in milking cups while the remaining bacteria had a low relative abundance. It is also worth noting that a third of the mesophilic bacteria recovered from the milking cups were not identified due to their lack of spoilage potential.

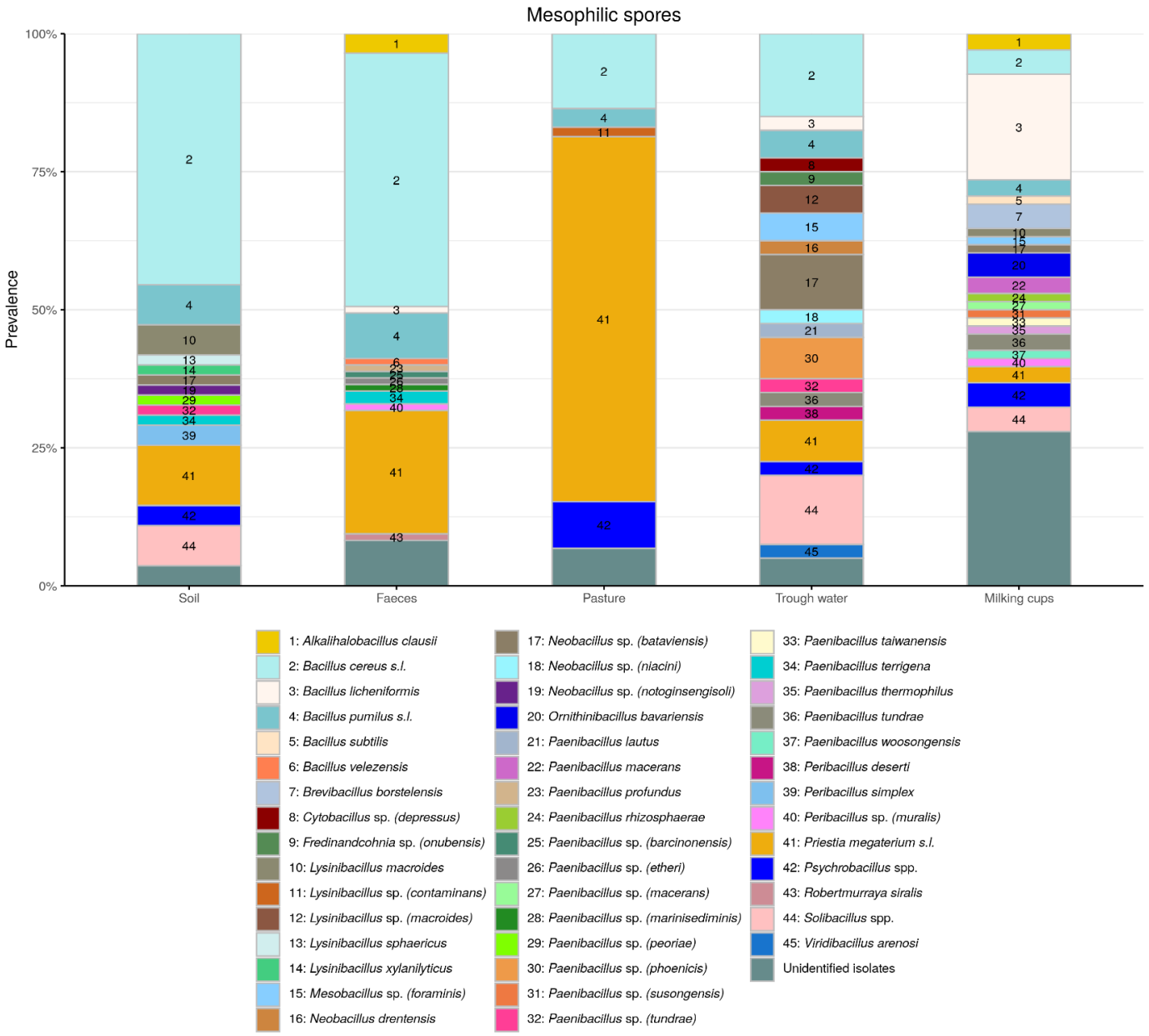


Figure 5.2. Diversity of mesophilic spores in the sources found in the farm environment of a pasture-based sheep dairy farm.

Coloured boxplots with a number represent the relative abundance of a bacteria in a given sample, the number referring to species in the legend. The proportion of unidentified isolates is visible as pale turquoise rectangles on the bottom of each bar graph. Isolates referred to as "sp." could not be confidently attributed to a type strain species based on their partial 16S rRNA gene sequence, further information on this isolates is available in Appendix 5.2.

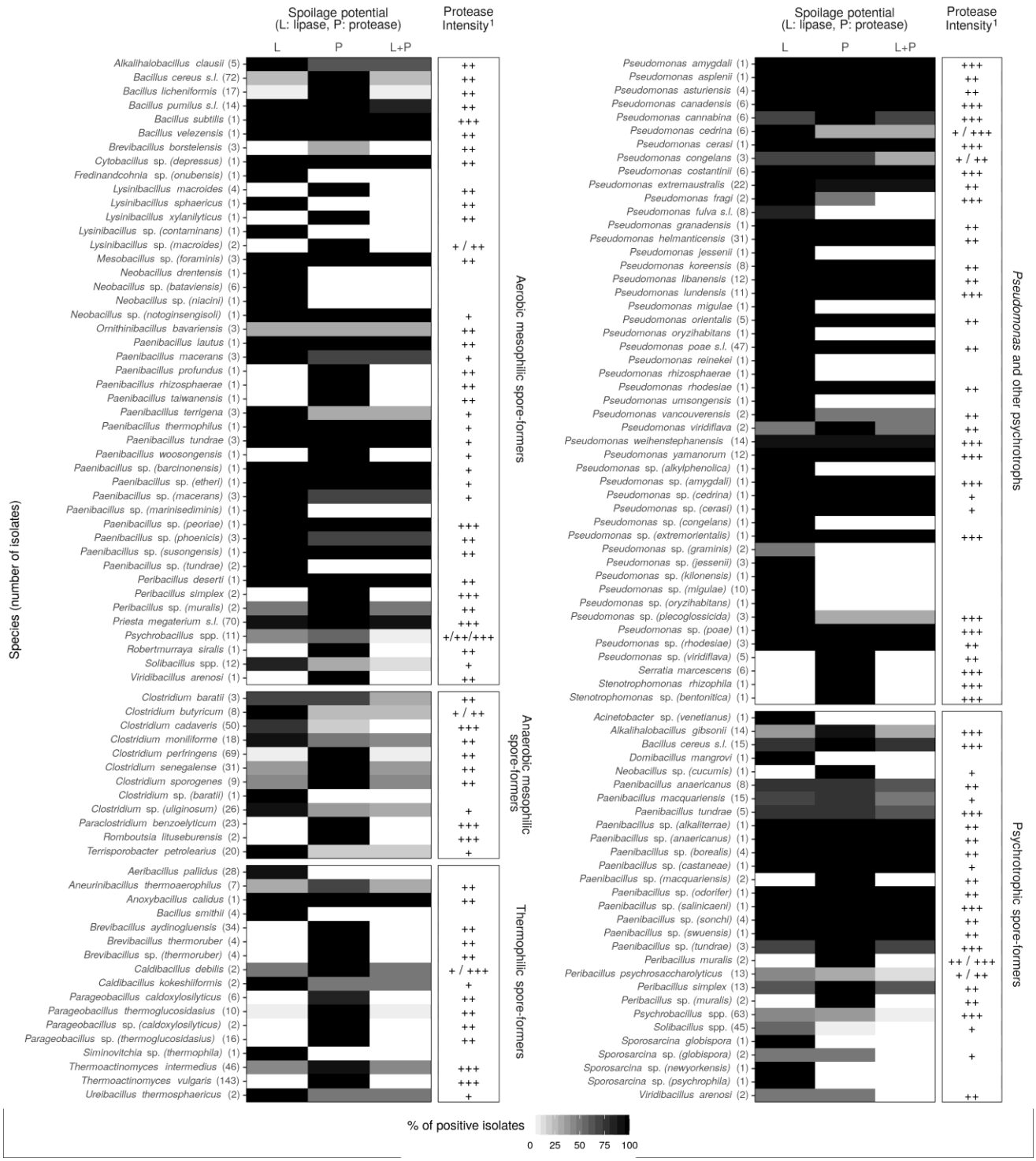


Figure 5.3. Spoilage potential of the spore-forming bacteria, *Pseudomonas* spp., and other psychrotrophs isolated from the environment of a pasture-based sheep dairy farm

For a given species or cluster, the proportion of isolates positive for lipase and/or protease activity is visible in shades of grey (the more isolates showing the corresponding enzymatic activity, the darker the shade). The numbers in the brackets next to each species or cluster shows the number of isolates screened for spoilage potential for that species.

[1]The intensity of the protease activity was ranked as weak (+, weak casein hydrolysis), medium (++, casein hydrolysis only directly under the colony) or strong (+++, diffused casein hydrolysis around the colony). The protease intensity displayed for each species or cluster corresponds to the most common protease intensity for that species.

Mesophilic spore-formers demonstrated strong spoilage potential. However, 28 of the 45 different species of bacteria were screened for spoilage potential in only one or two instances (i.e., when only one or two isolates were obtained). Proteolytic bacteria were highly prevalent, representing 76% of the isolates, and included *Bacillus cereus s.l.*, *Pr. megaterium s.l.*, *B. pumilus s.l.*, *B. licheniformis* and *Lysinibacillus macroides* (Figure 5.3). Lipase activity was recorded in 53% of the isolates, including in bacteria identified as *Pr megaterium s.l.*, *B. pumilus s.l.*, *Solibacillus* spp. and *Neobacillus* sp. (*bataviensis*). Simultaneous lipase and protease activity was observed in 37% of the isolates, mainly *Pr. megaterium* and *B. pumilus s.l.* but also *A. clausii*. No more than three isolates were screened for any given *Paenibacillus* species, but the members of this genus commonly displayed both protease and lipase activities, including for *P. macerans*, *P. tundrae* and *P. thermophilus*.

5.2.2.1.2 Aerobic thermophilic spore-formers

The diversity of thermophilic spore-formers spanned 10 genera and 17 species (Figure 5.4), including four clusters that could not be identified to the species level (Appendix 5.1). Thermophilic spore-formers were isolated from both SBA and MA, when possible, i.e., when the swarming and/or spreading of bacteria on agar media was limited. The thermophilic diversity was obtained after incubation on both MA and SBA for each sample type except for the soil sample (due to extensive swarming after incubation on MA at 65°C) and the milking cups sample (due to extensive swarming and spreading on SBA at 60 and 65°C). Overall, the predominant genera of thermophilic spore-formers included *Thermoactinomyces* (56% of the relative abundance), *Parageobacillus* (16%), *Brevibacillus* (9%) and *Aeribacillus* (6%). Apart from *Parageobacillus* which was only recovered from MA, these genera were isolated from both SBA and MA.

The thermophilic diversity recovered on SBA was low (H' < 1.000, Appendix 5.2), with only five different species being isolated: *Thermoactinomyces vulgaris*, *Brevibacillus aydinogluensis*, *Aeribacillus pallidus*, *Ureibacillus thermosphaericus* and *Caldibacillus kokeshiiformis* (Figure 5.4). *Thermoactinomyces vulgaris* dominated the thermophilic diversity after incubation on SBA. The five bacterial species that were recovered after incubation on SBA were also detected after incubation on MA, however, they were not isolated simultaneously on both media for any sample type. The thermophilic diversity on MA (H' > 1.400) was particularly higher than on SBA and included 12 additional bacteria. *Thermoactinomyces vulgaris* was ubiquitous and the predominant bacteria isolated from MA, but it was overall less abundant than on SBA. Other abundant bacteria included *Thermoactinomyces intermedius* and *Parageobacillus thermoglucosidasius s.l.* [i.e., *Parageobacillus*

thermoglucosidasius and *Parageobacillus* sp. (*thermoglucosidasius*)]. Across the environmental samples, the thermophilic diversity on MA was heterogeneous (BC > 0.72, Appendix 5.3) and the samples were scattered on the NMDS analysis plot (stress = 0.091, Appendix 5.4). Yet, several *Parageobacillus* species were detected in multiple samples, namely *Parag. caldoxylosilyticus* (faeces and trough water), *Parag. thermoglucosidasius* (faeces and pasture), *Parageobacillus* sp. (*caldoxysilyticus*) [pasture and trough water] and *Parageobacillus* sp. (*thermoglucosidasius*) [pasture and trough water]. The bacteria *Parag. caldoxylosilyticus* and *Parag. thermoglucosidasius* were particularly abundant in faeces while *Parageobacillus* sp. (*thermoglucosidasius*) was abundant in pasture.

In the milking cups, several bacteria were isolated at 60°C and 65°C: *Ae. pallidus, Aneurinibacillus thermoaerophilus Br. aydinogluensis, Caldibacillus debilis, T. intermedius* and *T. vulgaris*. *Thermoactinomyces vulgaris* was predominant at 65°C while *T. intermedius* was predominant at 60°C. Apart from *Ca. debilis* and *Siminovitchia* sp. (*thermophila*), all thermophilic bacteria isolated from the milking cups were also found in the environmental samples, namely *T. vulgaris* (ubiquitous), *Ae. pallidus* (soil, pasture and trough water), *B. smithii* (faeces and pasture), *Parag. caldoxylosiliticus* (faeces and trough water), *T. intermedius* (trough water), *Brevibacillus thermoruber* (pasture) and *Brevibacillus* sp. (*thermoruber*) [faeces]. According to the Bray-Curtis dissimilarity metrics (Appendix 5.3) and the NMDS analysis (Appendix 5.4), the thermophilic diversity of the milking cups at 65°C on MA was particularly similar to that of faeces (BC = 0.36).

Thermophilic spore-formers were often proteolytic but rarely lipolytic. Proteolytic bacteria represented 82% of the isolates and included *T. vulgaris, T. intermedius, Br. aydinogluensis, Parageobacillus* spp. and *An. thermoaerophilus* (Figure 5.3). Lipolysis was predominantly detected for *Ae. pallidus* and *B. smithii* while simultaneous protease and lipase activity was found in *Anoxybacillus calidus, Caldibacillus* spp. as well as in some *T. intermedius* isolates.

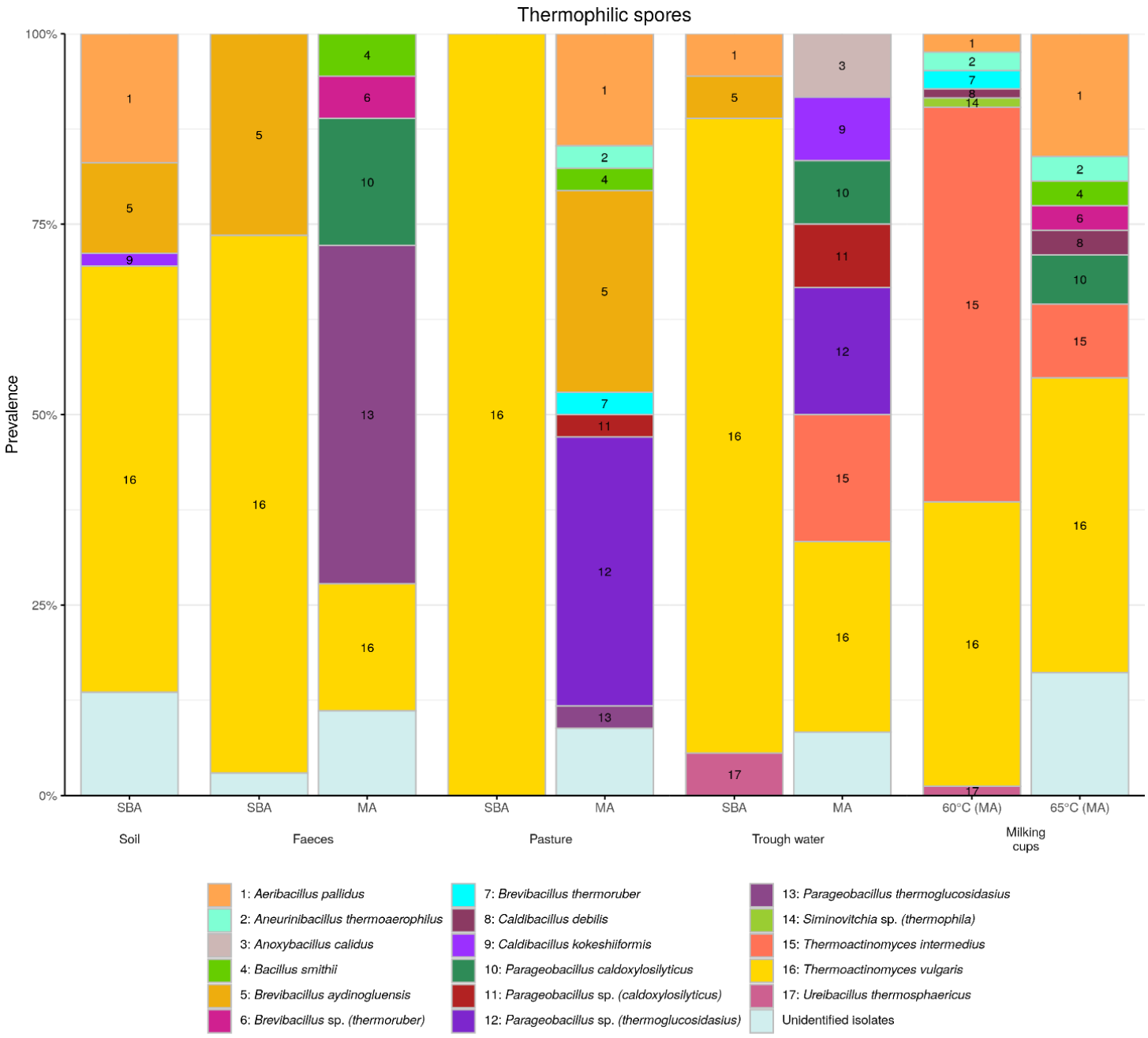


Figure 5.4. Diversity of thermophilic spores in the farm environment of a pasture-based dairy sheep farm.

Coloured rectangles with a number represent the relative prevalence of a bacteria in a given sample, the numbers referring to species in the legend. The proportion of unidentified isolates is visible as pale turquoise rectangles on the bottom of each bar graph. The isolates referred to as "sp." could not be confidently attributed to a type strain species based on their partial 16S rRNA gene sequence, further information on this isolates is available in Appendix 5.2.
The thermophilic diversity could not be recorded on MA for soil and on SBA for milking cups due to extensive swarming and spreading of the isolates on plate.

5.2.2.1.3 Aerobic psychrotrophic spore-formers

The diversity of psychrotrophic spore-formers spanned 11 genera and 29 species (Figure 5.5), including 16 clusters that could not be confidently identified (Appendix 5.1). With the exception of pasture (H' = 1.278, Appendix 5.2), a high bacterial diversity was recorded across the samples (H' > 1.500) and particularly in soil and trough water (H' > 2.000). The predominant genera included *Psychrobacillus* (23%), *Paenibacillus* (18%), *Solibacillus* (18%), *Alkalihalobacillus* (8%) and *Bacillus s.s.* (7%).

Although several bacteria were isolated across multiple samples, the diversity of the samples was particularly heterogenous (BC > 0.70, Appendix 5.3) apart from the pair soil/faeces (BC = 0.50). *Psychrobacillus* and *Solibacillus* spp., which could not be reliably identified to the species level by 16S rRNA gene sequencing, were predominant and nearly detected ubiquitously (Figure 5.5). *Psychrobacillus* were not recovered from faeces, but the phenotypical and morphological features of many unidentified psychrotrophic isolates suggest they were *Psychrobacillus* species. *Bacillus cereus s.l., Alkalihalobacillus gibsonii* and *Paenibacillus macquariensis* were particularly abundant in soil and faeces but most other bacteria were present at low relative abundance across the samples. *Paenibacillus* spp. were prevalent across the samples and were particularly abundant in soil but not in pasture.

Most of the bacteria isolated from milking cups were also recovered from the environmental samples, with the exception of *Paenibacillus* sp. (*macquariensis*), *Paenibacillus* sp. (*sonchi*) and *Sporosarcina globispora*. The diversity of psychrotrophic spores in the milking cups was most similar to that of trough water (BC = 0.62, Appendix 5.3). Bacteria detected both in the milking cups and the environmental samples included *Solibacillus* (ubiquitous), *Psychrobacillus* (soil, pasture, and trough water), *B. cereus s.l.* (soil, faeces and trough water), *P. anaericanus* (soil and faeces), *P. tundrae* (soil and trough water), *Paenibacillus* sp. (*tundrae*) [soil and trough water], *Peribacillus muralis* (pasture), *Peribacillus psychrosaccharolyticus* (trough water) and *Sporosarcina* sp. (*globispora*) [trough water].

Overall, the spoilage potential of psychrotrophic spore-formers was moderate but some genera demonstrated strong enzymatic potential. Bacteria with proteolytic activity represented 43% of the isolates and included species such as *B. cereus s.l., A. gibsonii, P. macquariensis, Peri. simplex* and *P. anaericanus* (Figure 5.3). Lipolytic activity was detected in 47% of the isolates, mainly *B. cereus s.l., P. macquariensis, Peri. simplex* and *P. anaericanus*.

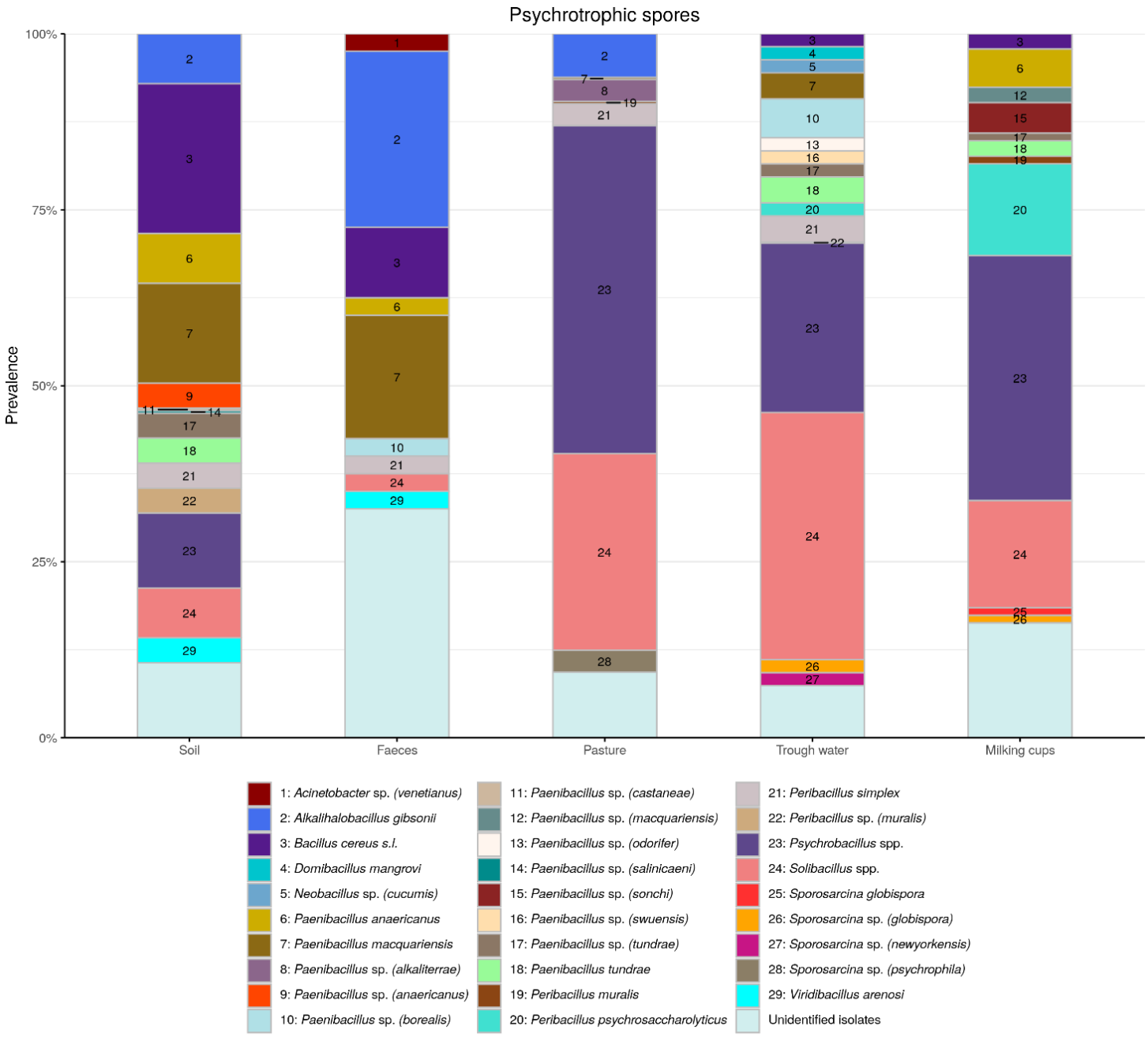


Figure 5.5. Diversity of psychrotrophic spores in the farm environment of a pasture-based sheep dairy farm.

Coloured rectangles with a number represent the relative prevalence of a bacteria in a given sample, the number referring to species in the legend. The proportion of unidentified isolates is visible as pale turquoise rectangles on the bottom of each bar graph. Isolates referred to as "sp." could not be confidently attributed to a type strain species based on their partial 16S rRNA gene sequence, further information on these isolates is available in Appendix 5.2.

Simultaneous lipase and protease activity was recorded in 24% of the isolates and predominantly for *B. cereus sensu lato*. *Paenibacillus* spp. also demonstrated strong spoilage potential and frequently produced both lipases and proteases.

5.2.2.1.4 Anaerobic spore-formers

Following CMG enrichment, the clostridial diversity spanned 4 genera and 12 species (Figure 5.6), including two clusters that could not be confidently assigned to a species (Appendix 5.1). The most commonly isolated clostridia after enrichment were *Clostridium perfringens*, *C. cadaveris* and *C. senegalense* which together accounted for half of the isolates. Soil showed the greatest clostridial diversity (7 species) while milking cups had the least diverse (4 species). Although no bacteria were found to be ubiquitous, a few were recovered from four out of five samples, namely *C. perfringens* (all apart from trough water), *Paraclostridium benzoelyticum* (all apart from faeces) and *Clostridium* sp. (*uliginosum*) [all apart from milking cups]. All clostridia isolated from the milking cups were also detected in the environmental samples, namely *C. perfringens* (soil, faeces, and pasture), *Paracl. benzoelyticum* (soil, pasture, and trough water), *C. butyricum* (soil and trough water) and *C. cadaveris* (faeces and pasture).

Proteolytic bacteria accounted for 57% of the isolates and included *C. perfringens*, *C. senegalense*, *Paracl. benzoelyticum* and *C. sporogenes* (Figure 5.3). Lipolytic clostridia represented 45% of the isolates and mainly comprised species such as *C. cadaveris*, *Clostridium* sp. (*uliginosum*), *T. petrolearius*, *C. moniliforme* and *C. butyricum*. Simultaneous lipase and protease activity was rare, but it was detected in some isolates of *C. sporogenes*, *C. senegalense* and *C. moniliforme*.

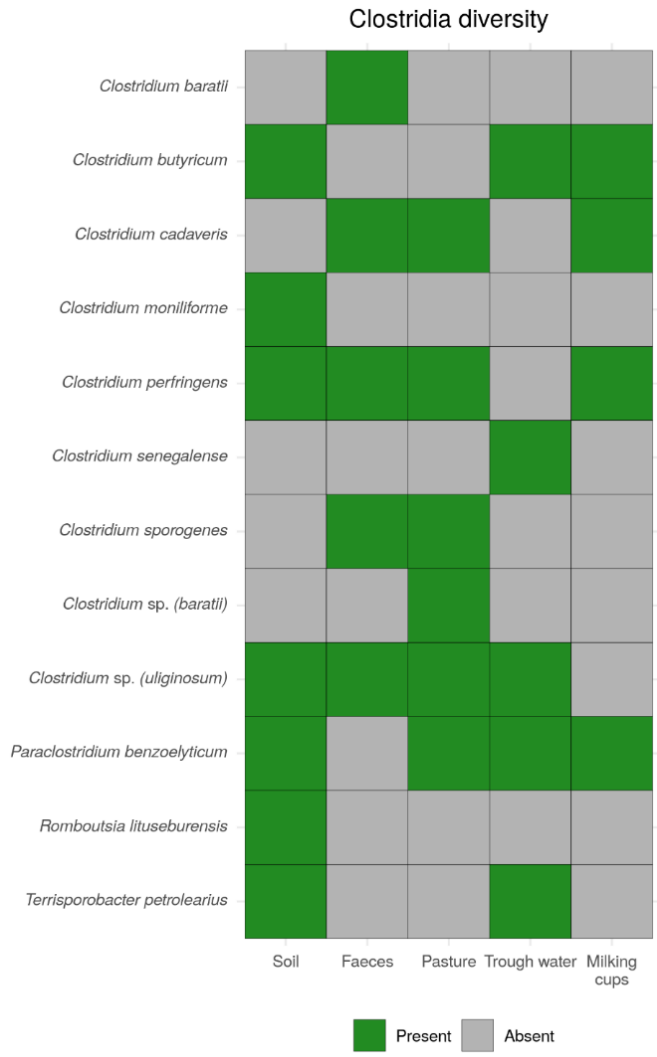


Figure 5.6. Clostridial diversity in farm samples collected at a pasture-based sheep dairy farm following cooked meat glucose starch broth (CMG) enrichment

The detection of at least one isolate of a specific clostridial species in a given sample is pictured as green squares while grey squares indicate the absence of detection of the organism. The isolates referred to as "sp." could not be confidently attributed to a type strain species based on their partial 16S rRNA gene sequence, further information on this isolates is available in Appendix 5.2

.

5.2.2.2 Prevalence, diversity and spoilage potential of *Pseudomonas* and other psychrotrophs

Pseudomonas spp. were successfully isolated from the farm samples using CFC agar. Bacteria recovered with this media included three genera: *Pseudomonas* (93% of the total relative abundance, Figure 4.7), *Stenotrophomonas* (< 1%) and *Serratia* (< 1%), with the remaining 6% representing unidentified isolates. A high bacterial diversity was recorded across the samples (H' > 1.500, Appendix 5.2), with the exception of trough water (H' =1.052), and particularly in soil and pasture (H' > 2.300). With only seven out of 46 species being detected in two or more samples, the bacterial diversity was also particularly heterogeneous (BC > 0.80). Overall, predominant bacteria included *Pseudomonas poae s.l.* (i.e., *Ps. poae* or *Ps. trivialis*), *Ps. helmanticensis, Ps. extremaustralis, Ps. lundensis, Ps. fulva s.l.* (i.e., *Ps. fulva* or *Ps. parafulva*), *Ps. weihenstephanensis, Ps. yamanorum* and *Ps. libanensis* (Figure 5.7). With the exception of *Ps. poae s.l.*, isolated from each sample but faeces,

and *Ps. extremaustralis*, isolated from each sample but trough water and milking cups, the bacteria mentioned above were each specific to a sample type. The predominant species varied across the samples: *Ps. libanensis*, *Ps. poae s.l.* and *Pseudomonas* sp. *(migulae)* were predominant in soil, *Ps. helmanticensis* and *Ps. weihenstephanensis* in faeces, *Ps. extremaustralis* and *Ps. cedrina* in pasture, *Ps. poae s.l.*, *Ps. yamanorum* and *Ps. costantinii* in trough water, *Ps. lundensis, Ps. fulva s.l.*, *Ps. poae s.l.* and *Ps. koreensis* in milking cups.

The bacterial diversity of the milking cups was particularly different to that of the environmental samples. There were only two bacteria isolated from both milking cups and other samples, namely *Ps. poae s.l.* (detected in soil, pasture and trough water) and *Ps. koreensis* (faeces and pasture), however, these bacteria only accounted for 25% of the bacteria in milking cups. The predominant bacteria in the milking cups were *Ps. fulva s.l.* and *Ps. lundensis*, while other bacteria included *Pseudomonas* sp. (*plecoglossicida*), *Ps. granadensis*, *Ps. rhizosphaerae* and *Serratia marcescens*.

Pseudomonas spp. were assigned to taxonomic groups (Figure 5.8) according to the classification defined by Garrido-Sanz et al. (2016). Members of the *Ps. aeruginosa* lineage represented 1% of the diversity and were only detected in soil and trough water. Across the samples, the *Ps. fluorescens* lineage was predominant, particularly members of the *Ps. fluorescens* subgroup, followed by the *Ps. koreensis* and *Ps. fragi* subgroups and the *Ps. syringae* group. The only taxonomic unit that was ubiquitous was the *Ps. fluorescens* subgroup whereas members of the *Ps. koreensis* and *Ps. fragi* subgroups were each predominant in faeces, milking cups and detected in pasture or trough water, respectively. Overall, the different groups under the *Ps. fluorescens* lineage were often sample-specific: members of the *Ps. mandelii* and *Ps. jessenii* subgroups were predominant in soil while the *Ps. koreensis* and *Ps. fragi* subgroups were particularly abundant in faeces. In trough water and pasture, the *Ps. gessardii* subgroup and the *Ps. syringae* group were predominant, respectively. Members of the *Ps. putida and Ps. rhizosphaerae* groups which were isolated from milking cups, were not found in any other samples.

Pseudomonas species had remarkable spoilage potential and only 6% (n = 18) of the total isolates recovered from CFC were not identified due to lack of protease or lipase activity. *Pseudomonas* spp. were often both proteolytic and lipolytic (71% of the isolates) and several species demonstrated consistent dual enzyme activity, including *Ps. poae s.l.*, *Ps. helmanticensis, Ps. extremaustralis, Ps. weihenstephanensis*, *Ps. libanensis*, *Ps. lundensis* and *Ps. koreensis*. In terms of taxonomic groups, bacteria with both enzymatic activities belonged to the *Ps. fluorescens, Ps. koreensis*, *Ps. fragi*, and *Ps. gessardii* subgroups. Few species of bacteria were exclusively proteolytic, such as *Serratia*

marcescens, *Ps. cannabina* and *Pseudomonas* sp. (*viridiflava*), or exclusively lipolytic, such as *Pseudomonas* sp. (*migulae*), *Ps. fulva s.l.* and *Ps. cedrina*.

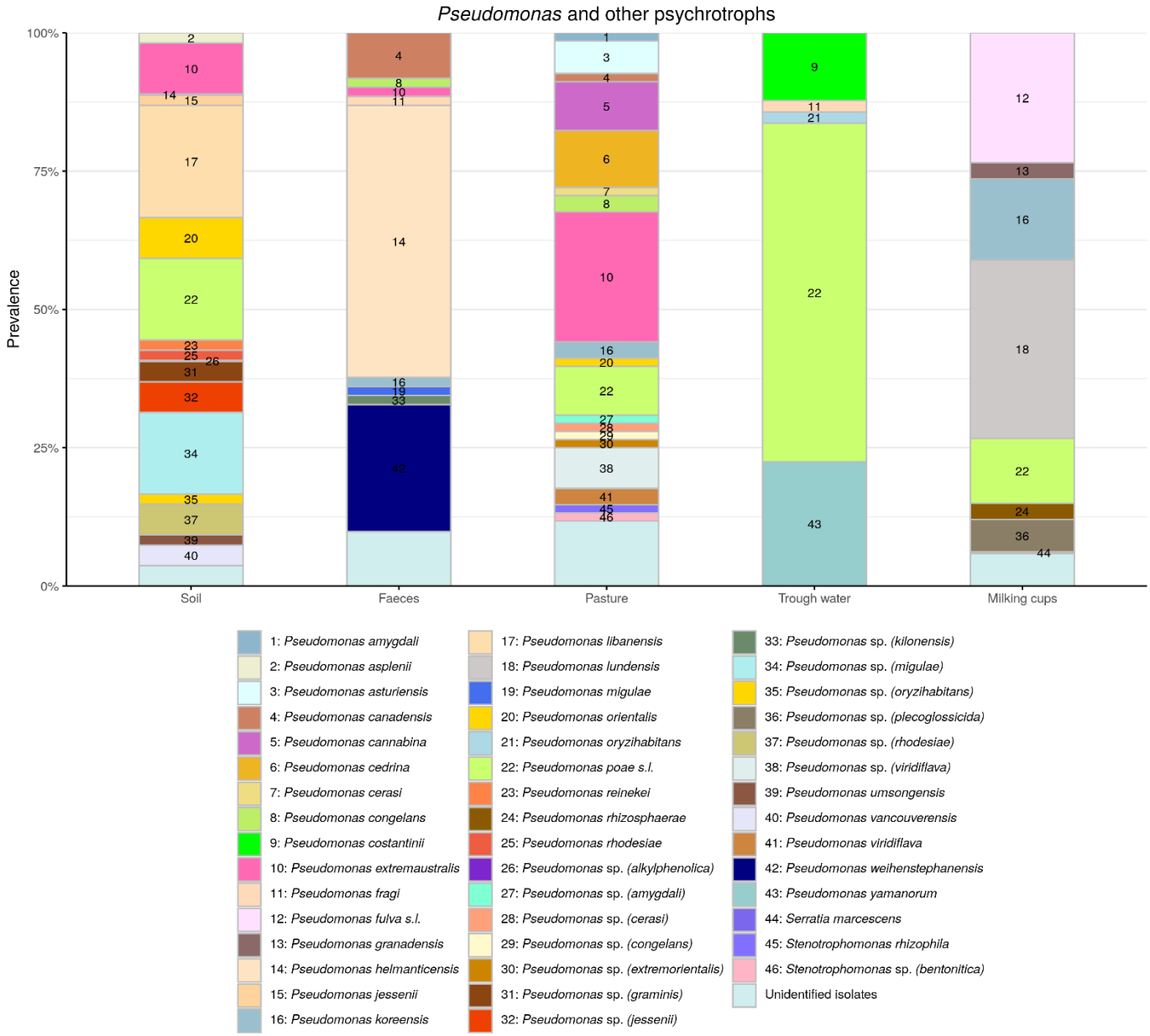


Figure 5.7. Diversity of *Pseudomonas* and other psychrotrophs in the farm environment of a pasture-based sheep dairy farm.

Coloured rectangles with a number represent the relative abundance of a bacteria in a given sample, the numbers referring to species in the legend. The proportion of isolates that were not identified is visible as pale turquoise rectangles on the bottom of each bar graph. Isolates referred to as "sp." could not be confidently attributed to a type strain species based on their partial 16S rRNA gene sequence, further information on these isolates is available in Appendix 5.2

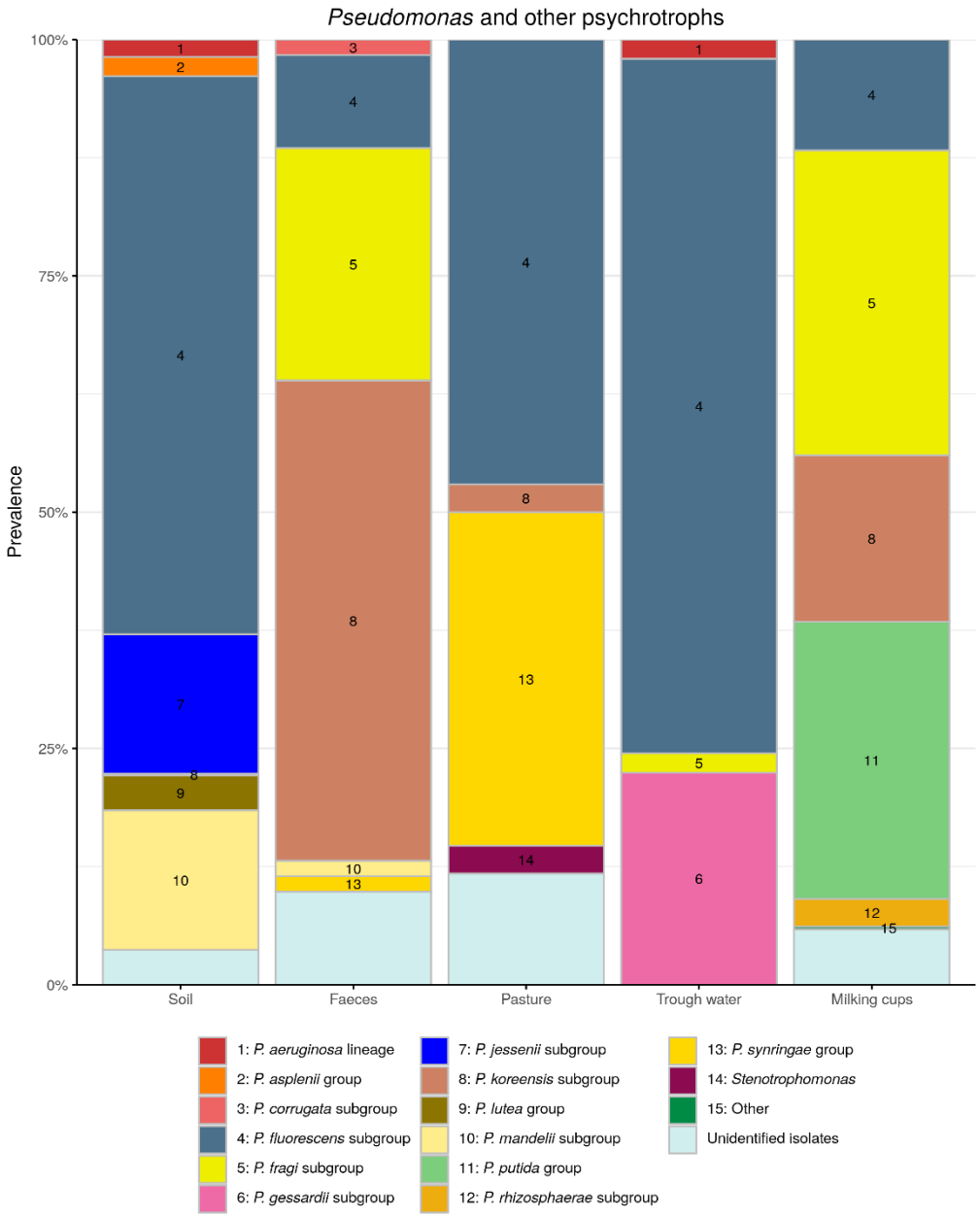


Figure 5.8. Diversity of *Pseudomonas*, sorted by phylogenetic groups, and other psychrotrophic bacteria in the environment of a pasture-based sheep dairy farm.

Coloured rectangles with a number represent the relative abundance of a bacteria in a given sample. The proportion of unidentified isolates is visible as pale turquoise rectangles on the bottom of each bar graph. No isolate was recovered after incubation of the silage samples on CFC agar. Isolates referred to as "sp." could not be confidently attributed to a type strain species based on their partial 16S rRNA gene sequence, further information on these isolates is available in Appendix 5.2. Phylogenetic grouping of the *Pseudomonas* species was done according to the phylogeny described by Garrido-Sanz et al. (2016).

5.3 Discussion

5.3.1 Approach

To initiate the design and development of farm practices and interventions that can improve the microbiological quality of ewe milk, we first need to improve our understanding of the ovine dairy farm microbiology. In Chapter 4, I demonstrated that the barn environment of a sheep dairy farm could contain high concentrations of *Pseudomonas* spp. and spores. In addition, the diversity of these bacteria, among which spoilage organisms were predominant, was well-conserved across the samples, suggesting the transmission of these bacteria in the environment. On dairy farms housing animals, the cycling and transmission of bacteria takes place in a confined space, which is often limited to the vicinity of the barn, and which may facilitate the passage of bacteria across different farm sources because of their proximity. However, on pastoral dairy farms, this cycling takes place over a shifting and much larger surface area (i.e., different paddocks and walkways).

On the farm studied here, the ewes were kept on pasture at all times throughout the year and followed a daily pasture rotation plan to provide quality grazing and optimise pasture growth. Because the environment of the animals changed daily, they may have been exposed to different populations of bacteria present on pastures. Dairy ewes were offered water in troughs ad libitum and grazed on ryegrass and clover while, during miking, they were also offered a mix of several grains which was similar to the one fed on Farm 1. Following ingestion of feed, water, or even soil when grazing, bacteria and their spores present in these sources may pass through the gastrointestinal tract of the ewes to be shed in faeces (Wu et al., 2007). When kept on pasture, sheep often defecate in the proximity of their night lying areas upon awakening (White and Hall, 1998) which may drive the faecal contamination of their udder. However, faeces laid during grazing may be less likely to contaminate the udders of the animals due to the large grazing area available and daily paddock rotation. In an outdoor farming set-up, the soil and faecal contamination of the udders of dairy cows drives the contamination of raw milk upon milking (Vissers et al., 2007c). On sheep dairy farms, other factors related to milking can also play a role in milk contamination such as dust, worker's hygiene, and the milking equipment (Arias et al., 2013, Weber et al., 2019). The collection of milking cup swabs enabled an overview of the bacteria transferred from the farm environment to raw milk, before it flows into the milking pipeline and potentially becomes contaminated by bacteria colonising the milking apparatus and the bulk tank (Scheldeman et al., 2005).

To capture a representation of the bacterial diversity to which the animals were exposed and obtain a more comprehensive overview of potential milk contaminants, two paddocks on which the flock had been grazing for the day were sampled and pooled into composite samples for analysis. My previous results suggested that the microbiota present throughout the barn environment of a sheep dairy farm could be heterogeneous for similar sample types (i.e., different concentrations of *Pseudomonas*, abundance of specific genera). Likewise, it is plausible that different bacterial loads and diversity may be found in samples collected from different paddocks. These variations may be the consequences of a wide range of factors that pertain to characteristics of individual paddocks, including local macroflora and soil properties such as composition, pH or drainage capacity (Palmer et al., 2019). Further studies may consider the sampling and analysis of individual locations coupled with the collection of their associated metadata to determine the microbiological profile of different types of farm soils and pastures. This may assist in the identification of soil types and plant species associated with a local microbiota that lacks dairy spoilage capabilities or which contribute to a reduction in the concentrations of spoilage bacteria found in these sources.

Due to the time required for analysis and due to the fact they were collected simultaneously, samples had to be preserved frozen at -20°C. Freezing of spores is thought to cause limited cell death as these structures enable bacterial survival in extreme environments (Jafari et al., 2016). It remains possible that upon freezing, some species of bacteria might have died at a greater rate than others as a result of lower tolerance to freezing, a phenotype known to vary across bacteria (Walker et al., 2006). Similarly, a general reduction of the bacterial counts in the samples, particularly for vegetative cells of *Pseudomonas,* may be expected as freezing can increase bacterial death and decrease culturability as a result of cellular damage. Nonetheless, high concentrations of vegetative cells of *Pseudomonas* were recovered from pasture (7.5 $\log_{10}$ cfu/g) after these samples were frozen as such in bags, while the concentrations of spores recorded here were generally similar or higher than in previous reports (Scheldeman et al., 2005, Martin et al., 2019). Therefore, although it was not directly assessed here, freezing did not appear to extensively impact the viability and culturability of the bacterial cells present in the different samples.

In this work, I used a culturomics approach to isolate 1,489 bacteria across five conditions of culture which targeted bacteria implicated in the spoilage of different dairy products, namely anaerobic mesophilic spore-formers, aerobic mesophilic, thermophilic and psychrotrophic spore-formers, as well as *Pseudomonas* species. These isolates spanned 34 genera and 145 species, pointing to the presence of a remarkable bacterial diversity in the environment of a sheep dairy farm. The spore detection methods used during this study were complementary and enabled the isolation of an

extensive diversity of aerobic spore-formers. *Bacillus* was the only genus isolated after mesophilic, thermophilic, and psychrotrophic aerobic incubation, but seven other genera (*Alkalihalobacillus*, *Neobacillus*, *Paenibacillus*, *Peribacillus*, *Psychrobacillus*, *Solibacillus* and *Viridibacillus*) were simultaneously isolated across mesophiles and psychrotrophs, while only one other genus was found common between mesophiles and thermophiles (*Brevibacillus*). No genus other than *Bacillus* was shared between thermophiles and psychrotrophs, demonstrating that these detection methods target an entirely different set of bacteria. At the species level, eight bacteria were isolated after both mesophilic and psychrotrophic incubation. Their detection, albeit redundant, provided additional insight in some instances. For example, mesophilic *P. tundrae* were isolated from trough water and milking cups while psychrotrophic isolates identified as *P. tundrae* were, in addition to these samples, also isolated in soil from which they are likely to originate (Nelson et al., 2009). Other species isolated after mesophilic and psychrotrophic incubation included *B. cereus s.l.*, *Peribacillus simplex*, *Viridibacillus arenosi*, *Solibacillus* spp. and *Psychrobacillus* spp., which have been isolated at 20°C and 37°C from the bovine farm environment (Scheldeman et al., 2005, Coorevits et al., 2008) and at 6° and 32°C from cow milk and dairy powders (Miller et al., 2015b). Surprisingly, I did not recover any psychrotolerant *B. pumilus s.l.* or thermotolerant *B. licheniformis*, although these bacteria were highly abundant in the bedding, faeces, and milking cups samples I analysed in Chapter 4.

Previously, I found *B. licheniformis* to be highly abundant in the barn environment of a sheep dairy farm, and these bacteria formed spreading colonies on agar after incubation at 55 or 60°C which prevented the reliable enumeration and isolation of thermophilic bacteria. In the present study, thermotolerant *B. licheniformis* were not isolated at 60 and 65°C, but mesophilic isolates of this bacterium were obtained from faeces, trough water, and particularly milking cups. Despite the absence of highly spreading *B. licheniformis*, few species of thermophilic spore-formers exhibited swarming or spreading plate motility after incubation on SBA and MA at 60°C and 65°C, which prevented the collection of bacterial diversity data on MA for soil and on SBA for milking cups. Swarming and spreading occurred irrespectively of incubation on SBA or MA, which contrasted with my previous observations identifying MA as able to partially limit these phenotypes (see Chapter 4). *Brevibacillus* spp. (*Br. aydonigluensis*, *Br. thermoruber*) and *Aneurinibacillus thermoaerophilus* were identified as bacteria able to swarm and spread on solid media. Motility on agar media is a common feature of thermophilic spore-formers; it can thwart the accurate study of these bacteria in the environment or in dairy products and therefore needs to be addressed (Murphy et al., 2021). Thermophilic *Brevibacillus* species have not been associated with dairy quality, but my results show they often display swarming and spreading plate phenotypes. Therefore, future work may consider

the formulation of agar media with inhibitory properties towards the growth of *Brevibacillus* spp. (e.g., antibiotics, complex carbon source), which would enable a focus on the thermophilic spore-formers that are associated with dairy spoilage.

The use of different agar media was reported to cause variations in the observed counts and diversity of spore-formers present in bovine raw milk and dairy powders (Kent et al., 2016, Murphy et al., 2021). In the present study, incubation of farm samples at 65°C on MA yielded a greater thermophilic diversity, but lower spore counts than after SBA incubation. These findings corroborate my previous observations and further demonstrate that the detection of thermophilic spore-formers is highly dependent on the conditions of culture. Here, MA enhanced the rate of isolation of *Parageobacillus*, *Aneurinibacillus* and *Anoxybacillus* spp. from the farm environment as compared to SBA which enhanced the rate of isolation of *Thermoactinomyces vulgaris*. *Parageobacillus, Aneurinibacillus* and *Anoxybacillus* spp. have been implicated in the colonisation of bovine dairy powder manufacturing plants (Murphy et al., 1999, Burgess, 2016) and the contamination of dairy powders from several animal species (Delaunay et al., 2021). Thermophilic spores are thought to originate from the farm environment where they contaminate raw milk at low levels. After their introduction in the dairy processing environment in raw milk, they can colonise pipelines using biofilms and persist in the environment, further proliferating in the high temperature sections of dairy plants (Phillips and Griffiths, 1986, Burgess et al., 2010). However, recent farm studies have had limited success in isolating the main contaminants of dairy powders, namely *Anoxybacillus* and *Geobacillus s.l.*, from the farm environment and raw milk (Phillips and Griffiths, 1986, Miller et al., 2015b). My findings demonstrate that these contaminants, or other species closely related to these bacteria, may be isolated from the dairy farm environment, which strengthen the idea that they are farm-borne. Therefore, to initiate actions and prevent the contamination of milk by *Geobacillus s.l.*, *Aneurinibacillus* and *Anoxybacillus*, there is a need to characterise the ecology of these bacteria in the farm environment. The formulation of growth media with selective properties towards the growth of *Geobacillus s.l.*, *Aneurinibacillus* or *Anoxybacillus* spp may enable their study by preventing the growth of bacteria that may be highly abundant in the environment (e.g., *T. vulgaris*, *B. licheniformis*). Because of the heterogeneous and sometimes complex growth requirements of *Geobacillus s.l.*, *Anoxybacillus* spp. and *Aneurinibacillus* spp (Heyndrickx et al., 1997, Goh et al., 2014, Burgess, 2016) it may be more suitable to isolate these genera separately.

Some of the isolates recovered during the present study could not be identified to the species level based on their 16S rRNA gene sequence. They were spread across 57 clusters: 20 clusters of mesophilic spore-formers, 4 of thermophilic spore-formers, 16 of psychrotrophic spore-formers

(including one common with mesophiles), 2 of anaerobic mesophilic spore-formers and 16 *Pseudomonas* and *Stenotrophomonas* species. Mesophilic and psychrotrophic isolates assigned to 17 of these clusters were most closely related to *Paenibacillus* spp., corroborating my previous findings (see Chapter 4) and those of previous studies on bovine dairy farms and products identifying a large set of yet uncharacterised and putatively novel *Paenibacillus* species in close association with the dairy production (Ivy et al., 2012, Doll et al., 2017). The majority of these bacteria demonstrated strong lipase and protease activity, pointing to their potential involvement in dairy spoilage. As an example, whole genome analysis of other isolates recovered during the study described in Chapter 4 revealed that bacteria with 16S rRNA similarity lower than 97% to type strains could in fact belong to the most closely related species displayed by RDP (Gupta et al., 2020c), but also to other species (Gupta et al., 2021), or represent novel species [i.e., with digital DNA-DNA hybridisation value, d4 < 70% against bacterial genomes contained in the Type Strain Genome Server (Tindall et al., 2010, Meier-Kolthoff and Göker, 2019)]. The characterisation of a novel bacterial species is a process that requires extensive cell phenotyping (e.g., microarrays, microscopy, fatty acid analysis) and genotyping (e.g., WGS, MLST) following guidelines set by the International Journal of Systematic and Evolutionary Microbiology (Pitt et al., 2018). This could not be completed during this PhD due to time and financial constraints, however the extensive cryobank generated by my work will enable the formal discovery of these putatively novel organisms in the future.

Overall, my findings demonstrate that the methods used in this study are minimally redundant and are able to capture a large diversity of spore-forming bacteria dwelling in the dairy farm environment. This complements the observations made during Chapter 4 and reinforces the suitability of SBA as an isolation media for spore-formers from dairy farm environments. My findings also point to the challenges associated with the isolation of thermophilic spore-formers from farm samples and to the need for future studies to formulate culture media tailored for the isolation of bacteria associated with dairy spoilage.

5.3.2 Concentrations of spores in the pasture-based farm environment

Primary sources of spore-formers in the farm environment consist of soil and plant- or animal-associated communities (Julien et al., 2008, Hong et al., 2009). Bacteria present in these sources may be passed on to other farm components by cross-contamination, travel throughout the farm environment and ultimately end up contaminating raw milk during milking, mainly via dirt present on the udders of dairy livestock. On bovine dairy farms, several hotspots of bacterial spores have

been identified, namely feed, faeces, soil, and bedding materials, frequently reaching numbers greater than 5-6 $\log_{10}$ cfu/g across different spore detection methods (Scheldeman et al., 2005, Martin et al., 2019). High spore concentrations in these matrices have been associated with higher spore counts in bovine bulk tank milk (Vissers et al., 2007c, Borreani et al., 2019, Murphy et al., 2019) but little is known about the spore burden of sources found on sheep dairy farms and about the factors governing sheep milk contamination. Here, the enumeration of aerobic mesophilic, psychrotrophic and thermophilic spores enabled the identification of pasture-associated farm sources with high spore concentrations and the potential to introduce substantial contamination into raw milk produced on pastoral sheep dairy farms.

Highest concentrations of mesophilic and psychrotrophic spores or thermophilic spores were recorded from soil, pasture, and trough water while faeces had lower spore counts. These findings complement those described in Chapter 4, by providing spore counts obtained with the same methods for analogous farm sources (i.e., faeces, trough water, milking cups) associated with a different farming system, as well as spore counts in additional sources (i.e., soil and pasture). In both of these studies, the collection, processing and analysis were performed with the same approach, isolation media and by the same operator, thus limiting laboratory-associated bias (Murphy et al., 2021) and enabling meaningful comparison between the spore load present on these sheep dairy farms.

In the present study, the abundance bacterial spores, and particularly thermophilic spores, was lower than previously reported on bovine dairy farms, in sources such as faeces, feed (i.e. silage or other feed types), trough (drinking) water and soil (Martin et al., 2019). Faecal TSC (2.96 $\log_{10}$/g) were remarkably lower than reported elsewhere for dairy cows [5.22 ± 0.71 $\log_{10}$/g (Martin et al., 2019)] and in the previous chapter (6.58 ± 0.11 $\log_{10}$/g, see Chapter 4). The low concentrations of spores and thermophilic spores recorded in the pasture environment may reflect (1) less interactions and transfer of bacteria between the different farm components as a result of their spatial organisation (i.e. different paddocks) and thereby limiting the transmission, cycling and accumulation of spores across farm sources, and/or (2) the involvement of fewer sources in which the growth of spore-forming bacteria may occur, or with high spore counts, leading to the dispersal of spores in the farm environment. The former hypothesis, although not directly tested here, may be plausible considering the larger living area that is typically available to grazed dairy ewes as compared to housed animals. Farm sources capable of supporting the growth of thermophilic spore-formers may include matrices in which moderate to high temperatures are reached, which is the case for self-heating decaying plant matter (i.e. composted or spontaneously composting), processed feed (e.g. pelleted feed or ensiled forages) and potentially the gastrointestinal tract (GIT) of the animals (Cross and Johnston, 1971,

Kikue et al., 2019, Murphy et al., 2019). With the exception of the animals, these sources are generally not present on pasture-based dairy farms, including on the farm considered here. Overall, these findings suggest that extensive sheep dairy farming (i.e., pasture grazing, no housing) may be associated with lower concentrations of bacterial spores and particularly thermophilic spores, in the farm environment. Because of the link between the microbiota of the farm environment and that of raw milk, these farms may also have a lower incidence of thermophilic spores in raw milk (Martin et al., 2019, Murphy et al., 2019). Raw milk with low thermophilic spore counts is highly desirable for producers and dairy powder manufacturers that aim to preserve product quality by limiting thermophilic growth and biofouling during long processing runs. Therefore, farms with a milk production dedicated to the manufacturing of dairy powders may consider placing particular emphasis on the selection of farming systems and feed to prioritise extensive farming practices and minimally processed feedstuffs that harbour low spore counts.

Soil was identified as an important potential reservoir of bacterial spores as it displayed the highest counts across the three aerobic spore detection methods. Martin et al. (2019) also recorded high mesophilic, psychrotrophic and thermophilic spore counts (5.68 ± 0.40; 5.04 ± 0.55; 4.61 ± 0.86 $\log_{10}$ cfu/g, respectively) in soil sampled on cow dairy farms. On pasture-based bovine dairy farms, the spore content of raw milk has been shown to be directly related to the contamination of teats with soil (Christiansson et al., 1999). Specifically, McKinnon and Pettipher (1983) identified the grazing period of dairy cows as a risk factor increasing concentrations of psychrotrophic spores in raw milk and also recorded high spore counts in pasture (2.81-4.74 $\log_{10}$ cfu/g) and particularly soil (5.01-5.46 $\log_{10}$ cfu/g). The contribution of soil to the spore contamination of raw milk on dairy farms is twofold: either direct, via udder contamination, or indirect, via soil ingestion and shedding in faeces followed by udder contamination (Vissers et al., 2007c, Vissers et al., 2007d, Arias et al., 2013). Livestock, and particularly sheep, are known to ingest soil when grazing, amounting to a sizeable portion of their daily intake in some instances [e.g. 220 g/day/sheep (Healy and Ludwig, 1965)]. Using the soil spore counts recorded here, an extreme scenario of 220 g of soil being ingested daily, as recorded by Healy and Ludwig (1965), would correspond to a daily intake and potential shedding of 9 $\log_{10}$ cfu of mesophilic spores, 8.7 $\log_{10}$ cfu of psychrotrophic spores and 6.4 $\log_{10}$ cfu of thermophilic spores by the animal. Soil ingestion is also detrimental for sheep health due to the potential uptake of chemicals present in soil, such as arsenic or iodine in some New Zealand soils (Healy, 1968, Gaw and McBride, 2010). Therefore, soil ingestion should be limited as it represents a considerable influx of spores that may be shed in faeces and with potential to contaminate raw milk (Heyndrickx, 2011). Soil contamination of the udders and soil ingestion are both governed by a similar set of factors, namely

those increasing the dispersal of soil particles onto pasture and the animals, and those causing strain on the integrity of the pasture, thus increasing the frequency of direct contact between barren soil, and the animals. The risk factors that exacerbate soil-animal contact are diverse and include intrinsic soil composition and structure (i.e. poor drainage and weak structure), high stocking rate, wet climate, poor pasture management and overgrazing (Healy and Ludwig, 1965, Healy, 1968). Spore-forming bacteria are intrinsically present in soil where they perform vital recycling and plant symbiotic functions, but they can also be introduced artificially (Garbeva et al., 2003, Hong et al., 2009). Jackson et al. (1997) showed that repeated ploughing of cattle manure increased the concentration of thermophilic actinomycetes spores in agricultural soils. Although spore-formers cannot be removed sustainably from soil, their abundance may be controlled by limiting the use of manure-based fertilisers which contain high concentrations of spores (Cross, 1968, Edesi et al., 2012, Gupta and Brightwell, 2017). When the animals are continuously kept on pasture, providing them a sheltered area during precipitations may also limit muddying of their udders (Gregory, 1995), but the maintaining of a dynamic pasture rotation plan (as opposed to set-stocking) and low stocking rate are key to limit damage on pasture and contact between soil and the animals (Healy, 1968). Dynamic pasture rotation is already a common feature of New Zealand bovine and ovine dairy farms, including the one considered here, as dairy farms generally possess numerous paddocks available for grazing. This practice is likely to contribute to reduce the soil-borne contamination of milk and help preserve pasture health. Soil contamination of the udders may also occur in access roadways leading to the milking platform or between paddocks (Christiansson et al., 1999). To prevent contamination of the udders, roadways should be kept from deteriorating and becoming muddy. Because soil in a fundamental part of pasture-based dairy farms, stringent hygiene during milking, including pre-milking cleaning of the udders and rigorous washing of the milking clusters, may be required to limit the transfer of soil particles —and the bacteria they contain— to raw milk (Magnusson et al., 2006, Elmoslemany et al., 2010).

5.3.3 Ecology of aerobic spore-forming bacteria in the pasture environment

Because of their unique diversity and versatility, spore-forming bacteria can successfully colonise a variety of processing environments and survive the stringent conditions found throughout the dairy continuum to ultimately contaminate dairy products in which they decrease product quality or cause premature spoilage. To prevent the transfer of these bacteria from their environmental reservoirs to milk, a better understanding of their ecology on dairy farms is required. Spore-forming bacteria

inhabit key ecological niches in the environment; they perform the recycling of organic matter and can establish symbiotic or parasitic relationships with plants, animals and insects (Hong et al., 2009). The production of hydrolytic enzymes displaying low substrate specificity is often central to these functions (Rao et al., 1998). Thus, many of the spore-formers present in the natural environment indirectly possess the ability to spoil dairy products by degrading their nutrients. This was confirmed here; most aerobic spore-forming isolates (89%) possessed some form of spoilage potential. Specifically, 46% and 66% were found to produce lipases or proteases, respectively, which is similar to the values previously recorded in the barn environment of a sheep dairy farm (44% and 55%, respectively, see Chapter 4) but higher than previous reports in bovine dairy products and raw materials [28% and 50%, respectively (Lucking et al., 2013)]. Across different spore detection methods, spoilage potential followed a similar pattern to what had been described in Chapter 4; namely mesophilic isolates generally displayed strong spoilage potential, producing proteases, lipases, and these enzymes in combination. Thermophiles were particularly proteolytic but rarely lipolytic, while psychrotrophs were proteolytic or lipolytic, albeit at a lower frequency than mesophiles.

The spore microbiota of sources associated with the pasture environment was found to be heterogeneous both in term of the general diversity and the relative abundance of the predominant species. This finding contrasted with my previous study, in which the predominant bacteria were consistent across different sources (i.e., bedding, feed, faeces, trough water) and locations (i.e., different pens) in the barn environment (see Chapter 4). These findings lend further support to the hypothesis that the transmission of bacteria across sources of the pasture environment is low as compared to the barn environment.

Bacillus s.l. were highly abundant across the aerobic mesophilic spore microbiota of the samples. The few mesophilic spore-formers found to be predominant were also the ones ubiquitously detected, namely *B. cereus s.l.*, *B. pumilus s.l.* and *Pr. megaterium s.l.* These taxonomical groups each include multiple species sharing high similarity in their 16S rRNA gene sequence and that could not be confidently distinguished based on my identification approach. However, the bacteria grouped under each of these taxonomical group have been documented to share similar ecological reservoirs and possess similar phenotypes.

Bacillus cereus s.l. is a highly ecologically diverse group comprised of at least 12 species involved in a variety of processes: pathogenesis (Ehling-Schulz et al., 2019), soil homeostasis (Akhtar et al., 2021), animal or plant symbiosis (Alexopoulos et al., 2001, Argôlo-Filho and Loguercio, 2013), and

dairy spoilage (Mehta et al., 2019). The species implicated in dairy spoilage mainly consist of *B. cereus* and *B. weihenstephanensis* but other members of this taxon are also frequently isolated from bovine dairy products, including *B. mycoides*, *B. pseudomycoides* and *B. thuringiensis* (Ivy et al., 2012). In the present study, all mesophilic and psychrotrophic *B. cereus s.l.* isolates were proteolytic while lipase activity was particularly common for psychrotrophic isolates. Lucking et al. (2013) identified *B. cereus s.l.* as some of the bacteria most often associated with bovine dairy spoilage while De Jonghe et al. (2010) also demonstrated a high frequency (> 70% positive isolates) of protease, lipase and phospholipase (lecithinase) production across 48 *B. cereus s.l.* isolates. Some members of the *B. cereus* group, including *B. cereus sensu stricto.*, *B. thuringiensis* and *B. cytotoxicus*, can produce toxins causing foodborne emetic or enteric illnesses (Dietrich et al., 2021). The spores of *B. cereus s.l.* generally survive pasteurisation (Necidová et al., 2014, Chaves et al., 2017), therefore, contamination of milk by *B. cereus s.l.* should be avoided at the farm level to reduce the impact of these bacteria on the safety and quality of sheep dairy products. The principal ecological niches and contamination sources of *B. cereus* on bovine dairy farms consist of soil, pasture, faeces, silage, and bedding materials (Christiansson et al., 1999, Vissers et al., 2007d). Here, according to the relative bacterial abundance and count data, soil, pasture, and faeces contained 6.30, 4.85 and 4.11 $\log_{10}$ cfu/g of mesophilic *B. cereus s.l.* spores, respectively. On bovine dairy farms, Vissers et al. (2007d) recorded lower levels of *B. cereus* in soil (5.0 $\log_{10}$ cfu/g) and faeces (2.5 $\log_{10}$ cfu/g) while Christiansson et al. (1999) also found lower levels (2.0 to 3.2 $\log_{10}$ cfu/g) in grass pasture. My approach did not specifically target *B. cereus s.s.* but also recovered other members of *B. cereus s.l.*, which may explain why I recorded higher counts of *B. cereus s.l.* in this study.

Because *B. cereus s.l.* are found in high concentrations in soil and pasture (Sutherland and Murdoch, 1994), the grazing period of dairy cows is associated with an increased incidence of these bacteria in raw milk (Christiansson et al., 1999). During the housing of dairy cows, bedding and feed (e.g., silage) were also identified as important sources of contamination (Magnusson et al., 2007a, Vissers et al., 2007d). With pasture-based dairy farms being at an increased risk of soilborne milk contamination, farmers should place particular emphasis on preventing the contamination of the animals udders with soil to limit the entry of *B. cereus s.l.* in milk and preserve its quality and safety. This may be achieved by reducing soil-animal contact, including by preventing overgrazing or stationing of the flock in muddy areas facilitating soil adhesion (Christiansson et al., 1999). Pre-milking cleaning of the udders may also help achieve a reduction of spores in milk, provided the udders are dried after the cleaning regime (Magnusson et al., 2006, Gibson et al., 2008). With the exception of the present study, the prevalence of *B. cereus s.l.* remains poorly documented on New Zealand dairy farms. Drean et al.

(2015) highlighted variations in the species and genotypes of *B. cereus s.l.* found across different bovine, ovine and caprine Australian dairy farms, and isolated *B. cereus s.s, B. weihenstephanensis, B. mycoides* and *B. pseudomycoides* from soil, feed, faeces, and milk filters. Future work in the New Zealand dairy farm environment is required to survey the incidence of *Bacillus cereus s.l* and assess the prevalence of species associated with food spoilage and safety.

Although most mesophilic isolates produced proteases or lipases, only few bacteria had been previously implicated in dairy spoilage, such as *B. pumilus s.l., B. licheniformis, B. subtilis, A. clausii, Peri. simplex* and *P. tundrae* (Lucking et al., 2013, Doll et al., 2017). Other bacteria positive for lipase or protease production may represent novel dairy spoilage bacteria but also bacteria unable to efficiently contaminate raw milk and/or failing to proliferate in milk, and therefore not implicated in dairy quality. Bacteria identified as *Priestia megaterium s.l.*, which includes *Pr. megaterium* and *Pr. aryabhatthai* [*Pr. aryabhatthai* has been proposed as a heterotypic synonym of *Pr. megaterium* (Manik et al., 2019)], were often found to be both proteolytic and lipolytic, and were found to be highly abundant in soil, faeces and pasture. Members of this taxon are considered to be cosmopolitan and have been isolated from a wide variety of environments including soil, livestock faeces, human clinical samples and insects (Glaser, 1914, Garbeva et al., 2003, Bhatt and Maheshwari, 2019). *Priestia megaterium s.l.* are considered important endophytes which thrive in association with plants, performing plant growth promotion, bioremediation, and biocontrol (Ortíz-Castro et al., 2008, Elarabi et al., 2020, Antil et al., 2021). Despite their ubiquity in the farm environment, *Pr. megaterium s.l.* have seldom been associated with dairy spoilage or isolated from milk (Lucking et al., 2013). The cells of these bacteria are amongst the largest known in the prokaryotic kingdom [about 4 x 1.5µm (Bunk et al., 2010)] and thus may be retained more efficiently by milk filters partially obstructed with larger particles (e.g. soil, straw) with size comparable to that of the milk filter pores (i.e. 100 to 250 µm). Recent research interest has unravelled the probiotic potential of several *Bacillus s.l.* (e.g. *B. licheniformis, B. cereus s.l., B. pumilus s.l., A. clausii*) including *Pr. megaterium s.l.* in livestock (Deng et al., 2021). When *Bacillus* spp. are used as probiotics (e.g., fed to livestock or as bedding inoculants), it remains unknown if their prevalence and abundance can increase in the environment, and thus possibly in raw milk, as a result of their artificial introduction on farm. If these probiotics consist of species associated with dairy spoilage (e.g., *B. licheniformis, B. cereus s.l.*) increasing their abundance in the environment could cause dairy quality concerns. Probiotic potential of *Pr. megaterium s.l.* to improve animal health and production has been highlighted in several animal species (Afrilasari et al., 2016, Deng et al., 2021). Because these bacteria are not associated with dairy

spoilage, their use as probiotics may be better suited than other bacteria historically associated with dairy spoilage (e.g., *B. licheniformis*, *B. cereus s.l.*).

The spores of *Thermoactinomyces* spp. have been recently increasingly detected in dairy powders of ovine and bovine origin (Sadiq et al., 2018, Delaunay et al., 2021) but their presence in soil and the faeces of livestock had been documented long before (Cross and Johnston, 1971). Whether *Thermoactinomyces* spp. are involved in the alteration of dairy powders quality remains unclear, however, they possess several factors of dairy spoilage: they form highly resistant spores, secrete proteases and lipases that may impact dairy quality during processing (Sadiq et al., 2016b) (as confirmed by my findings), and may have the ability to form biofilm (Demirci et al., 1993). In addition, the strong proteolytic potential of *Thermoactinomyces* spp. suggest they could enable the growth of other spoilage bacteria such as *Parag. thermoglucosidans* that may require exogenous proteolytic activity to proliferate during powder manufacturing (Zhao et al., 2013). *Thermoactinomyces vulgaris* is associated with agricultural activity and the natural environment (i.e., soil, decaying plants), therefore, the farm and raw milk are likely to represent sources of these bacteria in dairy powders. Prior to my work, these bacteria had been rarely isolated in direct association with the dairy farm environment. Their remarkably high abundance on New Zealand sheep dairy farms could point to the prevalence of these bacteria in the New Zealand environment, or to the efficacy of SBA in isolating these bacteria. Although MA is commonly used to isolate thermophilic spore-formers, the use of this media in my study yielded markedly lower counts of *T. vulgaris* (range: 0.66 to 3.05 $\log_{10}$ cfu/g or /l) than on SBA (range: 2.81 to 3.82 $\log_{10}$ cfu/g or /l), suggesting previous studies may have underestimated the abundance of *T. vulgaris*.

The spores of *Thermoactinomyces* spp. were particularly abundant in the farm environment, with *T. vulgaris* being the most abundant species and *T. intermedius* being also detected. These results corroborate my previous observations identifying the spores of *T. vulgaris* as being the most abundant thermophilic spores in the barn environment of a sheep dairy farm (see Chapter 4). Several other studies have captured high concentrations of *Thermoactinomyces* in agricultural environments [i.e. > 6 $\log_{10}$/g of substrate, including soil and faeces (Unsworth et al., 1977, Brinkmann et al., 2014)] with some proposing livestock faeces to be the major source of these bacteria (Cross and Johnston, 1971). Here, soil had the highest concentrations of *T. vulgaris* spores (3.82 $\log_{10}$ cfu/g) but they were lower than previously reported [4.84-5.60 $\log_{10}$ cfu/g (Cross, 1968)]. Because *Thermoactinomyces* are particularly abundant in the faeces of livestock, organic fertilisation (e.g. dairy effluents, manure) has the potential to increase the spore content of soil (Jackson et al., 1997). The farm considered in my study did not use manure-based fertilisers such as dairy effluents on pasture, which may contribute

to explaining the relatively low thermophilic spore counts recorded. *Thermoactinomyces* spores may also be more abundant on dairy farms housing animals resulting from an increased faecal exposure of the different environmental sources, including barned animals, and which may facilitate the cycling of spores and their accumulation [see Chapter 4, (Coorevits et al., 2008)]. The next step to identify ways of disrupting the contamination of raw milk by *Thermoactinomyces* and other thermophilic spores will be to identify matrices in which they are able to proliferate and monitor the spore load of these sources or prevent them from coming into contact with animals.

Geobacillus s.l. (i.e., *Geobacillus* and *Parageobacillus* spp.), *Anoxybacillus* and *Aneurinibacilus* represent some of the most common thermophilic spore-formers isolated from dairy powders across the world (Sadiq et al., 2018, Delaunay et al., 2021). These fast-growing and highly heat-resistant bacteria are capable of proliferating in high-heat sections of the manufacturing chain and can form biofilms on the equipment surface, enabling them to persist in the processing environment (Burgess et al., 2010). The source of these contaminants in milk remains unclear but several species of *Geobacillus* s.*l.* and *Aneurinibacillus* have been isolated from fodder, the milking equipment and bovine raw milk (Phillips and Griffiths, 1986, Scheldeman et al., 2005, Miller et al., 2015b). Here, I isolated *Parag. caldoxylosilyticus* and *Parag. thermoglucosidasius* from faeces, pasture, trough water and milking cups while *Anox. calidus* and *Aneu. thermoaerophilus* were isolated from trough water or pasture and the milking equipment, respectively. Although I did not recover these genera from soil, they have been frequently isolated from soils and sediments (Ahmad et al., 2000, Obeidat et al., 2012, Bezuidt et al., 2015). In my study, extensive swarming and spreading on MA for the soil sample prevented the obtention of the thermophilic spore diversity on this media, which may explain the absence of detection of these bacteria in soil as they were only recovered after incubation on MA in other sample types. The same species of *Parageobacillus* and *Aneurinibacillus* have been isolated from feed (fodder and green crops) and raw milk on bovine dairy farms (Scheldeman et al., 2005) while *Anox. calidus* was isolated from soil in the vicinity of a powerplant (Cihan et al., 2014). Eijlander et al. (2019) isolated *Parag. caldoxylosilyticus* and *Aneu. thermoaerophilus* from bovine dairy powders. The detection of these bacterial species in dairy powders highlights their possible entry in raw milk at the farm level and their persistence in the dairy manufacturing environment. In my study, *Geobacillus s.l.*, *Anoxybacillus* and *Aneurinibacillus* demonstrated strong spoilage potential, producing majority proteases but also lipases in some instances. Protease activity is common in members of *Geobacillus s.l.* and has been reported for *Parag. thermoglucosidasius* (Suzuki et al., 1983) and *Parag. caldoxylosilyticus* [(Fortina et al., 2001), Chapter 4]. *Aneurinibacillus thermoaerophilus* was also previously shown to produce thermostable proteases and

lipases that may retain activity post-processing and thus impact the quality of reconstituted milk (Rahman et al., 2009, Ebrahimpour et al., 2011). Koc et al. (2015) identified *Anox. calidus* as the most lipolytic bacterial species in a set of thermophilic spore-formers which included the dairy powder contaminants *G. thermodenitrificans* and *G. stearothermophilus*. These findings suggest that several species of *Geobacillus s.l.*, *Anoxybacillus* and *Aneurinibacillus* with potential to spoil dairy products are present in the sheep dairy farm environment and thus may be transferred to raw milk. My work identifies farm sources that may function as reservoirs of these bacteria, and which may represent sources of thermophilic spore contamination in raw milk. However, the concentrations of spores recorded in the pasture environment were particularly low.

The diversity of psychrotrophic spore-formers was high and heterogeneous across the different farm samples. The most abundant bacteria were identified as *Psychrobacillus* and *Solibacillus* and they displayed weak spoilage potential. *Psychrobacillus* and *Solibacillus* have been originally isolated from soil and river water (Larkin and Stokes, 1967, Abd El-Rahman et al., 2002) as well as from wildlife [e.g. ants (Shen et al., 2017) and fox faeces (Rodríguez et al., 2020)], highlighting their association with the natural environment. *Solibacillus* and *Psychrobacillus* demonstrated weak to moderate spoilage potential, which corroborated previous findings reporting that these bacteria lack the ability to spoil dairy products [Chapter 4, (Rodríguez et al., 2020)]. *Solibacillus* and *Psychrobacillus* have been isolated from bovine dairy farms, the udders of dairy cows and raw milk (Huck et al., 2008, Kent et al., 2016) but they are rarely isolated from processed dairy products, and have not been linked to dairy spoilage (Lucking et al., 2013, Buehler et al., 2018). In a study evaluating the sporulation, germination and growth of several species of psychrotrophic spore-formers, three species of *Psychrobacillus* failed to sporulate in laboratory conditions (Buehler et al., 2018). However, in my study high concentrations of their spores were found throughout the pasture-based farm environment, and even higher concentrations were also recorded in the barn environment of a sheep dairy farm (see Chapter 4). These results suggest that farm-associated factors may promote sporulation of *Solibacillus* and *Psychrobacillus* spp. while laboratory- or milk-associated factors could increase spore germination and/or the presence of vegetative cells of these bacteria. If these bacteria are mostly present in milk as vegetative cells, they would be more efficiently removed by heat processing such as pasteurisation, which could explain the rare accounts of their detection in dairy products. In order to test this hypothesis, future work may consider enumerating vegetative cells of *Psychrobacillus* and *Solibacillus* in freshly drawn milk and at different timepoint before and after processing to monitor changes in the populations of these bacteria.

The principal psychrotrophic spore-formers implicated in the quality of refrigerated dairy products are *Bacillus* and *Paenibacillus* spp. which originate from raw milk (Huck et al., 2008) or post-processing recontamination (Alles et al., 2018) and proliferate in refrigerated fluid milk. By understanding the ecology of these bacteria in their natural environment, including on dairy farms, systemic interventions may be designed to efficiently prevent their entry in milk and subsequent persistence in the dairy continuum. On bovine dairy farms, several sources are thought to be implicated in the contamination of raw milk by psychrotrophic *Paenibacillus*, including bedding materials, feed, manure, soil and water (Huck et al., 2008) but this has not been studied on sheep dairy farms. In the present study, psychrotrophic *Paenibacillus* were highly abundant in faeces, trough water and particularly soil, but not in pasture. Interestingly, the *Paenibacillus* counts found in this study for pasture (3.71 $\log_{10}$ cfu/g) are similar to those recorded in silage analysed in Chapter 4 (3.79 $\log_{10}$ cfu/g). Soil had the highest concentrations of psychrotrophic *Paenibacillus* spp. (5.83 $\log_{10}$ cfu/g), suggesting it may represent an important reservoir of these bacteria on dairy farms. Many of the recently discovered *Paenibacillus* spp. have been isolated from soil and found to be involved in key biological processes [e.g., plant symbiosis, biocontrol and bioremediation (Berge et al., 2002, Grady et al., 2016, KIm et al., 2021). I isolated several species simultaneously from soil, faeces, trough water and/or milking cups, including *P. anaericanus*, *P. macquariensis* and *P. tundrae.* These bacteria, and the vast majority of *Paenibacillus* isolates recovered during my study, demonstrated strong spoilage potential and produced proteases, lipases, or both in combination, as well as the ability to grow at low temperature (7°C) for psychrotrophic isolates. The presence of *Paenibacillus* in faeces and water may point to the transfer of bacteria from soil to these sources, either through the ingestion of soil by livestock during grazing, or the contamination of the water troughs by animal-borne or wind-borne soil particles, respectively. These results suggest that soil may represent an important source of *Paenibacillus* contamination in raw milk produced on pasture-based sheep dairy farms. Practices that reduce the soil contamination of raw milk either directly (e.g., pre-milking conditioning of the udders) or indirectly (e.g., limiting soil-animal contact) may therefore be effective at mitigating the entry of *Paenibacillus* spores in raw milk and prevent the impact of these bacteria on the quality of pasteurised fluid dairy products.

5.3.4 Ecology of anaerobic spore-forming bacteria in the pasture environment

Clostridial spores were ubiquitous in the pasture-based farm environment, being isolated from soil, pasture, dairy sheep faeces, trough water and milking cups. In my previous study (see Chapter 4), *Clostridium s.l.* were also found to be ubiquitous in the barn environment of a sheep dairy farm which demonstrates the presence of these bacteria on New Zealand sheep dairy farms. The most widespread species included the lipolytic clostridia *C. butyricum* and *C. cadaveris* as well as the proteolytic bacteria *C. perfringens* and *Paracl. benzoelyticum*, all of which were also detected in the milking cups, thereby highlighting their possible transfer to raw milk.

Several *Clostridium s.l.* that have been associated with late blowing in cheeses (*C. sporogenes, C. butyricum*) or documented to have cheese-spoiling potential (*Paracl. benzoelyticum, C. perfringens*) were isolated. *Clostridium sporogenes* and *C. butyricum* have been frequently isolated from sheep milk and cheeses with late-blowing defects (Arias et al., 2013, Turchi et al., 2016). Out of 223 clostridia isolated from Manchego cheeses with late blowing defects, 78.9% and 1.8% consisted of *C. sporogenes* and *C. butyricum*, respectively (Garde et al., 2011). *Clostridium sporogenes* and *C. butyricum* belong to the proteolytic group and *C. butyricum* group, respectively, based on their metabolic capabilities (Pahlow et al., 2003). Lactate fermentation (the main cause of late blowing defects in cheese) and proteolysis are generally only found in proteolytic clostridia which are able to grow at relatively high pH [pH > 5 (Driehuis, 2013)]. Members of the *C. butyricum* group can grow at lower pH (pH > 4.5) and can ferment a wider range of monosaccharides, but they often lack proteolytic enzymes and the ability to ferment lactate. The proteolytic isolates of *C. butyricum* that were recovered in the present study (2 isolates) and in the one described in Chapter 4 (1 isolate) are to the best of my knowledge, the first reports of proteolytic *C. butyricum* in the literature. In a study, the manufacturing of cheese with milk artificially contaminated with monocultures of *C. sporogenes* and *C. butyricum* produced cheese showing mild symptoms of late blowing after 55 and 60 days, respectively, while cheese produced with milk inoculated with *C. tyrobutyricum* showed the same symptoms after only seven days (Gómez-Torres et al., 2015). The predominant clostridia known to impact both silage and cheese quality include *C. tyrobutyricum*, *C. beijerinckii*, *C. sporogenes* and *C. butyricum* (Driehuis, 2013, Li et al., 2020), while the cheese-spoilage potential of other *Clostridium s.l.* remains unclear. In the present study, several of these bacteria, including *C. perfringens, C. cadaveris, C. baratii*, *Paracl. benzoelyticum* and *T. petrolearius*, were isolated from farm sources.

Because these bacteria are present in the sheep dairy farm environment, they may gain entry to raw milk and ultimately contaminate cheeses. My results suggest that these bacteria possess the enzymes to contribute to the development of organoleptic defects in cheeses through proteolysis and lipolysis. To evaluate the potential impact of these bacteria on cheese quality *in situ*, they should be screened for other cheese-associated spoilage factors such as lactate fermentation, acid tolerance and salt resistance. Artificial inoculation of these bacteria in cheese milk would also provide an evaluation of their individual spoilage potential, however, both monocultures and polycultures should be tested as their simultaneous presence may be synergistically detrimental to cheese quality.

In the present study, the species of clostridia recovered from the pasture-based farm environment were similar to the ones isolated from the barn environment of a sheep dairy farm. Prevalent clostridia on both farms were similar, including *C. perfringens, Paracl. benzoelyticum, C. cadaveris*, *C. butyricum* and *Clostridium* sp. (*uliginosum*). However, some bacteria were found to be less abundant in the pasture environment than in the barn environment, such as *Te. petrolearius, C. sporogenes* and *C. senegalense*. In addition, two species isolated in the present study were not recovered from the barn environment (*C. baratii* and *Romboutsia lituseburensis*). Both the diversity and concentration of *Clostridium* spp. in milk are important factors that affect its quality and safety. My results suggest they have the potential to be affected by farming practices and systems.

Soil *Clostridium s.l.* have been implicated in clinical pathologies such as bacteraemia and enteric diseases in human as well as in livestock (McClane et al., 2006). Clostridial bacteraemia are generally rare, but they can be caused by a large set of species, (Repaci, 1910, Li et al., 2021). These bacteria are, in majority, opportunistic pathogens that take advantage of disruptions in the host tissues (e.g., following surgery or trauma) or of an immunocompromised state [e.g., cancer patients (Paula et al., 2001)] to cause disease. Several clostridia can cause illnesses in healthy individuals after these bacteria, or their toxins are ingested alongside food or contaminated water. These include *C. perfringens, C. botulinum* and *C. difficile,* but strains of other species have also been shown to produce toxins in laboratory and clinical environments, such as *C. butyricum, C. baratii* and *Paracl. bifermentans* (McClane et al., 2006, Hale et al., 2016). Multiple proteases and lipases produced by clostridia can also act as toxins. The *Clostridium perfringens* phospholipase C is the main virulence factor causing gas gangrene (Urbina et al., 2011) while protease activity in *C. perfringens* and *C. botulinum* increase pathogenicity by either activating protoxins or disrupting host tissues (Tjaberg, 1974, Hatheway et al., 1980). Because several *Clostridium* spp. documented to be associated with pathogenesis were detected in the present study, my results indicate that limiting the contamination

of milk by clostridia will not only help improve cheese quality but also product safety, including for powder-based dairy products in which the spores of clostridia may persist (Barash et al., 2010).

In the present study, CMG enrichment provided a unique look at the clostridial diversity. However, the enrichment may have favoured the growth of specific species of *Clostridium s.l.*: the predominant clostridia isolated in the present study and the one described in Chapter 4 were similar. In particular, SFP agar was originally designed for the isolation of *C. perfringens* (Shahidi and Ferguson, 1971), which could contribute to explain why these bacteria were abundantly recovered. In Australia, Drean et al. (2015) isolated *C. perfringens* from soil, feed, faeces milk filters and raw milk collected at bovine, ovine and caprine dairy farms. Because *C. perfringens* is relevant to both dairy quality and safety, there is a need to accurately survey the incidence of these bacteria in the New Zealand farm environment.

5.3.5 Ecology of *Pseudomonas* spp. in the pasture environment

In spite of their heat lability, *Pseudomonas* spp. constitute perhaps the most important group of dairy spoilage bacteria as they can impact the quality of refrigerated raw milk due to the production of thermostable and sometimes cold-active spoilage enzymes that can remain active after processing and during refrigeration. Because these bacteria are efficiently removed by processing, the presence of *Pseudomonas* spp. in heat treated dairy products is generally indicative of post-processing contamination (Alles et al., 2018) but the *Pseudomonas* found in raw milk originate mainly from the farm environment.

In my work, a wide array of *Pseudomonas* species (n = 43) were isolated from samples associated with a pasture-based sheep dairy farm using CFC media. Several studies have used CFC agar to enumerate and isolate *Pseudomonas* spp. from foods, including milk (Bruzaroski et al., 2020), as well as from different farms [e.g. fisheries and broiler farms (Tryfinopoulou et al., 2001, Maes et al., 2019)], but not in the dairy farm environment. My research constitutes the first account of isolation of such a diverse set of *Pseudomonas* spp. from environmental dairy farm samples using CFC media. Many species of *Pseudomonas* are telluric rhizobacteria or saprophytes that are involved in symbiotic relationships with plants, bioremediation and biocontrol (Silby et al., 2011). Because soil is an oligotrophic environment, heterotrophic telluric bacteria can lack the ability to grow on nutrient-rich media, including those specifically designed for the isolation of *Pseudomonas* such as CFC agar or Gould's S1 medium (Mead and Adams, 1977, Gould et al., 1985). To address this, nutrient-poor

media with selective properties for *Pseudomonas* spp. (e.g. NAA media) have been designed and are considered to be more suitable for the isolation of telluric species (Aagot et al., 2001). Here, the isolation of heterotrophic *Pseudomonas* spp. may possibly have been enhanced through the use of nutrient-poor media, however, my focus for bacterial isolation was on those species able to proliferate in milk, a media with high nutrient content. Li et al. (2013) used 454 pyrosequencing of the 16S rRNA gene to highlight the presence of more than 50 putative different species of *Pseudomonas* in soil samples. Using the selective NAA growth medium, the same study was only able to culture between 1.1 to 3.5% of the *Pseudomonas* cells present in soil samples, as enumerated by qPCR. In my present work, the isolation of *Pseudomonas* from soil using CFC media yielded at least 17 different species of *Pseudomonas*, with the majority displaying spoilage potential, including several putative novel bacteria. Across the samples, two other genera were isolated from CFC media: *Stenotrophomonas* (pasture and milking cups) and *Serratia* (milking cups) but together they represented less than 1% of the total bacterial diversity. The former genus comprises bacteria closely related to *Pseudomonas* both ecologically and phenotypically, although they have not been linked to dairy quality (Ryan et al., 2009). Many Proteobacteria such as members of *Serratia*, *Acinetobacter*, and *Brucella* can display natural multidrug resistance to antibiotics, including to those contained in the formulation of CFC agar [cetrimide, fusidic acid and cephaloridine (Stock et al., 2003)]. In my previous study (Chapter 4), *Pseudomonas* were also the predominant genus recovered after incubation on CFC media (90% of the diversity) although *Stenotrophomonas* and other bacteria (*Serratia*, *Advenella*, *Brucella* and *Phyllobacterium* spp.) were also detected. Natural antibiotic resistance can enable the growth of some bacteria on CFC media and thwart the recovery of P*seudomonas* isolates that may be outnumbered on isolation agar (Tryfinopoulou et al., 2001). Maes et al. (2019) isolated 13 species of *Pseudomonas*, two species of *Stenotrophomonas* as well as bacteria belonging to eight non-*Pseudomonas* genera from the water systems of five broiler farms using CFC agar. My results suggest that CFC media may be used to isolate *Pseudomonas* spp. from the pasture-based dairy farm environment with minimal interference from other genera. Further evaluation of this media should be undertaken to confirm its validity, including on farm samples with a microbiota that might display a high incidence of natural or acquired antibiotic resistance (e.g., farms with systemic antibiotic use, waste milk, soils contaminated with heavy metals).

The paucity of information pertaining to the ecology of *Pseudomonas* spp. on dairy farms represents a major hurdle to the identification of farm-level sources of these bacteria, and consequently to the design of interventions aiming at preventing their entry into raw milk. The *Pseudomonas* counts reported here provide an overview of the abundance of these bacteria in sources common to sheep

dairy farms. Pasture was identified as an important reservoir of *Pseudomonas*, while soil, faeces and trough water had relatively lower counts of these bacteria. This is consistent with other studies identifying *Pseudomonas* to be abundant in the rhizosphere of pastures (Ruscoe et al., 2021) and to be particularly abundant in the phytosphere of plant species, including grasslands and miscanthus (Aydogan et al., 2018, Purahong et al., 2018, Grady et al., 2019). Ruscoe et al. (2021) recorded *Pseudomonas* counts of 5.5 $\log_{10}$ cfu/g in bulk grassland soil using CFC media while other studies have reported concentrations ranging between 3.89 to 5.94 $\log_{10}$ cfu/g using Gould's S1 medium, and 4.62 to 6.41 $\log_{10}$ cfu/g using NAA media. After being ingested, the bacteria present in feed may be shed in faeces, which can contaminate the udders of the animals, and subsequently milking cups. The populations of *Pseudomonas* present in the faeces of livestock are feed- or waterborne and likely transient; although *Pseudomonas* spp. are frequently isolated from the faeces and GIT of animals, they are not considered to be functional members of their gut microbiota (Lutgendorff et al., 2008, Mani et al., 2021). However, the abundance of *Pseudomonas* in the GIT of livestock can be modulated by extrinsic and intrinsic factors. Mani et al. (2021) recorded an increase in the abundance of *Pseudomonas* in the rumen of sheep fed a diet supplemented with lactic acid bacteria or antibiotics, while a higher abundance of these bacteria was found in human patients with dysbiosis-related illnesses (Wu et al., 2013). Because of their intrinsic spectrum of antibiotic resistance, enteric populations of *Pseudomonas* may not be impacted by antibiotic treatment of dairy livestock (e.g., for mastitis), which could indirectly increase their relative abundance in the gut microbiota of the animals during antibiotic treatment. In the present study, faeces had the lowest *Pseudomonas* counts across the samples, and they were also lower than those recorded in my previous study (6.04-7.29 $\log_{10}$ cfu/g, see Chapter 4). The diet of livestock can impact the abundance and diversity of the microbiota of their faeces (Wu et al., 2007), yet I found that despite recording the highest concentration of *Pseudomonas* in pasture, faecal counts were remarkably low. Conversely, in my previous work the faeces of dairy sheep fed a silage-based diet had higher *Pseudomonas* counts although silage contained < 1 $\log_{10}$ cfu/g of culturable *Pseudomonas*. These results suggest that the abundance of *Pseudomonas* in the faeces of dairy sheep may be modulated by factors others than feed and that these bacteria are not shed in large amounts from pasture to animal faeces. The literature suggests that these factors may relate to the state of the gut microbiota of the animals and to the chemical and biological composition of their gut environment (Mani et al., 2021).

Most of the bacteria recovered after incubation on CFC were found to display strong spoilage potential and produced with high frequency both lipases (86% of the isolates), proteases (78%), or these enzymes in combination (42%). This is consistent with previous surveys of spoilage potential

in *Pseudomonas* isolates recovered from raw milk (Meng et al., 2017, Yuan et al., 2018b) and raw milk cheeses (Arslan et al., 2011). However, in the dairy processing environment, a lower frequency of spoilage potential was recorded by Dogan and Boor (2003) [51% protease production; 172/338 isolates), resulting from the presence of strains with weak spoilage potential identified as *Ps. putida*, *Ps. alcaligenes* and *Ps. pseudoalcaligenes* (Dogan and Boor, 2003, Arslan et al., 2011). In the present study, *Ps. graminis* was the only species with weak spoilage potential (≤ 50% isolates with lipase or protease), while only 21 unidentified putative *Pseudomonas* spp. (7.5% of the isolates) lacked both lipase and protease activity. The remaining bacteria exhibited strong spoilage potential, particularly amongst members of the *Ps. fluorescens*, *Ps. koreensis* and *Ps. fragi* subgroups, as well as of the *Ps. syringae* group, almost all of which were both lipolytic and proteolytic.

Because of their high genetic resemblance, the demarcation of closely related *Pseudomonas* species is often challenging during laboratory identification of these bacteria. Strain misidentification is historically common in *P. fluorescens*-like bacteria that share a variety of phenotypic and genotypic similarities, and which require a polyphasic approach for reliable identification (Marchand et al., 2009, Garrido-Sanz et al., 2016). As a result, many studies in the dairy production have highlighted strong spoilage potential in isolates identified as *Ps. fluorescens* (Dogan and Boor, 2003, Arcuri et al., 2008, Arslan et al., 2011) although these isolates were in fact likely to represent several other species belonging to the *Ps. fluorescens* complex (Marchand et al., 2009). Meng et al. (2017) identified seven members of the *Ps. fluorescens* subgroup and four members of the *Ps. fragi* subgroup to be proteolytic and to possess the *aprX* gene coding for a thermostable protease. Yuan et al. (2018b) highlighted the simultaneous production of proteases and lipases in members of the *Ps. fluorescens*, *Ps. fragi*, *Ps. gessardii* and *Ps. jessenii* subgroups, as well as the *Ps. putida* group. My work expands our understanding of the *Pseudomonas* capable of producing spoilage enzymes by assessing a large number of isolates that were identified at the species level using a polyphasic identification approach. In addition to proteases and lipases, *Pseudomonas* can also produce other types of spoilage-associated compounds, including β-galactosidases, lecithinases and other phospholipases (Yuan et al., 2018b) as well as several pigments (Circella et al., 2020, Carrascosa et al., 2021). Although the production of these compounds was not assessed in my study, the isolates have been preserved and may be further characterised in the future.

The *Pseudomonas* diversity recovered across the different farm samples was high (46 different species were identified) and particularly heterogeneous (eight species isolated from two or more samples). Only two bacteria could be considered somewhat prevalent: *Pseudomonas poae s.l.* (i.e., *Ps. poae* or *Ps. trivialis*) and *Ps. extremaustralis* were isolated from four and three different samples,

respectively. These bacteria belong to the *Ps. fluorescens* subgroup which constitutes one of the predominant taxa of dairy spoilage-associated *Pseudomonas* worldwide (Yuan et al., 2018b, Kumar et al., 2019). In the present study, members of the *Ps. fluorescens* subgroup (10 different species, see Appendix 5.5) were prevalent and particularly abundant in the environmental samples: soil, pasture, and trough water, suggesting that the natural environment may represent an important source of these pseudomonads. Another taxon of *Pseudomonas* commonly associated with dairy spoilage is the *Ps. fragi* subgroup (Marchand et al., 2009), which included *Ps. fragi*, *Ps. lundensis*, and *Ps. weihenstephanensis* in the present study. Contrary to *Ps. fluorescens*-like bacteria, *Ps. fragi*-like isolates were mainly recovered from faeces and milking cups. These results contrasted with the findings of my previous study in which members of the *Ps. fragi* subgroup were abundant in the environmental samples: bedding, trough water and particularly faeces, while *Ps. fluorescens* subgroup isolates were predominantly recovered from milking cups. My results also suggest that some *Pseudomonas* taxa may be specifically associated with the pasture-based farm environment, namely the *Ps. syringae*, *Ps. putida* and *Ps. lutea* groups. Members of the *Ps. syringae* and *Ps. lutea* groups are associated with the phyllosphere of pastures (Behrendt et al., 1999, Monika et al., 2020), while *Ps. putida*-like bacteria have been identified to be soil saprophytes (Behrendt et al., 1999). Some taxa of *Pseudomonas* may be particularly abundant in specific sources, and because the degree of exposure of the animals to these sources may differ across farming systems, the species of *Pseudomonas* present in the environment and raw milk may differ across farming systems. The results of my present work, corroborated by the findings presented in Chapter 4, suggest that contamination by members of the *Ps. fragi* subgroup, particularly *Ps. weihenstephanensis*, may be originating from the faeces of dairy sheep. *Pseudomonas weihenstephanensis* is an important milk contaminant that was recently shown to proliferate during the pre-processing storage of raw milk and has the ability to produce strong spoilage enzymes (Liang et al., 2022). In contrast, *Ps. fluorescens*-like bacteria appeared to be associated with natural environmental sources: soil and pasture.

In the present study, the *Pseudomonas* diversity found across the farm samples was highly heterogeneous: *Pseudomonas* species, and higher taxa (i.e., groups and subgroups), were often specific to a sample type. Members of the *Ps. mandelii* and *Ps. jessenii* subgroups were specific to soil, members of the *Ps. gessardii* subgroup were found in trough water and those of the *Ps. syringae* group were particularly abundant in pasture. In faeces and the milking cups, the predominant taxa consisted of the *Ps. fragi* and the *Ps. koreensis* subgroups, as well as the *Ps. putida* group for the milking cups only. Species that are grouped under a same taxon often share similar ecologies and biological functions (Silby et al., 2011, Garrido-Sanz et al., 2016), but the inter-taxon variability

found across the -as of May 2022- 297 validly published species of the *Pseudomonas* genus is notoriously high. Various species of *Pseudomonas* are likely to thrive in different farm reservoirs because of their ability to utilise environmental resources and interact with local macro- and microbiota. The predominant *Pseudomonas* taxa associated with dairy spoilage consist mainly of the *Ps. fragi* and *Ps. fluorescens* subgroups (Marchand et al., 2009, Reichler et al., 2018), but other subgroups in the *Ps. fluorescens* complex (e.g. the *P. koreensis* and *P. gessardii* subgroups), as well as members of other taxonomical units in the *Ps. fluorescens* lineage (e.g., the *Ps. putida* and *P. rhizosphaerae* groups) have been isolated from dairy products and the processing environment (He et al., 2009, Van Tassell et al., 2012, Chiesa et al., 2014, Reichler et al., 2021). Chiesa et al. (2014) identified *Ps. koreensis* (*Ps. koreensis* subgroup) as the predominant species isolated from environmental sources as well as from raw materials (raw milk) and finished products (mozzarella cheese) sampled in a cheese manufacturing plant affected by discoloration defects. Reichler and colleagues (Reichler et al., 2018, Reichler et al., 2020) isolated members of the *Ps. koreensis* and *Ps. chlororaphis* subgroups, as well as members of the *Ps. putida*, *Ps. lutea* and *Ps. rhizosphaerae* groups from a pasteurised milk manufacturing plant. The same authors later demonstrated the production of spoilage enzymes (proteases and lipases) and compounds (yellow pigments), in a cluster-dependent fashion by these bacteria, highlighting their potential to impact dairy quality through different processes (Reichler et al., 2021). In my study, isolates belonging to the *Ps. mandelii* and *Ps. jessenii* subgroups, as well as those of the *Ps. putida* group, were almost exclusively lipolytic while isolates identified as part of the *Ps. gessardii* and *Ps. koreensis* subgroups as well as the *Ps. syringae* group demonstrated strong and simultaneous production of lipases and proteases. These results are consistent with the spoilage assessment presented in my previous work for isolates belonging to the *Ps. mandelii*, *Ps. jessenii*, *Ps. gessardii* and *Ps. koreensis* subgroups (see Chapter 4). My findings also corroborate previous studies that found similar production of spoilage enzymes in members of these taxonomic groups (Meng et al., 2017, Yuan et al., 2018b, Zarei et al., 2020, Reichler et al., 2021). Zarei et al. (2020) identified *Ps. jessenii* and *Ps. gessardii* as the predominant contaminants of bovine milk in Iran and demonstrated the production, and subsequent deleterious effect on the quality of UHT milk, of potent thermostable AprX proteases. The presence of genes coding for hydrolytic spoilage enzymes has also been reported in *Ps. syringae*-like bacteria (Patel et al., 2017) but they have, to the best of my knowledge, not been isolated from milk or dairy products, nor has the functionality of their enzymes been investigated prior to my work. My results provide clues to the capability of different species and taxonomic units to produce spoilage enzymes. Future work may build on these findings to evaluate the ability of these bacteria to impact dairy quality *in situ*. For example, despite the strong and frequent production of spoilage enzymes by Ps. *syringae*-like

Pseudomonas, these bacteria have been almost exclusively identified as plant pathogens, and the presence of these enzymes is likely to only serve scavenging- or pathogenesis-related functions, rather than pertain to the spoilage of dairy products (Patel et al., 2017). Conversely, the *Ps. putida* group is highly versatile and contains species that may be animal or plant pathogens and symbionts, as well as telluric saprotrophs (Nishimori et al., 2000, Dabboussi et al., 2002, Abanda-Nkpwatt et al., 2006). Members of this taxonomical group have been repeatedly isolated from milk and dairy products and found to produce lipases (Reichler et al., 2021), which suggests that they might be involved in dairy spoilage.

5.3.6 Possible sources of milk contamination during grazing

Milking cups represent the interface where the bulk of milk contamination takes place. Environmental bacteria that are present in the milking cups, or those that have gained access to the surface of the udders may flow into raw milk during milking. Contamination may also stem from the milking equipment as a result of equipment deterioration, inadequate cleaning-in-place procedures, and the colonisation of the pipeline by biofilm-forming bacteria (Weber et al., 2019). By analysing miking cups, I was able to assess the presence of contaminants that were being transferred from the environment or that were present on the cups before milking, while overlooking the bacteria that enter raw milk in the pipeline of the milking equipment.

The collection of milking cup swab samples is prone to variations across studies as several types of swabs and buffer are used, which can impact the recovery rate of microorganisms (Goverde et al., 2017). In addition, bacterial counts of milking cup swabs are often reported with different units (e.g., $\log_{10}$ cfu /ml of buffer, $\log_{10}$ cfu /cm^2 of cups surface or $\log_{10}$ cfu /swab) that may not be easily translatable from one study to another without additional sampling metadata (Scheldeman et al., 2005, Capodifoglio et al., 2016). Together, these factors limit the comparison of bacterial counts in milking cups across studies in which different approaches are used. The milking cup swabs from Farm 1 (barned animals, see Chapter 4) and Farm 2 (grazed animals, reported in the current study) were collected and analysed with the same technique, equipment, and methods, which contributed to limit sampling- or handling-associated bias and thus enable a more meaningful comparison between the bacterial counts obtained for the milking cup swabs of these studies. The spore counts recorded in the present study for the milking cups of a pasture-based sheep dairy farm (2.26-3.39 $\log_{10}$ cfu/swab) are lower than those I previously recorded in milking cups collected at a barn-based farm (range 3.22-4.52 $\log_{10}$ cfu/swab, see Chapter 4). Milk-contaminating spores originate from environmental dirt

particles (i.e., bedding materials, soil, faeces) that are present on the udders of dairy livestock and which may be dislodged during milking, thereby entering raw milk (Vissers et al., 2007c). Therefore, low concentrations of spores in the grazing environment may cause the transfer of fewer bacteria to the milking equipment and raw milk, as compared to a housing set-up. Vissers et al. (2007c) proposed an equation to estimate the quantity of spores that may be transferred in the form of dirt from the environment to raw milk. The concentration of bacteria present in environmental sources that compose this dirt (in $\log_{10}$ cfu/g), in our case soil and faeces, is multiplied by the quantity of dirt entering raw milk (in g/l) to provide an estimation of the concentration of bacteria transferred to raw milk (in $\log_{10}$ cfu/l). Vissers et al. (2007c) found an average of 59 mg/l of dirt being transmitted to raw milk during the milking of dairy cows, and other studies have reported comparable results on bovine dairy farms where animals are barned (Stadhouders and Jørgensen, 1990, Martin et al., 2019). The quantification of the transmission of dirt to raw milk has, to the best of my knowledge, not been evaluated on sheep dairy farms, nor on farms that exclusively rely on pasture grazing, however this process is likely to follow similar principles than on bovine dairy farms. Using the concentrations of bacteria found in the present study for faeces and soil, and assuming an average of 59 mg/l of dirt being transmitted to raw milk, estimates of spore counts in raw milk are as follow for mesophilic (3.22 to 5.46 $\log_{10}$ cfu/l), psychrotrophic (2.86 to 5.09 $\log_{10}$ cfu/l) and thermophilic spores (1.73 to 2.85 $\log_{10}$ cfu/l). These estimates are similar to the ones calculated in Chapter 4 for mesophilic (5.46 to 5.69 $\log_{10}$ cfu/l) and psychrotrophic spore counts (2.97 to 5.77 $\log_{10}$ cfu/l) in raw milk produced by barned dairy ewes. However, the estimate of thermophilic spore counts in sheep raw milk produced on a pasture-based farm is particularly lower than that of milk produced in a barn-based environment (4.18 to 5.35 $\log_{10}$ cfu/l, see Chapter 4)) These results suggest that the spore contamination of raw milk may be higher on sheep dairy farms that harbour high concentrations of spores in the environment, which corroborates previous observations in the bovine farm environment (Vissers et al., 2007b, Vissers et al., 2007d, Martin et al., 2019). Specifically, my results suggest that the environment of pasture-based farms may harbour lower concentrations of thermophilic spores than barn-based farms, which could in turn contribute to limit the transfer of these bacteria to raw milk.

In the present study, the *Pseudomonas* counts in the milking cups (8.50 $\log_{10}$ cfu/swab) were particularly high and particularlyhigher than the spore counts, indicating the possible transmission of a large amount of *Pseudomonas* to raw milk. *Pseudomonas* counts of the milking cups were also higher than previously recorded in a barn-based sheep dairy farm (7.44 $\log_{10}$ cfu/swab, see Chapter 4). Using the aforementioned equation described by Vissers et al. (2007c), the estimated *Pseudomonas* counts in raw milk for the present study would range between 4.11 to 4.64 $\log_{10}$ cfu/l.

This range is lower than my previous estimates for raw milk produced at a barn-based sheep dairy farm (5.78-6.21 $\log_{10}$ cfu/l), despite *Pseudomonas* counts of the milking cups being higher in the present study. This result suggests that the dynamics of the transmission of *Pseudomonas* to milking cups and raw milk differ to that of spores and possibly involve other sources of contamination. Because *Pseudomonas* are particularly abundant on pastures (Dignam, 2016), pasture-based dairy farms may be at risk of increased milk contamination by these bacteria. In addition, several other factors can contribute to the contamination of milking cups and raw milk, including milking staff, wash water quality and hygiene of milking and the milking parlour (Gibson et al., 2008, Vidal et al., 2017). *Pseudomonas* spp. are known to thrive as aquatic organisms and they can colonise water supplies and the pipeline of water systems through biofilm formation (Maes et al., 2019), which can lead to ongoing farm-level contamination of the wash water source, the milking equipment and subsequently of raw milk in the absence of adequate cleaning and monitoring (Sun et al., 2022).

Whereas the species of spore-formers recovered from the milking cups were, in majority, also isolated from the environmental samples (35/49 species), most of the *Pseudomonas* species found in the milking cups were not isolated from other samples (5/7 species). The *Pseudomonas* that were predominant in the milking cups were, with the exception of *Pseudomonas poae s.l.*, either not detected in the environment (*Ps. fulva s.l.*, *Ps. lundensis*, *Ps. rhizosphaerae*) or only found at low abundance in faeces and pasture (*Ps. koreensis*). Because the environmental samples and milking cups were sampled with an interval of six weeks, variations in the environmental microbiota at these two time points could explain the fact that few species isolated from the miking cups were present in the environmental samples. Another hypothesis involves the contribution of additional sources that were not assessed in my work, but which can contribute to milking cups contamination, including initial equipment contamination, wash water, milking staff and hygiene of milking and the milking parlour (Gibson et al., 2008, Vidal et al., 2017). To provide additional answers, future work may consider surveying environmental populations of *Pseudomonas* both temporally (i.e., weekly) and spatially (i.e., different browsing areas available to the animals) on dairy farms to assess farm-level variations in the bacterial diversity. By sampling and analysing milking cups simultaneously to environmental sources, one would be able to assess the extent of environmental contamination. Indirectly, this will also provide an assessment of the contaminants that may originate from other sources, which could then enable the set-up of targeted monitoring and interventions on sheep dairy farms.

Most of the species of spore-forming bacteria that were isolated from the milking cups were also isolated from the farm environment, indicating the possible transfer of these bacteria from the farm

to raw milk. This was particularly true for aerobic thermophilic (10/11 of the bacteria isolated from milking cups) and psychrotrophic spore-formers (9/12) as well as anaerobic spore-formers (4/4) but only about half of the mesophilic bacteria isolated from milking cups were also detected in the environment (12/22). This result demonstrates that the diversity of the spores that contaminate milking cups consists in majority of the populations found in the environment, particularly for thermophilic and psychrotrophic spore-formers. The detection of several contaminating mesophilic spore-forming bacteria that were not isolated from elsewhere in the environment may suggest that additional sources are contributing to the contamination of milking cups.

The bacteria that were predominant in the environment were consistently isolated from the milking cups, namely *B. cereus s.l.*, *Pr. megaterium s.l.*, *Solibacillus* spp., *Psychrobacillus* spp. and *B. pumilus s.l.* amongst mesophiles, *T. vulgaris* for thermophiles and *B. cereus s.l.*, *Psychrobacillus* spp. and *Solibacillus* spp. for psychrotrophs. These results suggests that the predominant spore-forming bacteria in the environment are most likely to be transmitted to milking cups. This is in agreement with my previous findings (Chapter 4), and, although the bacterial species that were predominant were similar in some instances (e.g., *B. pumilus s.l.*, *Solibacillus* spp., *T. vulgaris*), they also varied (e.g., *B. licheniformis*, *Alkalihalobacillus clausii*, see Chapter 4). Subtyping of the isolates will be undertaken to allow for source-tracking of the milking cups contaminants and assess the findings of this study.

When the different spores were considered per isolation method (i.e., mesophiles, thermophiles and psychrotrophs), the main sources of milking cups contamination were found to vary. For both mesophilic and psychrotrophic spore-formers, soil and trough water had the most bacterial species common with milking cups. These contaminants included spoilage bacteria (e.g., *B. cereus s.l.*, *P. tundrae*), but also in majority a diverse set of bacteria that have not or not yet been associated with dairy quality, though some possessed strong spoilage potential (i.e., *Lysinibacillus*, *Mesobacillus*, *Neobacillus*, *Psychrobacillus*, *Solibacillus*, *Peribacillus* and *Sporosarcina* species). Faeces were identified as another potential source of spoilage-associated mesophilic and psychrotrophic bacteria (e.g., *B. licheniformis*, *B. pumilus s.l.*, *B. cereus s.l.*) while the contribution of pasture was found to be minimal. These results corroborate previous observations that the mesophilic and psychrotrophic spore contamination of raw milk mainly originate from soil and faeces during the grazing period (Vissers et al., 2007c, Vissers et al., 2007d). However, my results also demonstrate that contamination may simultaneously arise from multiple environmental sources and is frequently species dependent.

Across mesophilic, thermophilic and psychrotrophic spore-formers, trough water was identified as the farm component contributing the most to the contamination of the milking cups. Overall, the trough water sample shared 21 common species of spore-forming bacteria with milking cups, which was markedly more than soil (15 species), and faeces or pasture (12 species). The quality and palatability of drinking water impacts the drinking habits of dairy livestock and can also affect milk production (LeJeune et al., 2001). Animal drinking water has been included in the microbiological analysis of several studies, but its contribution to the spore contamination of raw milk has seldom been highlighted, perhaps because it generally contains low spore counts (Borreani et al., 2019, Martin et al., 2019). Martin et al. (2019) found that the concentration of highly heat resistant thermophilic spore-formers in livestock drinking water was a variable of importance with regards to the concentration of these spores in raw milk. However, the same study recorded low spore counts in water and could not establish the same link between the levels of mesophilic, psychrotrophic and thermophilic spores in drinking water and in raw milk. Some bacteria can proliferate in trough water by using the nutrients deposited during cross-contamination by other sources [e.g., faeces, feed, plant matter (LeJeune et al., 2001, Brillard et al., 2015)]. In addition, this cross-contamination provides an influx of bacteria that contributes to shape the microbiological composition of trough water. Using modelling, (Ayscue et al., 2009) highlighted trough water as a reservoir of pathogenic *E. coli* O157:H7 in the barn environment and suggested that these bacteria could be transmitted from other sources and proliferate in the water over time, maintaining stable populations. On dairy farms, the occurrence and diversity of spore-formers in trough water remains largely unexplored. My results suggest that the drinking water of dairy livestock may represent a source of milking cups and thus of raw milk contamination. The water supplied to the troughs consisted of clean, potable water which underwent filtration and UV-light treatment at the farm. However, trough drinking water also contains, in majority, bacteria originating from cross-contamination by other sources, particularly soil, faeces, and the animals (Van Eenige et al., 2013). It would be interesting to assess if the similarity in bacterial diversity between trough water and milking cups indicates transmission of bacteria between these two components, or if it reflects the presence of environmental bacteria in trough water that are originating from other farm sources. Poor management of the drinking system will be likely to increase the microbial load of trough water and thus possibly exacerbate its contribution to the cycling and transmission of bacteria in the farm environment (Ayscue et al., 2009, Van Eenige et al., 2013). Several practices can help improve the microbiological quality of trough water, including frequent cleaning of the troughs, monitoring and maintenance of the water supply system and a moderate stocking rate which prevents over-soiling of drinking troughs.

5.4 Conclusions

In Chapter 4, a comprehensive microbiological survey of an indoor sheep dairy farm revealed the presence of high concentrations of spoilage bacteria in sources associated with the barn environment during housing of the animals. These bacterial communities, characterised by a core set of species with strong spoilage potential, were pervasive across barn components and milking cups, indicating a high degree of bacterial transmission within the indoor setting. Extending this investigation, the study described here adopts a similar methodology to examine microbial communities associated with the grazing environment of dairy sheep. This approach complements the findings of Chapter 4 and contributes to improve our understanding of the dairy spoilage-associated microbiota found on sheep dairy farms with different farming systems. The results of this study reveal that the pasture-based sheep dairy farm environment represents an important source of bacteria with the enzymatic capability to adversely impact the quality of sheep milk products. However, contrasting with the findings of Chapter 4, the bacterial communities associated with the grazing environment and corresponding milking cups exhibited significant diversity and heterogeneity, suggestive of a lower degree of bacterial transmission during grazing. These findings lay the groundwork for emphasising and further exploring potential differences in the distribution, diversity, and transmission of spoilage bacteria on sheep dairy farms operating under different farming systems.

Overall, low spore counts were recorded across the pasture environment and particularly in faeces, typically a key source of spore contamination in milk. Soil was the source containing the highest concentrations of spores, indicating that, when present on the udders of dairy ewes, it could contribute to the transfer of large amounts of these bacteria to raw milk. *Pseudomonas* species were particularly abundant in pasture which agreed with previous assessments of their ecology. However, the detection of low concentrations of *Pseudomonas* in faeces suggested that the populations present on pasture were not proportionally shed in faeces. Nonetheless, the *Pseudomonas* contamination of the milking cups was particularly high, which could suggest that the grazing environment somehow favours the transfer of these bacteria to miking cups and raw milk.

In each of the five detection methods, the array of bacterial species that were identified to have spoilage potential encompassed bacteria previously associated with the spoilage of bovine and ovine dairy products, but also several environmental commensal bacteria as well as many bacteria isolated for the first time in a dairy farm environment. The culturable bacterial associated with the pasture environment were notably diverse and there were only few bacterial species found ubiquitously across

the grazing environment. This stark contrast to the findings of Chapter 4 suggests a low degree of bacterial cross-contamination in the grazing environment. This observation is further supported by the distinct population structure of the bacterial communities in milking cups, underscoring the disparity between the microbial composition of environmental samples and that of the milking equipment.

In addition, this work uncovered a substantial set of isolates whose identity remain unclear, some of which are likely to represent novel bacterial species. Future phenotypic and genotypic characterisation of these bacteria will enable an assessment of their possible involvement in dairy spoilage or in other processes (e.g., bioremediation, biocontrol), and will permit their formal discovery.

My results also draw attention to the variations in bacterial diversity associated with the use of different isolation media in farm samples. Specifically, the diversity and counts of thermophilic spore-formers varied across sheep blood agar and milk agar, which corroborated my previous observations. Thermophilic spore-formers are important contaminants of dairy powders that may originate from raw milk and the farm environment. The study of these bacteria in the farm environment requires a set of updated culture methods that would enable a focus on the taxa associated with dairy spoilage, such as *Geobacillus s.l.* and *Anoxybacillus*, which are often not predominant. Additionally, the further characterisation of *T. vulgaris*, a thermophilic bacterium highly predominant in the environment and found in dairy powders, should be undertaken to assess its dairy spoilage potential.

In New Zealand, the sheep dairy industry operates a combination of farms with grazed and/or barned animal. Yet, the implications of these farming systems on the microbiology of the farm and of raw milk were unknown, which thwarted any preventive attempts at mitigating the impact they might have. My work contributes to address this lack of data, but it will need to be complemented by the study of additional farms, something which was not possible at the time of this project due to low farm availability. With the success of sheep dairy farming in New Zealand, many new farms become producers of sheep milk and join existing ventures handled by larger operators. This increase in the number of sheep dairy farms will provide a larger set of farms with homogeneous general farm practices and on which some of the hypotheses formulated in my work may be more conveniently assessed.

Chapter 6 - Tracking spoilage bacteria in ovine dairy farm environments: possible sources of milking cups contamination

6.1 Introduction

In prior chapters, I explored the diversity and abundance of spoilage bacteria within the farm environments of both a barn-based (Chapter 4) and pasture-based (Chapter 5) sheep dairy farms. These studies revealed not only a highly diverse set of bacteria inhabiting sheep dairy farms, including numerous species implicated in dairy spoilage, but also identified key reservoirs of these bacteria at the farm level, such as feed, bedding materials, soil, and faeces. The coincident presence of bacteria in both milking cups and the broader farm environment indicated that the latter could potentially serve as a significant source of contaminants in milking cups. However, to comprehensively understand the transmission of these bacteria at the farm level, it is essential to investigate and compare the genetic relatedness of isolates across different sources. A specific emphasis is placed on *Thermoactinomyces* species, which were identified as highly abundant in both environments, by sequencing the genomes of representative isolates and studying their phylogeny using several genome-based analyses. The outcomes of this research aim to consolidate the ecological insights gained in Chapters 4 and 5, contributing to a comprehensive understanding of potential routes of milk contamination in both indoor and outdoor sheep dairy farms. This knowledge will lay the groundwork for future research endeavours focusing on the study of specific routes of milk contamination on sheep dairy farms.

Raw milk accounts for most of the contaminants found in dairy products, however it is the farm environment that represents the principal source of bacteria in raw milk (Miller et al., 2015b, VanderKelen et al., 2016, McHugh et al., 2020). Spore-formers and *Pseudomonas* spp. are ubiquitous on dairy farms and can be particularly abundant in a variety of sources, including soil, plants, feed

and wildlife (Gould et al., 1985, Hong et al., 2009). The production of compounds that are associated with dairy spoilage (e.g., hydrolytic exoenzymes) is particularly common across the microbiota of the natural environment because the initial function of these molecules serves saprotrophic, symbiotic or pathogenic purposes in these bacteria (Hong et al., 2009). Previously, I showed that bacteria displaying spoilage potential represented more than 80% of the isolates of spore-formers and *Pseudomonas* (n = 2,932, see Chapter 4 & 5) that were recovered from sources present on two New Zealand sheep dairy farms. Because of the intrinsic relationship between the components of dairy farms, bacteria may be transmitted across sources such as soil, feed, and the animals, which contributes to their ubiquity in the environment of dairy livestock and can ultimately lead to their entry in milk (Vissers et al., 2007d). Several studies in the bovine dairy farm environment have highlighted a set of key routes of bacterial transmission and raw milk contamination, which form together the basis for what is termed the dairy farm contamination cycle (Pahlow et al., 2003, Gleeson et al., 2013). This schematic representation pictures the global transmission of bacteria taking place across sources of the dairy farm environment, as well as their possible routes of entry into raw milk (see Figure 2.1). The general management and functioning of bovine and ovine dairy farms remain, in essence, similar, but differences pertaining to physiological and morphological features of the species of farmed livestock, or to their management, have the potential to affect the dynamics of the dairy farm contamination cycle. For example, bovine milk is generally found to contain higher concentrations of the spores of butyric acid bacteria (*Clostridium* spp. and related species capable of cheese spoilage) during the winter season (Vissers et al., 2007b). Evidence from research on these farms suggests that the increased spore contamination of raw milk during winter can be attributed to a change in husbandry practices, including barning of the cows, and particularly to the feeding of ensiled forages with high spore counts (Vissers et al., 2007b, Borreani et al., 2019). Despite similarities between the seasonality of the feeding regime of sheep and cows (i.e., reliance on pasture availability), peak butyric acid bacteria spore counts in ewe milk generally occur during summer time (Garde et al., 2011). Even on farms that are not feeding silage to dairy ewes, butyric acid bacteria spore counts in milk are higher in summer, suggesting this effect may be, unlike on bovine dairy farms, primarily driven by factors other than feed (Turchi et al., 2016).

Most of the currently available knowledge pertaining to the dynamics of raw milk contamination on dairy farms has been generated by studies conducted in the bovine dairy farm environment (Vissers et al., 2007d, Gleeson et al., 2013). Recently, mathematical modelling has been used to provide additional insight on the transfer of bacteria throughout specific nodes of the bovine dairy farm contamination cycle, including around bedding materials or feed (Martin et al., 2019, Murphy et al.,

2019), or to study the population dynamics of bacteria representing key dairy contaminants such as *B. cereus s.s.* (Vissers et al., 2007e). On European sheep dairy farms and as a result of the unique cultural and financial implications of sheep milk cheeses in this area, several studies have focused on studying the transmission of butyric acid bacteria spores to raw milk (Arias et al., 2013, Turchi et al., 2016). However, the ecology and population dynamics of other spore-forming bacteria, as well as of *Pseudomonas* species in the ovine dairy farm environment, remain largely unknown, in spite of their potential impact on dairy quality should they enter raw milk.

In the food industry, phylogenetic tools are commonly utilised to compare the genetic material of foodborne bacteria and environmental isolates, with the goal of identifying the sources of organisms responsible for outbreaks (Chiaverini et al., 2021) or food spoilage events, during both primary production (te Giffel et al., 2002), and processing (Chiesa et al., 2014). Currently, the highest degree of phylogenetic resolution is provided by genome-scale phylogenetic analysis [e.g., using the Genome-to-Genome Distance Calculator tool available at https://tygs.dsmz.de/ (Meier-Kolthoff and Göker, 2019)], while other techniques such as multilocus sequence typing (MLST) of housekeeping genes can also provide results comparable to genome-scale comparisons (Garrido-Sanz et al., 2016). However, these approaches require sequencing of individual genes, or alternatively access to the genome sequence of the isolates. To enable quick, affordable and strain-level source-tracking of the environmental and contaminating bacteria present in our food systems, several techniques relying on fingerprinting of the bacterial DNA – and therefore which do not require sequencing of DNA fragments – have been developed, including randomly amplified polymorphic DNA PCR [RAPD-PCR, (Welsh and McClelland, 1990, Williams et al., 1990)], repetitive element sequence-based PCR [rep-PCR, (Versalovic et al., 1998)], amplified ribosomal DNA restriction analysis (ARDRA, (Grimont and Grimont, 1986)] and enterobacterial repetitive intergenic consensus PCR (ERIC-PCR, (Versalovic et al., 1991)]. RAPD has been widely used for the identification and typing of bacteria isolated from bovine dairy products (Rueckert et al., 2004, Sadiq et al., 2016a), or for the source-tracking of contaminants present in bovine raw milk and the farm environment (te Giffel et al., 2002). ERIC-PCR has been successfully used to type several bacteria including *Pseudomonas* spp. (Han et al., 2013, Zarei et al., 2018), as well as aerobic spore-formers isolated from dairy effluents (Gupta and Brightwell, 2017). Brightwell and Horváth (2018) used ARDRA to type *Clostridium* species isolated from red meat and its processing environment in New Zealand. ERIC-PCR and ARDRA have not been extensively used for the typing of bacterial isolates associated with the dairy production, but the resolution of these approaches, as reported in the aforementioned work as well as

when I used them across Chapters 4 & 5, suggest they are suitable to use for studying the phylogeny of dairy farm bacterial isolates.

In this Chapter, I used ERIC-PCR and ARDRA fingerprinting to carry out source tracking of the bacteria that were previously isolated from two sheep dairy farms and identify possible farm-level sources of milking cups and raw milk contamination. To further probe the phylogeny of *Thermoactinomyces* spp. with an improved resolution, the genomes of the predominant environmental ERIC-PCR phylotypes of these bacteria on both farms were sequenced and utilised in a set of phylogenetic analyses.

6.2 Results

6.2.1 Fingerprinting study data and quality of the analysis

The goal of this study was to perform source-tracking of raw milk contaminants by investigating the potential clonality of isolates recovered from different environmental sources and those found in milking cups. The bacterial strains included in this work had been previously isolated from two sheep dairy farms and identified to the species level by partial 16S rRNA gene sequencing, as described in Chapter 4 for Farm 1 (F1, see section 4.2.2) and in Chapter 5 for Farm 2 (F2, see section 5.2.2). Selection of the bacterial species to be included for phylogenetic analysis was performed on a farm basis and according to two criteria: (1) detection in both milking cups and the environmental samples of the farm considered, and (2) bacterial species with isolates found to exhibit strong spoilage potential (i.e., lipase and/or protease activity). Based on these criteria, 24 different species of bacteria (F1, n = 15; F2, n = 17, including eight species found on both F1 and F2) comprising a total of 693 bacterial isolates (F1, n = 351; F2, n = 342) were subtyped. Typing of the aerobic isolates (i.e., aerobic mesophilic spore-formers, thermophilic and psychrotrophic spore-formers as well as *Pseudomonas* spp.) was performed with ERIC-PCR as detailed in section 3.2.1. As the typing of anaerobic isolates using ERIC-PCR was unsuccessful, (often producing few to no amplified DNA fragments), ARDRA was used instead to type these bacteria (see section 3.2.2). A summary of the bacterial dataset and of the associated phylogenetic dataset generated by this study is available in Appendix 6.1.

For each bacterial species, distance matrices were built based on homologies between DNA fragments present on the gel electrophoresis profiles obtained after PCR and/or DNA restriction analysis (see section 3.2.3). An estimation of the resolution and depth of each analysis could be obtained through

the different metrics associated with each analysis (Appendix 6.1). Compared to ARDRA, ERIC-PCR analysis generally produced a more sophisticated phylogeny. Across the different bacteria, ERIC-PCR fingerprinting profiles were varied, yet with frequent identical matches between the profiles of environmental isolates and those from the milking cups. In comparison, ARDRA subtyping generated fingerprinting profiles that were particularly similar (67% to 100% similarity) among the isolates of a given bacteria.

6.2.2 Source tracking of the bacteria found in the barn-based sheep dairy farm environment

The phylogenetic survey conducted on the isolates of Farm 1 included analysis of four species of aerobic mesophilic spore-formers (Appendix 6.1), three species of thermophilic spore-formers, three species of psychrotrophic spore-formers, three *Pseudomonas* spp. and two species of *Clostridium sensu lato.* The DNA fingerprint of bacterial strains that had been isolated from milking cups was compared to that of environmental isolates originating from bedding materials, silage, trough water and silage. In the first instance, the phylogeny of these bacteria was studied by grouping isolates according to the sample type from which they had been isolated (e.g., faeces), to identify sources in the barn environment that may be involved in the transfer of bacteria to milking cups. The isolates were subsequently sorted by sample set (i.e., if they were isolated from sample collected in pens with either "old" or "new" bedding materials, see Chapter 4) to study the contribution of the different pens to the contamination of milking cups.

The results of the source tracking for F1 at the sample type level are presented by groups of isolation: mesophilic spore-formers (Figure 6.1), thermophilic spore-formers (Figure 6.2), psychrotrophic spore-formers (Figure 6.3), *Pseudomonas* spp. (Figure 6.4), and anaerobic spore-formers (Figure 6.5) The same analysis, albeit considering sample sets (i.e., "old" and "new") as opposed to sample types, is presented in Figure 6.6. For each species of bacteria, phylogenetic trees were built according to the distance matrices generated by the DNA fingerprinting analysis and using the unweighted pair group method with arithmetic mean (UPGMA) clustering algorithm (Appendices 6.2 – 6.17).

Potential sources of spoilage-associated mesophilic spore-formers contamination in milking cups were identified as mainly faeces, but also silage and bedding materials (Figure 6.1). The contribution of trough water was less substantial: across the four species of mesophilic spore-formers, there was no isolate from trough water which had an ERIC-PCR profile with a perfect match to one of the

milking cups isolates. On the dendrograms, isolates of *B. licheniformis* (Appendix 6.2), *A. clausii* (Appendix 6.3) and *B. pumilus s.l.* (Appendices 6.4 & 6.5) recovered from the milking cups showed an ERIC-PCR fingerprint profile analogous to that of isolates obtained from faeces, as well as from silage and bedding materials for *B. pumilus s.l.* only (Figure 6.1). Across the *B. pumilus s.l.* isolates, three clusters showed particularly distinct ERIC-PCR profiles (i.e., similarity < 70 %) and were denoted type A (Appendix 6.5), type B (Appendix 6.4), and type C (Appendix 6.5). *Bacillus pumilus s.l.* types A and C consisted exclusively of mesophilic isolates while there were both psychrotrophic and mesophilic isolates identified as *B. pumilus s.l.* type B. These results, together with the results of 16S rRNA identification suggested that type A *B. pumilus s.l.* isolates were likely to consist of species such as *B. pumilus* and *B. safensis* (henceforth referred as *B. safensis s.l.*). *Bacillus pumilus s.l.* type B isolates represented the most common phylotype and were identified as the species *B. zhangzhouensis* and *B. australimaris* (henceforth referred as *B. zhangzhouensis s.l.*), while type C isolates were identified as *B. altitudinis*, *B. aerophilus* or *B. stratosphericus* (henceforth referred as *B. altitudinis s.l.*). Isolates identified as *B. safensis s.l.* were not recovered from the milking cups but they were isolated from faeces and bedding materials. *Bacillus zhangzhouensis s.l.* were particularly prevalent across the samples, and those recovered from the milking cups had an ERIC-PCR profile matching that of environmental isolates from bedding materials, faeces and silage (100% similarity) but also from trough water (> 95% similarity), highlighting the ubiquity of this phylotype in the barn environment. *Bacillus altitudinis s.l.* isolates were found in both milking cups and the environmental samples, although they shared relatively low similarity in their fingerprinting profile (80-90% similarity). The ERIC-PCR profile of *B. subtilis* isolates originating from the milking cups did not have an exact match across environmental isolates of these bacteria, but a trough water isolate showed high similarity to an isolate from milking cups (> 95%, Appendix 6.6).

On the barn-based farm, silage and faeces were identified as the main sources of thermophilic spore contamination in milking cups. The only thermophilic spore-forming bacteria recovered from the milking cups with an exact match for their ERIC-PCR profile amongst the environmental isolates were strains of *Thermoactinomyces vulgaris* isolated from silage and faeces (Figure 6.2). Other strains of *T. vulgaris* isolated from bedding and trough water also showed high similarity to the milking cups isolates (> 95%). A few isolates from faeces and trough water (n = 3) clustered away from the bulk of the isolates, suggesting they may represent other species of *Thermoactinomyces* (Appendix 6.7). The ERIC-PCR profile of *Aer. pallidus* isolates from silage and faeces, the only environmental samples from which these bacteria were isolated, showed high similarity to that of the milking cups isolates (> 95%, Appendix 6.8), while strains of *Cald. kokeshiiformis* from faeces and trough water

(> 95% similarity, Appendix 6.9), but not silage (< 80%) were particularly similar to the milking cups isolates.

The ERIC-PCR profiles of isolates of psychrotrophic spore-formers from milking cups, which included *Paenib. xylanedexens* (Appendix 6.10), *Perib. psychrosaccharolyticus* (Appendix 6.11) as well as *Oceanobacillus chungangensis* (Appendix 6.12) were particularly different to those of the environmental strains. The highest similarity to the ERIC-PCR profile of milking cups isolates was found for *Perib. psychrosaccharolyticus* (< 85%, Figure 6.3).

Environmental *Pseudomonas* spp. were also different to the milking cups isolates according to ERIC-PCR fingerprinting (Figure 6.4). Faecal isolates of *Ps. koreensis* represented the bacteria most similar to the milking cups isolates (92% similarity, Appendix 6.13) while environmental isolates of *Ps. azotoformans* (Appendix 6.14) and *Ps. libanensis* (Appendix 6.15) respectively shared < 70% and < 90 % similarity of their ERIC-PCR profile with the milking cups isolates.

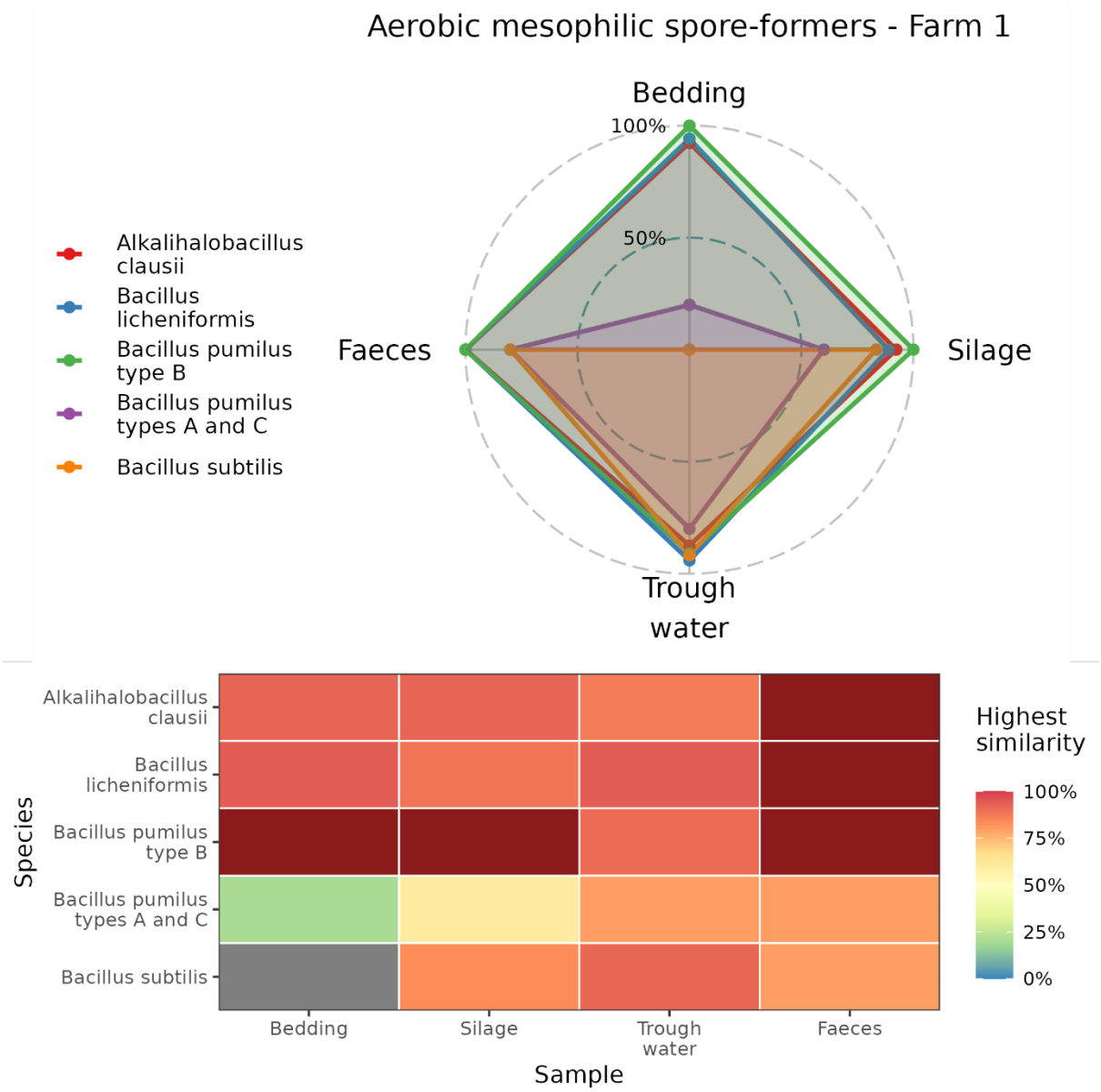


Figure 6.1. Radar chart comparing similarities in the ERIC-PCR profile of Farm 1 isolates of aerobic mesophilic spore-forming bacteria isolated from the environment, as compared to milking cups isolates.

The highest similarity values between the ERIC-PCR profiles of environmental and milking cups isolates are visible as coloured datapoints on the radar chart, and simultaneously presented under, in a table in which this value is pictured as a colour gradient. Grey squares indicate the absence of any isolates of the specific bacteria in a given sample while brown squares indicate the presence of at least one isolate with an ERIC-PCR profile identical to that of a milking cup isolate.

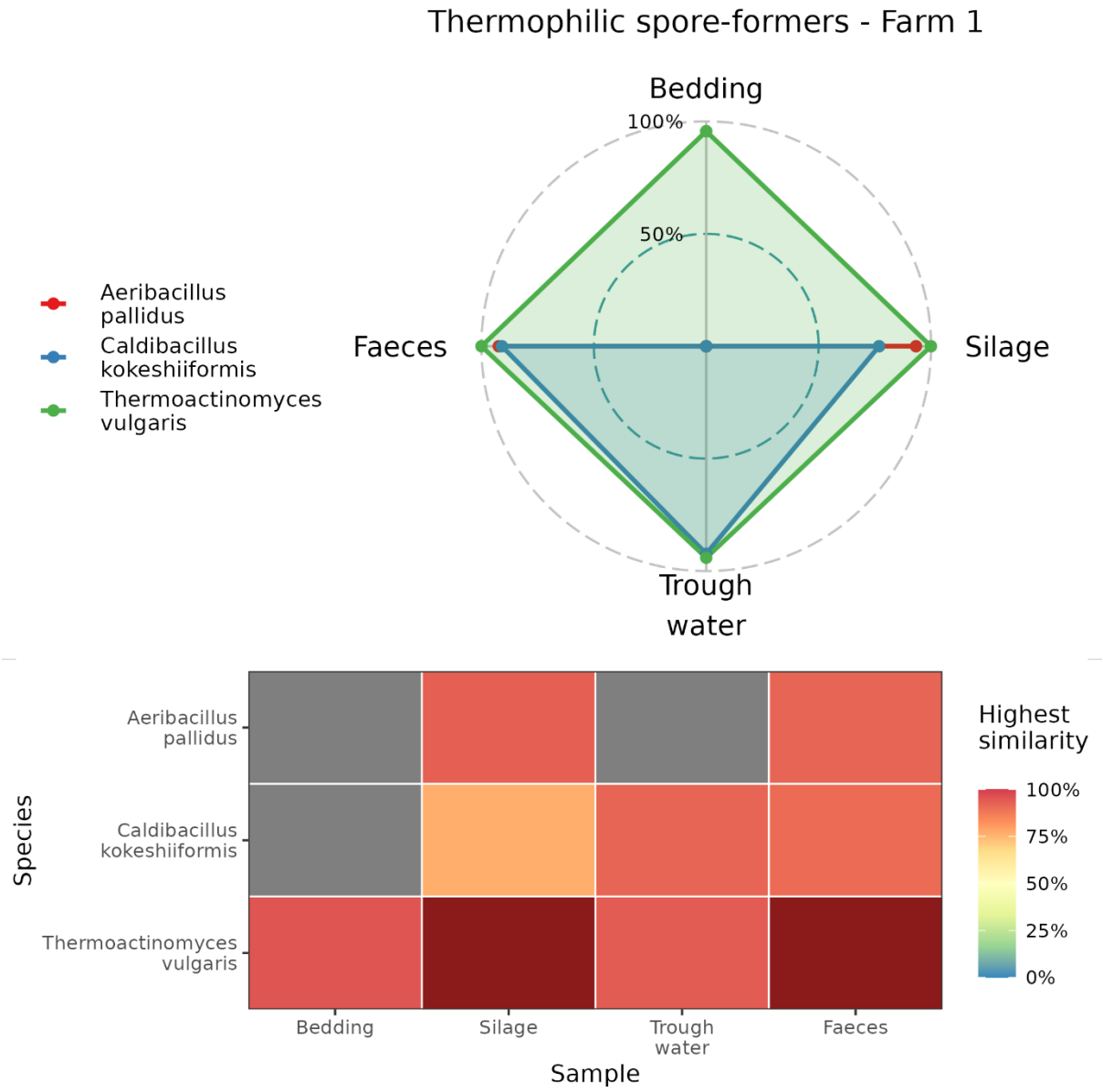


Figure 6.2. Radar chart comparing similarities in the ERIC-PCR profile of Farm 1 isolates of thermophilic spore-forming bacteria isolated from the environment, as compared to milking cups isolates.

The highest similarity values between the ERIC-PCR profiles of environmental and milking cups isolates are visible as coloured datapoints on the radar chart, and simultaneously presented under in a table in which this value is pictured as a colour gradient. Grey squares indicate the absence of any isolates of the specific bacteria in a given sample, while brown squares indicate the presence of at least one isolate with an ERIC-PCR profile identical to that of a milking cup isolate.

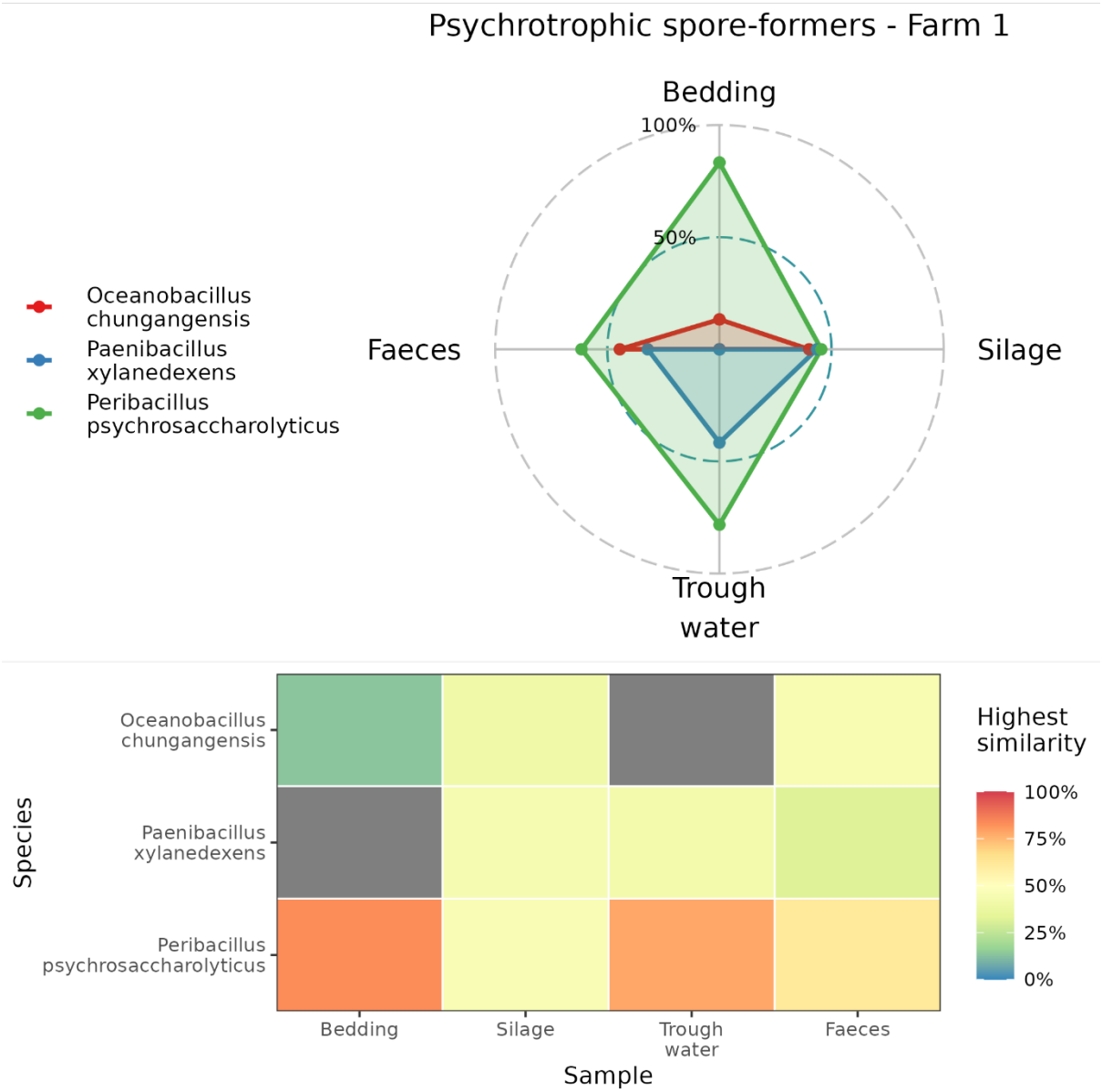


Figure 6.3. Radar chart comparing similarities in the ERIC-PCR profile of Farm 1 isolates of aerobic psychrotrophic spore-forming bacteria isolated from the environment, as compared to milking cups isolates.

The highest similarity values between the ERIC-PCR profiles of environmental and milking cups isolates are visible as coloured datapoints on the radar chart, and simultaneously presented under in a table in which this value is pictured as a colour gradient. Grey squares indicate the absence of any isolates of the specific bacteria in a given sample, while brown squares indicate the presence of at least one isolate with an ERIC-PCR profile identical to that of a milking cup isolate.

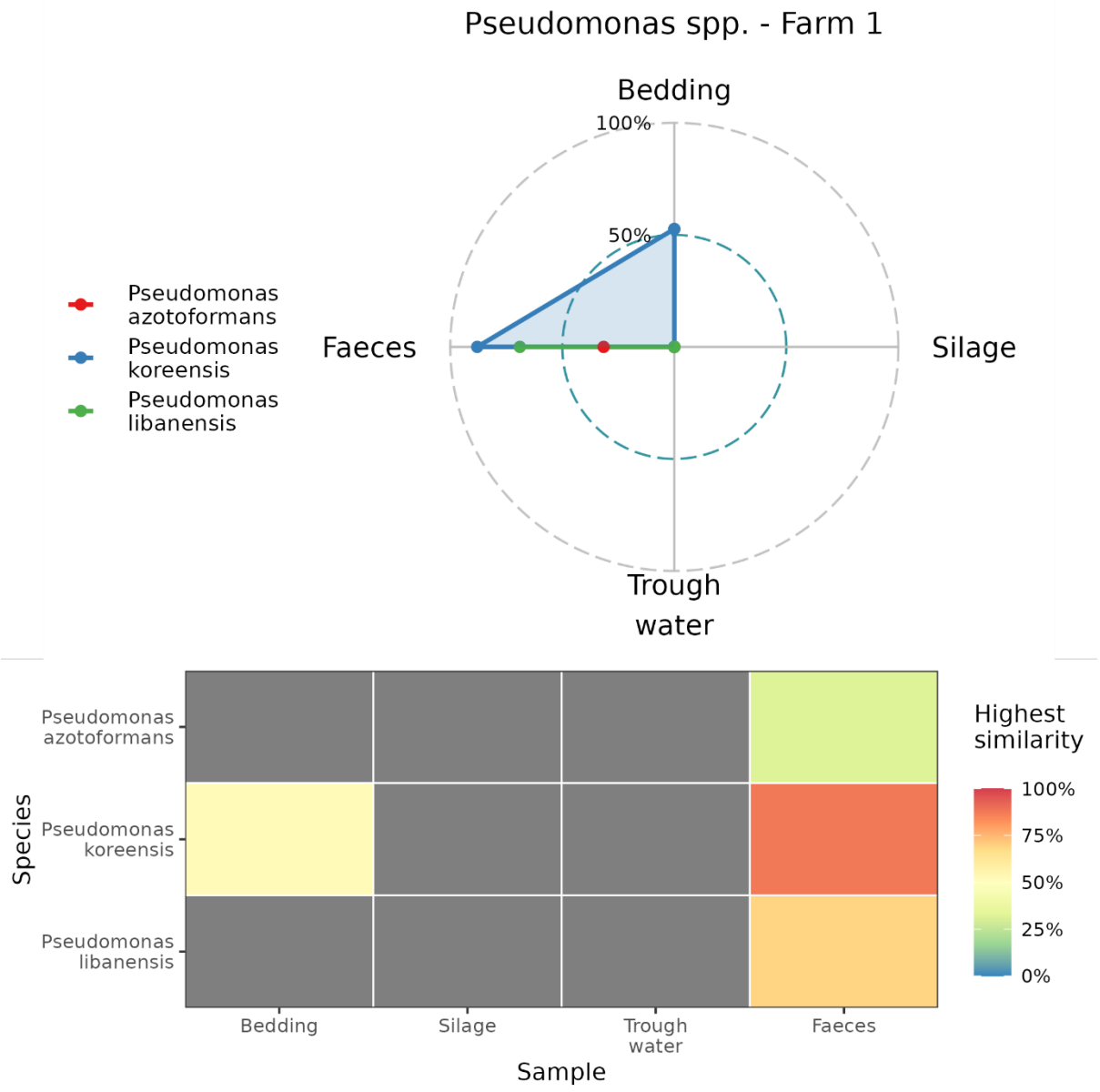


Figure 6.4. Radar chart comparing similarities in the ERIC-PCR profile of Farm 1 isolates of Pseudomonas spp. isolated from the environment, as compared to milking cups isolates.

The highest similarity values between the ERIC-PCR profiles of environmental and milking cups isolates are visible as coloured datapoints on the radar chart, and simultaneously presented under in a table in which this value is pictured as a colour gradient. Grey squares indicate the absence of any isolates of the specific bacteria in a given sample, while brown squares indicate the presence of at least one isolate with an ERIC-PCR profile identical to that of a milking cup isolate.

ARDRA of *Paraclostridium benzoelyticum* and *Clostridium perfringens* isolates revealed two distinct profiles of transmission for these bacteria (Figure 6.5). Several isolates of *C. perfringens* from bedding materials, faeces, silage and trough water had ARDRA profiles identical to that of the milking cups isolates (Appendix 6.16), but all isolates of *C. perfringens* shared at least 90% similarity between their ARDRA profiles. On the dendrogram, four clusters could be distinguished based on a 95% similarity threshold (Appendix 6.16). Each of these clusters contained an isolate from the milking cups, as well as an isolate from silage. The strains originating from bedding and trough water were present across two clusters, while faecal isolates were only found in one cluster. There was no environmental isolate of *Paracl. benzoelyticum* with an ARDRA profile identically matching that of the milking cups isolates (Figure 6.5). The fingerprint profiles of *Paracl. benzoelyticum* isolates were overall similar (> 88%, Appendix 6.17) and six different clusters could be distinguished at the 95% similarity level. Only two clusters contained isolates from the milking cups, one of them being specific to faeces and milking cups, and the other being specific to milking cups. Other clusters did not contain any milking cups isolates and they were instead specific to faeces, faeces and silage, or faeces, silage and bedding materials.

When comparing the contribution of each sample set (i.e., "new" or "old") to the contamination of the milking cups, a similar profile was observed for both sample sets across the different bacterial species (Figure 6.6). However, in the case of *A. clausii* and *B. subtilis*, isolates from the "new" samples (rather than those from the "old" samples) were more similar to the isolates from the milking cups.

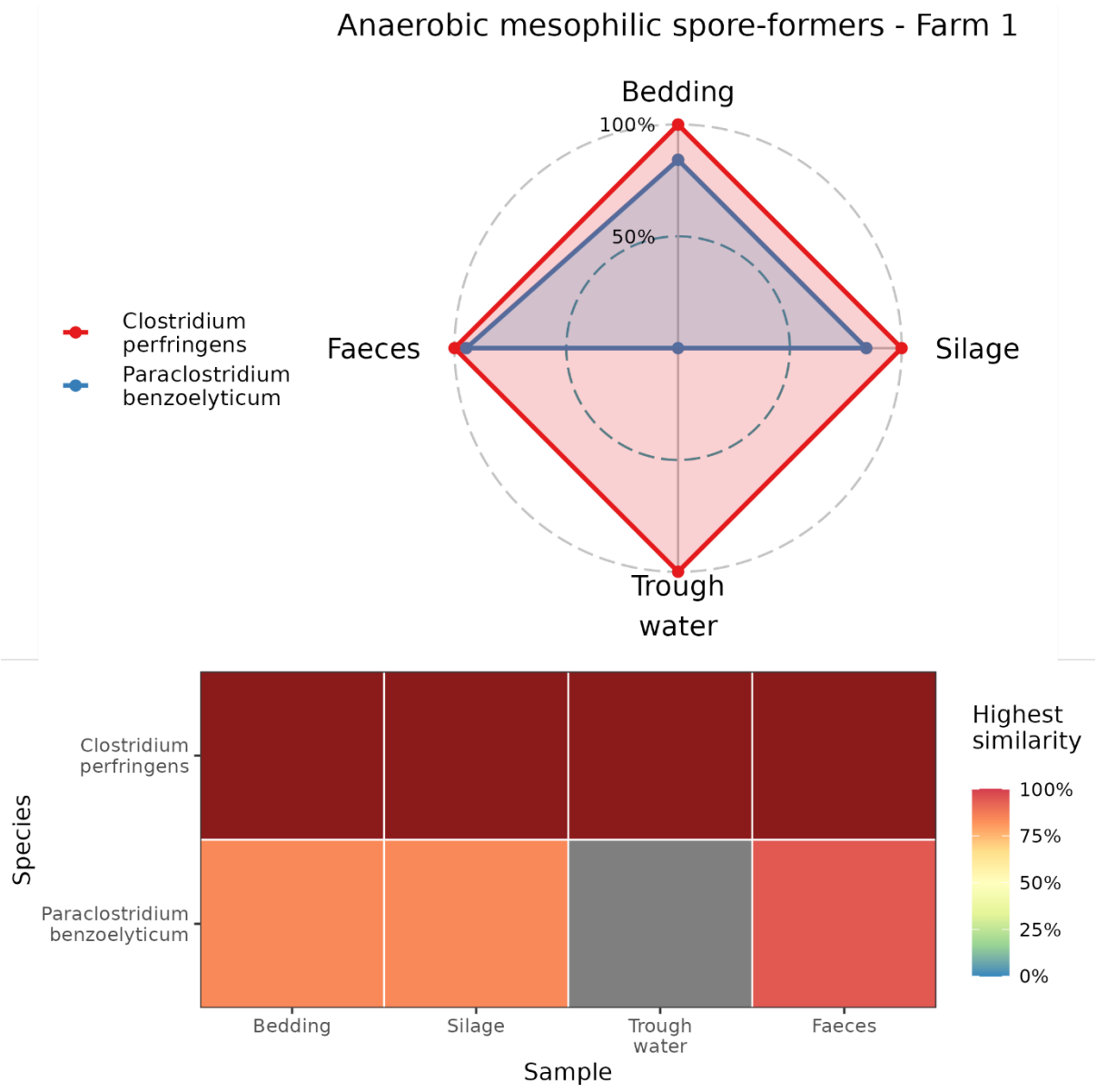


Figure 6.5. Radar chart comparing similarities in the ARDRA profile of Farm 1 isolates of anaerobic mesophilic spore-forming bacteria isolated from the environment, as compared to milking cups isolates.

The highest similarity values between the ARDRA profiles of environmental and milking cups isolates are visible as coloured datapoints on the radar chart, and simultaneously presented under in a table in which this value is pictured as a colour gradient. Grey squares indicate the absence of any isolates of the specific bacteria in a given sample, while brown squares indicate the presence of at least one isolate with an ARDRA profile identical to that of a milking cup isolate. The enzyme *HhaI* was used for the restriction analysis.

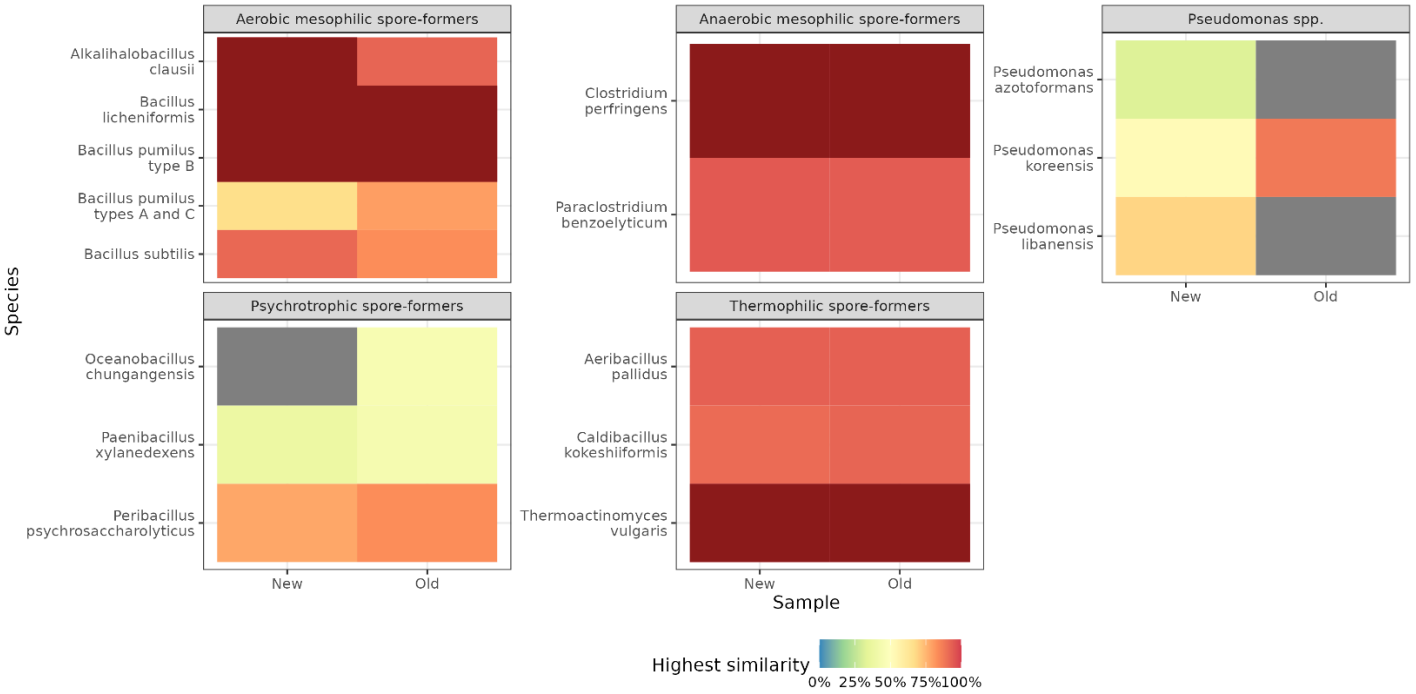


Figure 6.6. Similarity matrices between the DNA fingerprinting profile of Farm 1 bacterial isolates isolated from samples collected in pens with 3-month-old ("old") or 3-week-old ("new") bedding materials.

The highest similarity values between the DNA fingerprinting profiles of environmental and milking cups isolates are pictured as a colour gradient. Grey squares indicate the absence of any isolates of the specific bacteria in a given sample, while brown squares indicate the presence of at least one isolate with an ERIC-PCR profile identical to that of a milking cup isolate.

6.2.3 Source tracking of the bacteria found in the pasture-based sheep dairy farm environment

The phylogenetic analysis conducted on the isolates of F2 included five species of aerobic mesophilic spore-formers, five species of thermophilic spore-formers, three species of psychrotrophic spore-formers, two *Pseudomonas* spp. and three species of *Clostridium sensu lato*. The DNA fingerprint of bacteria which had been isolated from milking cups was compared to that of environmental isolates originating from soil, pasture, trough water and faeces, in order to assess the potential transfer of bacteria from the pasture-based farm environment to milking cups.

The results of the source tracking for F2 are presented by groups of isolation: mesophilic spore-formers (Figure 6.7), thermophilic spore-formers (Figure 6.8), psychrotrophic spore-formers (Figure 6.9), anaerobic spore-formers (Figure 6.10) and *Pseudomonas* spp. (Figure 6.11). For each bacterial

species, phylogenetic trees were built according to the distance matrices generated by the results of ERIC-PCR or ARDRA typing and using the UPGMA clustering algorithm (Appendices 6.18 – 6.34).

Overall, the ERIC-PCR profiles of the environmental mesophilic spore-forming bacteria recovered from F2 were noticeably different to that of the milking cups isolates, with only a few exceptions. Faeces, pasture and trough water were identified as potential sources of milking cups contamination by mesophilic spore-forming bacteria such as *B. licheniformis*, *Priestia megaterium* and *A. clausii* (Figure 6.7). The only environmental strains with an ERIC-PCR profile identical to that of the milking cups isolates were *B. licheniformis* from trough water and faeces (Appendix 6.18), as well as *Pr. megaterium s.l.* from pasture (Appendix 6.19). The *Pr. megaterium s.l.* isolates were distributed across nine different clusters based on a threshold of 80% similarity between their ERIC-PCR profile, and only two of these clusters included milking cups isolates. Most clusters of *Pr. megaterium s.l.* were specific to, and ubiquitous in the environment. One of the *A. clausii* strains isolated from trough water also showed high similarity to the milking cups isolates (> 95% similarity, Appendix 6.20). However, the ERIC-PCR profile of the remaining environmental bacteria was different to that of the milking cups isolates, including for *L. macroides* (Appendix 6.21) and particularly for *B. cereus s.l.* (< 73% similarity, Appendix 6.22). The ERIC-PCR profiles of *B. cereus s.l.* were highly variable across the isolates; there were six different clusters at the 65% similarity level, and 16 different clusters at 70% similarity. Milking cups isolates of *B. cereus s.l.* were found across four different clusters at the 65% similarity threshold. Unlike in the case of *B. pumilus s.l.* isolates, as mentioned above, species-level identification could not be confidently performed for *B. cereus s.l.* isolates based on the results of the ERIC-PCR phylogeny and partial 16S rRNA gene sequencing. However, the detection of several ERIC-PCR phylotypes across *B. cereus s.l.* with low similarity (< 70%) suggests that different species of *B. cereus s.l.* are present in the pasture environment.

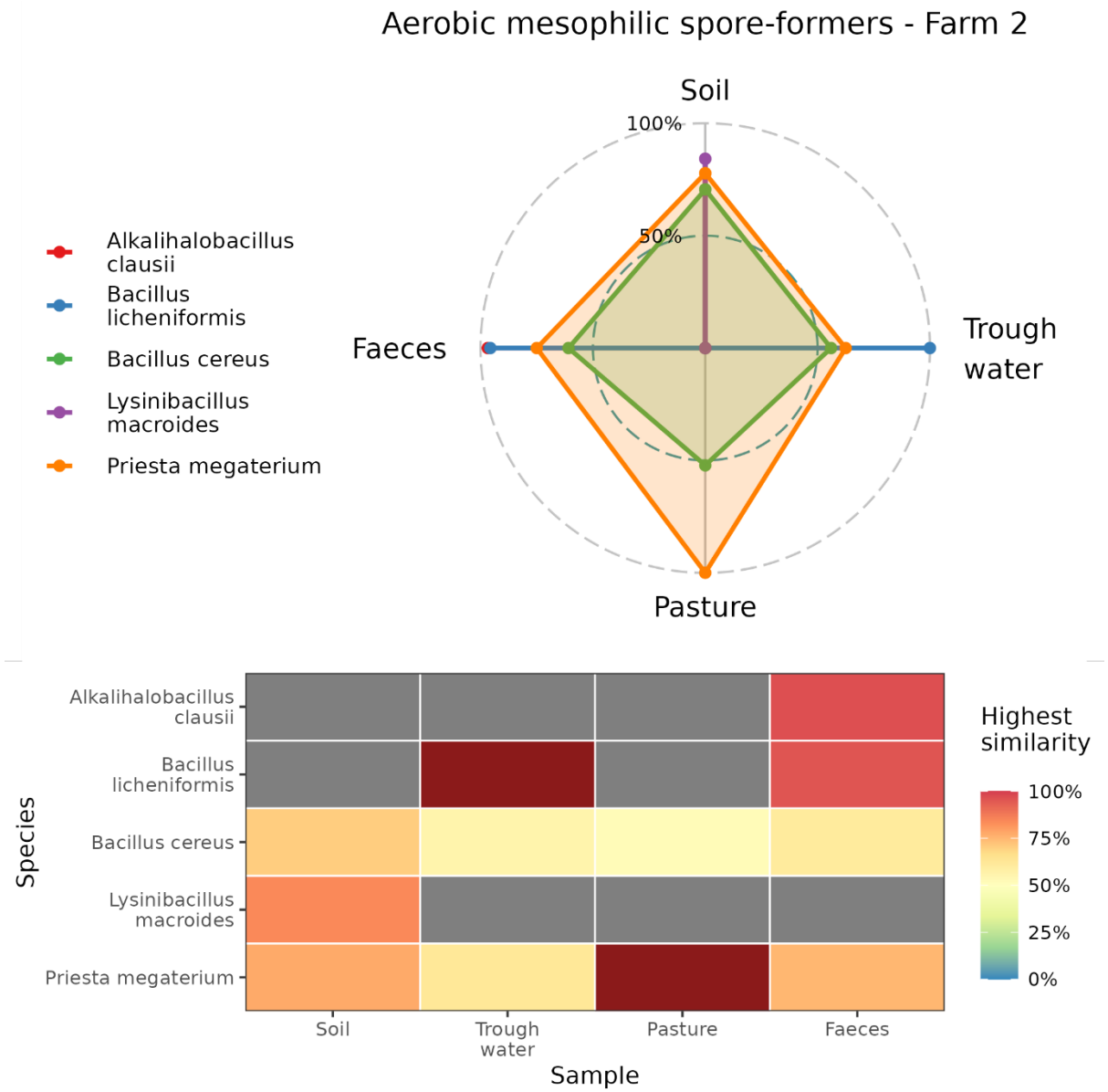


Figure 6.7. Radar chart comparing similarities in the ERIC-PCR profile of Farm 2 isolates of aerobic mesophilic spore-forming bacteria isolated from the environment, as compared to milking cups isolates.

The highest similarity values between the ERIC-PCR profiles of environmental and milking cups isolates are visible as coloured datapoints on the radar chart, and simultaneously presented under in a table in which this value is pictured as a colour gradient. Grey squares indicate the absence of any isolates of the specific bacteria in a given sample while brown squares indicate the presence of at least one isolate with an ERIC-PCR profile identical to that of a milking cup isolate.

With the exception of *B. smithii*, which environmental isolates showed particularly dissimilar ERIC-PCR profiles to that of the milking cups isolates (< 80% similarity, Appendix 6.23), potential sources of the thermophilic spore-formers *Aer. pallidus*, *Parag. caldoxylosilyticus* and *Thermoactinomyces* spp. in milking cups could be identified (Figure 6.8). For each of these bacteria, respectively, an isolate from the milking cups showed the same ERIC-PCR profile as other isolates from pasture, faeces, and trough water. The ERIC-PCR profiles of *Aer. pallidus* isolates were heterogeneous and distributed across four clusters according to a threshold of 70% similarity (Appendix 6.24). The main cluster contained two third of the isolates (n = 16) and included *Aer. pallidus* from milking cups, pasture, soil and trough water. Two other clusters were milking cups- or soil-specific, respectively, while several isolates from milking cups and soil clustered away from the bulk of the isolates. Fingerprinting profile similarities < 70% between strains of *Aeribacillus pallidus* may indicate that some isolates represent other species of *Aeribacillus*, or subspecies of *Aer. pallidus*, even though they were identified by partial 16S rRNA gene sequencing with confidence (> 800 bp fragments and RDP seqscore > 0.970). Potential source of *Parag. caldoxylosilyticus* in milking cups was identified as faeces (Figure 6.8). The isolates clustered in two groups (< 70% similarity, Appendix 6.25); the first cluster included an isolate from the milking cups and another from trough water (90% similarity) while the second cluster was made up of a milking cup and three faecal isolates, one of which had an ERIC-PCR profile matching that of the milking cups isolate. There were two principal ERIC-PCR profiles for *Thermoactinomyces* spp. which shared 70% similarity and corresponded to the isolates identified as *T. intermedius* (upper clade, Appendix 6.26) and *T. vulgaris* (lower clade). Within the cluster corresponding to *T. intermedius*, the different isolates were relatively dissimilar (< 90% similarity), with the exception of a pair of isolates from trough water and milking cups (> 95% similarity). The potential sources of *T. vulgaris* in milking cups were identified as trough water, but also faeces and pasture. Across the *T. vulgaris* isolates, 10 different clusters could be differentiated at the 85% similarity level (Appendix 6.26), with milking cups isolates being found in seven of these clusters, three of which were specific to milking cups. The predominant cluster of *T. vulgaris* was found ubiquitously, being isolated from soil, faeces, trough water, pasture and milking cups, and the phylotype of the isolates members of this clusters also shared high similarity one to another (up to 96 – 100% similarity).

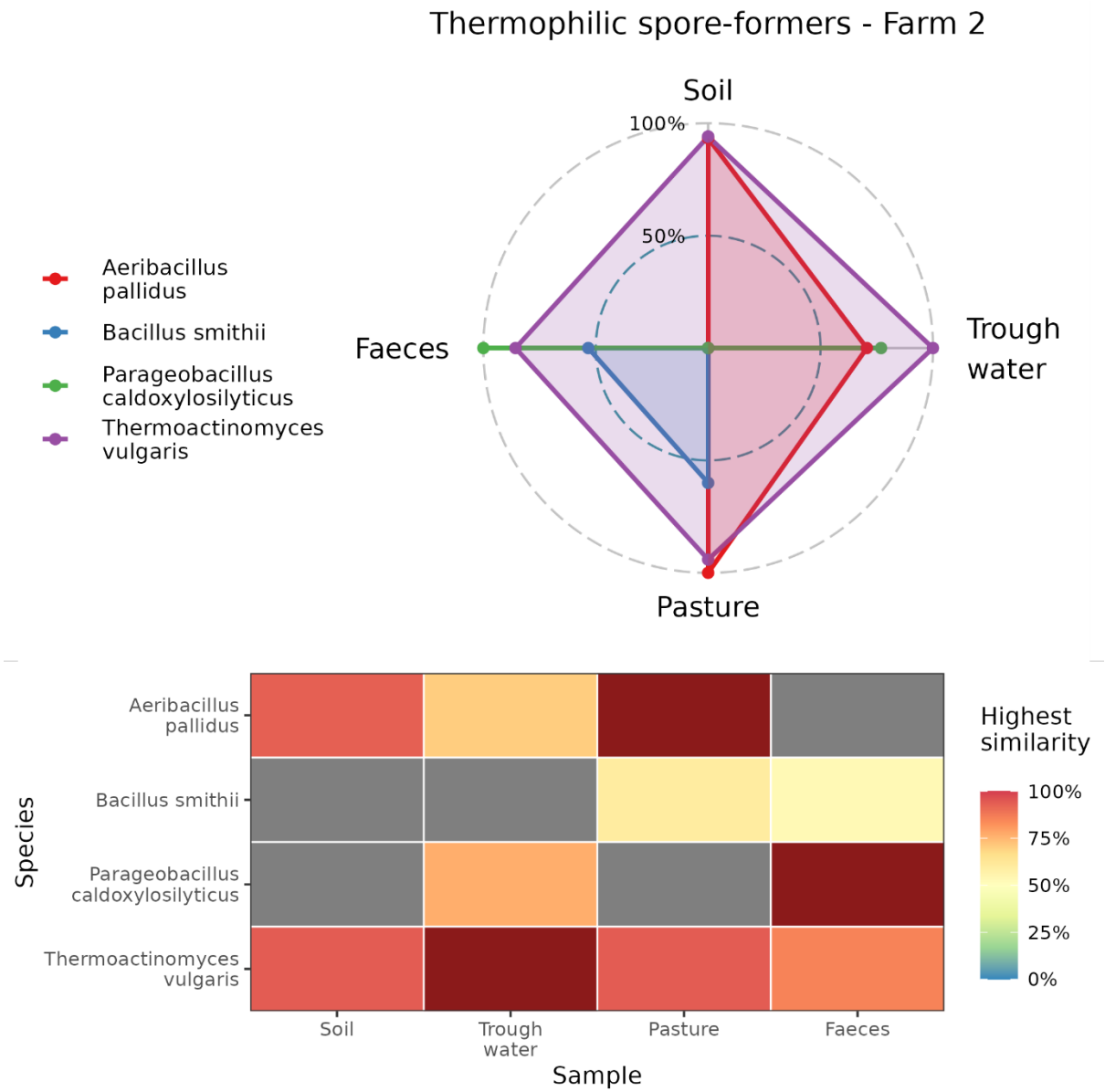


Figure 6.8. Radar chart comparing similarities in the ERIC-PCR profile of Farm 2 isolates of aerobic thermophilic spore-forming bacteria isolated from the environment, as compared to milking cups isolates.

The highest similarity values between the ERIC-PCR profiles of environmental and milking cups isolates are visible as coloured datapoints on the radar chart, and simultaneously presented under in a table in which this value is pictured as a colour gradient. Grey squares indicate the absence of any isolates of the specific bacteria in a given sample while brown squares indicate the presence of at least one isolate with an ERIC-PCR profile identical to that of a milking cup isolate.

The resolution of the ARDRA typing of *Clostridium s.l.* was found to vary greatly across the three species considered (Figure 6.9). Using the *HhaI* restriction enzyme, *C. perfringens* isolates could be typed with sufficient resolution (80-100% similarity, Appendix 6.27). However, restriction analysis using *HhaI* showed respectively very little to no divergence in the ARDRA profile of the different isolates of *C. butyricum* (96-100% similarity, Appendix 6.28) and *Paracl. benzoelyticum* isolates (100% similarity across all isolates). Subsequently, the restriction enzyme *AluI* was used for ARDRA which slightly improved the profiling of *C. butyricum* (93-100% similarity, Appendix 6.29). However, the ARDRA profiles of *Paracl. benzoelyticum* were identical across all isolates. *Clostridium perfringens* isolates were clustered across three different groups at the 90% similarity threshold (Appendix 6.27). Phylotypes of the milking cups isolates were only found in one cluster, and they had matching profiles to isolates from soil and pasture, as well as faeces and trough water. According to the two types of ARDRAs performed here, one isolate of *C. butyricum* from soil had a profile similar to the milking cups isolates. In addition, there were matching ARDRA profiles between trough water and soil isolates.

Similar to what was found on F1, environmental isolates of psychrotrophic spore-formers and *Pseudomonas* spp. were particularly different to those isolated from the milking cups (Figure 6.10 and 6.11). The only species of psychrotrophic spore-formers with a relatively high similarity between the environmental and milking cups isolates was *Paeni. anaericanus* (at most < 95% similarity, Figure 6.10). On the dendrogram and at the 80% similarity level, three different clusters were observed across the *P. anaericanus* isolates (Appendix 6.30). The first clusters included *P. anaericanus* isolates from milking cups and soil, the second comprised isolates from faeces, pasture and milking cups, and the third was specific to milking cups. Farm environmental isolates of both *Paenib. tundrae* (Appendix 6.31) and *Peri. psychrosaccharolyticus* (Appendix 6.32) shared less than 80% and 70% similarity, respectively, with the strains of these bacteria isolated from the milking cups. Environmental *Ps. poae s.l.* (Appendix 6.33) and *Ps. koreensis* (Appendix 6.34) showed particularly different ERIC-PCR profiles compared to that of the milking cups isolates (< 83% similarity, Figure 6.11).

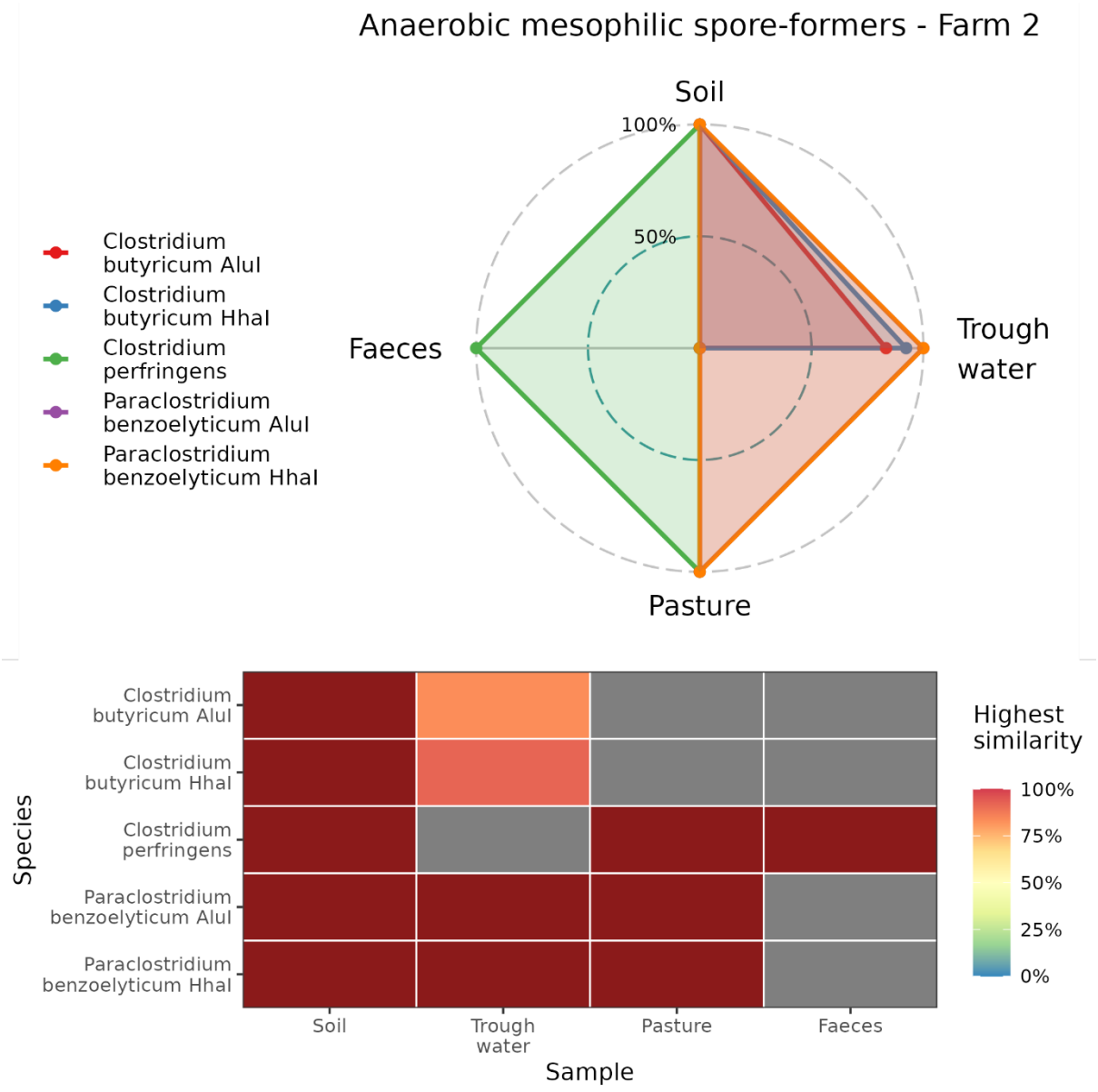


Figure 6.9. Radar chart comparing similarities in the ARDRA profile of Farm 2 isolates of anaerobic mesophilic spore-forming bacteria isolated from the environment, as compared to milking cups isolates.

The highest similarity values between the ARDRA profiles of environmental and milking cups isolates are visible as coloured datapoints on the radar chart, and simultaneously presented under in a table in which this value is pictured as a colour gradient. Grey squares indicate the absence of any isolates of the specific bacteria in a given sample while brown squares indicate the presence of at least one isolate with an ARDRA profile identical to that of a milking cup isolate. *Clostridium butyricum* and *Paraclostridium benzoelyticum* were typed with ARDRA using both *HhaI* and *AluI* restrictions enzymes, while *C. perfringens* was only typed with *HhaI*.

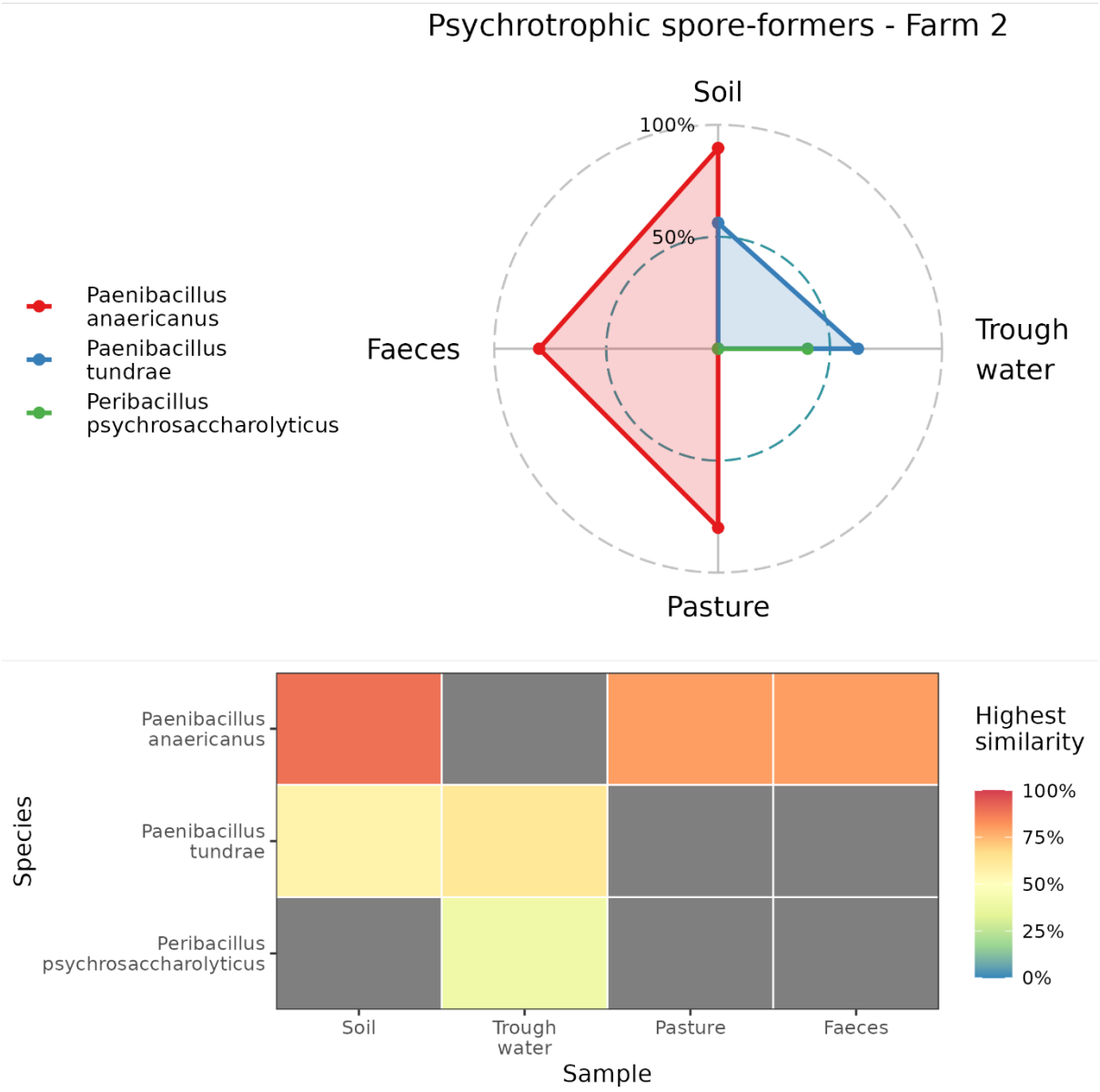


Figure 6.10. Radar chart comparing similarities in the ERIC-PCR profile of Farm 2 isolates of aerobic psychrotrophic spore-forming bacteria isolated from the environment, as compared to milking cups isolates.

The highest similarity values between the ERIC-PCR profiles of environmental and milking cups isolates are visible as coloured datapoints on the radar chart, and simultaneously presented under in a table in which this value is pictured as a colour gradient. Grey squares indicate the absence of any isolates of the specific bacteria in a given sample while brown squares indicate the presence of at least one isolate with an ERIC-PCR profile identical to that of a milking cup isolate.

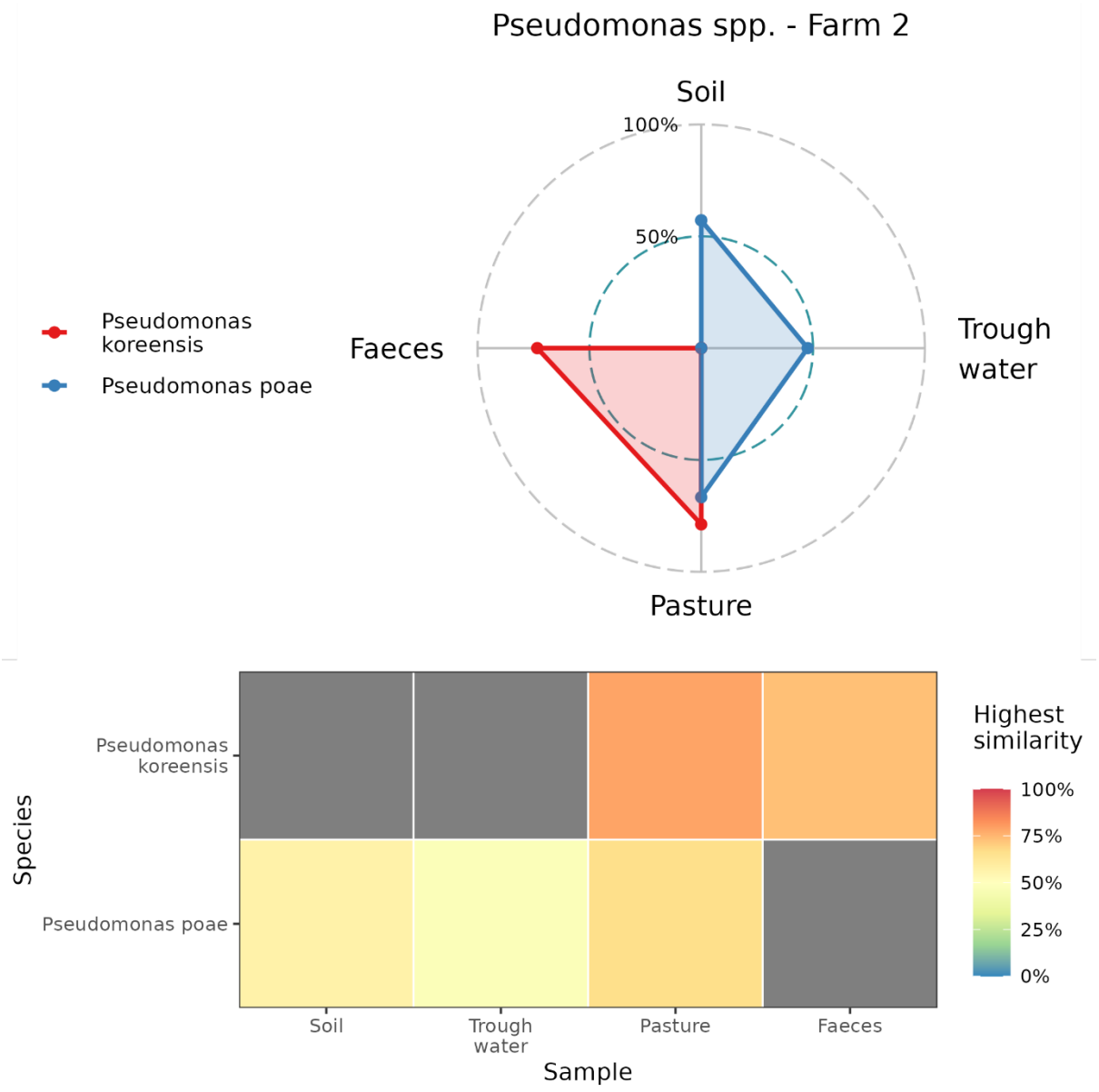


Figure 6.11. Radar chart comparing similarities in the ERIC-PCR profile of Farm 1 isolates of *Pseudomonas* spp. isolated from the environment, as compared to milking cups isolates.

The highest similarity values between the ERIC-PCR profiles of environmental and milking cups isolates are visible as coloured datapoints on the radar chart, and simultaneously presented under in a table in which this value is pictured as a colour gradient. Grey squares indicate the absence of any isolates of the specific bacteria in a given sample while brown squares indicate the presence of at least one isolate with an ERIC-PCR profile identical to that of a milking cup isolate.

6.2.4 Comparison of sources contributing to milking cups contamination during housing or grazing of dairy ewes

There were eight different bacteria for which a phylogenetic analysis was conducted on both F1 and F2: *A. clausii*, *B. licheniformis*, *Aer. pallidus*, *T. vulgaris*, *C. butyricum*, *Paracl. benzoelyticum*, *Peri. psychrosaccharolyticus* and *Ps. koreensis* (Figure 6.12).

On both farms, the main source of the mesophilic spore-formers, *A. clausii* and *B. licheniformis* in milking cups was identified to be faeces (Figure 6.12B and 6.12C). However, in the barn environment these bacteria were particularly ubiquitous, while they were only found in faeces and trough water in the pasture-based farm environment. Thermophilic spore-forming bacteria from the milking cups appeared to be linked to feed (silage or pasture) on both farms (Figure 6.12A, 6.12H). In the barn environment, faeces were also identified as a key source of thermophilic spore contamination in milking cups, while on the pasture-based farm, trough water was identified as a key potential source of *Thermoactinomyces* species. On both farms, potential sources of *C. perfringens* in milking cups were ubiquitous in the farm environment (Figure 6.12E). However, the sources of *Paracl. benzoelyticum* were found to vary across the two farms. Milking cups isolates of *Paracl. benzoelyticum* on F1 were associated with the faecal isolates, while on F2 the sources were soil, pasture and trough water (Figure 6.12F). Although the ERIC-PCR profiles of *Peri. psychrosaccharolyticus* and *Ps. koreensis* were overall particularly different at the farm level, isolates with the highest similarity to those of the milking cups were similar on both farm: faeces in the case of *Ps. koreensis* (Figure 6.12D), and trough water for *Peri. psychrosaccharolyticus* (Figure 6.12G).

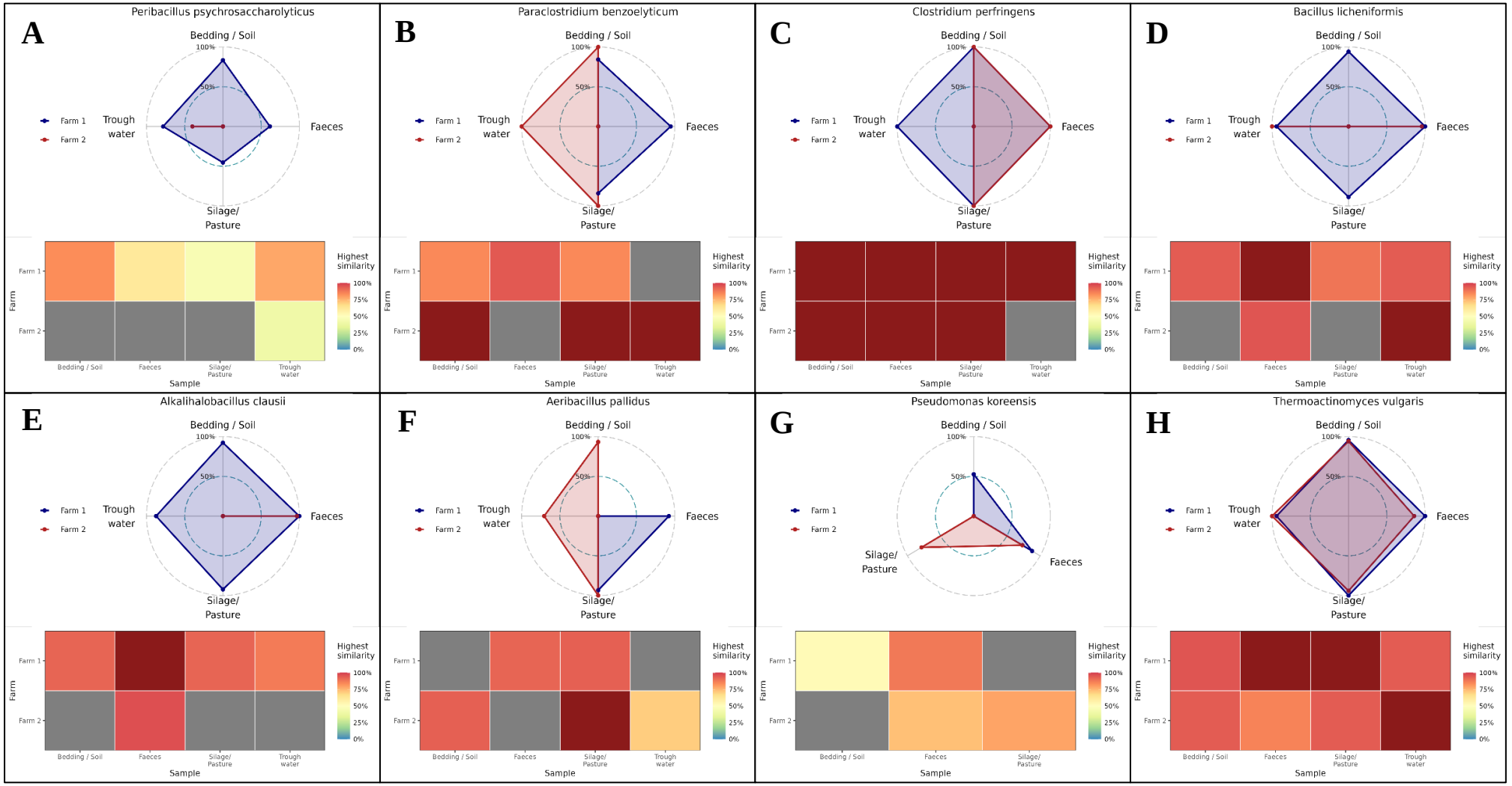


Figure 6.12. Radar chart comparing similarities in the ERIC-PCR profile of bacteria isolated from the environment of Farm 1 and Farm 2 as compared to milking cups isolates.

For each bacterial species, the highest similarity values between the DNA fingerprinting profiles of farm environmental and milking cups isolates are visible as coloured datapoints on the radar chart, and simultaneously presented under in a table in which this value is pictured as a colour gradient. Grey squares indicate the absence of any isolates of the specific bacteria in the environmental samples (faeces, silage/pasture, bedding/soil, or trough water) of a given farm while brown squares indicate the presence of at least one isolate with an ERIC-PCR profile identical to that of a milking cup isolate in that farm. F1 = Farm 1 (barn-based farm); F2 = Farm 2 (pasture-based farm).

6.2.5 Phylogeny and ecology of the predominant populations of *Thermoactinomyces spp.* present on sheep dairy farms

In Chapters 4 & 5, the thermophilic spore-formers *Thermoactinomyces* spp. were found to be highly abundant in a variety of farm samples associated with both indoor and outdoor sheep dairy farming. To obtain a more comprehensive understanding of the phylogeny of farm *Thermoactinomyces* spp. isolates and provide a higher resolution assessment of their ecology in the sheep dairy farm environment, the genomes of 12 strains of *T. vulgaris* and three strains of *T. intermedius*, which had been isolated from F1 (*T. vulgaris*, n = 6) and F2 (*T. vulgaris*, n = 6 and *T. intermedius*, n = 3), were sequenced (see sections 3.2.4 and 3.2.5). A series of phylogenetic analyses was subsequently performed using whole-genome sequences of these isolates to assess and compare the results obtained with the different methods, namely 16S rRNA gene-based phylogeny, genome-based phylogeny, ribosomal multi-locus sequence typing (rMLST), and another multi-protein sequence analysis (MPST) designed during this work and described in Chapter 3 (see 3.2.7.2).

The isolates that were selected for sequencing consisted of strains displaying the most common ERIC-PCR profile in each sample and for each farm (Table 6.1). The phylogenetic position of these isolates according to ERIC-PCR and as compared to other strains of *Thermoactinomyces* spp. isolated from F1 or F2 can be seen in Appendix 6.7 and 6.26, respectively.

Average genome size was 2.65 Mb for the *T. vulgaris* isolates and 2.79 Mb for the *T. intermedius* isolates (Table 6.1). The genomes were of overall good quality (average contigs n = 49), with the exception of F2TSHDT2 which genome was particularly fragmented (256 contigs). To compare the genetic material of the farm isolates with that of publicly available strains of *Thermoactinomyces* spp., some analyses also included the genome sequences of reference strains (Table 6.2).

The first approach was based on the 16S rRNA gene sequences of the isolates which was extracted from their respective genomes and aligned as part of the TYGS analysis pipeline, as described in section 3.2.6.1 (Meier-Kolthoff and Göker, 2019, Meier-Kolthoff et al., 2022). The phylogenetic tree produced by this analysis revealed respective clustering of *T. intermedius* and *T. vulgaris* isolates (Figure 6.13). The 16S rRNA gene sequence of farm *T. intermedius* isolates were identical, and also similar to that of the type strain (pairwise distance (PD) < 0.00160). Based on their 16S rRNA gene sequence, most *T. vulgaris* isolates were closely related (0.00059 < PD < 0.00060). Farm isolates of *T. vulgaris* clustered apart from the two external strains, both of which shared the same 16S rRNA gene sequence. The 16S rRNA

Table 6.1. Genomic features of *Thermoactinomyces* spp. farm isolates

Genome	**F1NBHDT1**	**F1NWHDT6**	**F1NPHDT35**	**F1NSHDT14**	**F1MC60HDT27**	**F1MC65HDT1**	**F2MC65HDT1**	**F2MC60HDT51**	**F2MC65HDT1**	**F2MC60HDT7**	**F2TFHDT17**	**F2TPHDT3**	**F2TSHDT2**	**F2TWHDT18**	**F2TWHDT2**
Origin of the isolate	Bedding	Trough water	Faeces	Silage	Milking cups	Milking cps	Milking cups	Milking cups	Milking cups	Milking cups	Faeces	Pasture	Soil	Trough water	Trough water
Species	*T. vulgaris*	*T. vulgaris*	*T. vulgaris*	*T. vulgaris*	*T. vulgaris*	*T. vulgaris*	*T. vulgaris*	*T. intermedius*	*T. intermedius*	*T. vulgaris*	*T. vulgaris*	*T. vulgaris*	*T. vulgaris*	*T. intermedius*	*T. vulgaris*
G+C content	48.00%	47.62%	47.64%	47.89%	48.01%	48.06%	47.93%	47.94%	47.90%	47.95%	47.91%	48.04%	47.91%	47.93%	48.03%
Main genome contig total	28	42	33	32	31	32	44	45	28	29	35	28	256	41	27
Main genome contig sequence total (mb)	2.684	2.696	2.723	2.648	2.573	2.551	2.782	2.785	2.739	2.637	2.591	2.593	2.754	2.785	2.597
Main genome contig N/L50 (kb)	2/566.014	2/620.102	537.647	2/605.693	2/566.098	2/563.785	4/263.596	3/534.479	2/622.561	2/538.11	3/538.152	2/606.72	2/582.419	2/570.351	2/606.369
Main genome contig N/L90 (kb)	6/94.111	8/86.378	6/132.576	5/101.574	5/132.806	6/132.692	14/40.305	11/67.622	7/98.623	7/71.559	8/71.695	5/93.77	10/46.233	8/67.457	5/118.275
Max contig length (kb)	1.045	749.664	1.126	1.025	946.547	752.544	550.261	659.862	842.485	814.094	626.086	1.005	805.43	867.332	1.124
Number of contigs > 50 kb	8	10	8	7	7	8	12	11	10	8	8	7	9	9	6
% main genome in contigs > 50 kb	96.69%	95.08%	98.00%	96.58%	98.11%	98.35%	88.21%	90.70%	98.31%	92.52%	94.35%	96.62%	89.08%	92.64%	98.50%
CDS	2786	2815	2824	2744	2620	2605	2880	2885	2830	2691	2649	2642	2788	2881	2639
rRNA	7	8	4	6	7	8	10	13	15	12	11	13	10	11	14
tmRNA	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
tRNA	63	55	64	64	63	63	79	80	73	72	73	74	75	78	72

Table 6.2. Reference strains used for comparison in the study

Reference strain	Genome GenBank accession number	Reference
***T. vulgaris* DSM43016^{T}**	GCA_003688725.1	(Goeker et al., 2018)
***T. intermedius* DSM43846^{T}**	GCA_016055025.1	(Cheng, 2020)
***T. vulgaris* CDF**	GCA_001187615.2	(Li et al., 2019)
***T. vulgaris* 2H**	CP039710.1	(Mankai et al., 2019)
***T. intermedius* s-9**	GCA_013867715.1	(Feng, 2020)
***B. subtilis* 168^{T}**	GCA_002055965.1	(Arbor, 2017)

gene sequence of the six isolates from F1, which consisted of the predominant clones of ERIC-PCR types in each sample type, was identical. In addition, the soil isolate from F2 (F2TSHDT2) also had an identical gene sequence to these bacteria, hinting at their environmental provenance. However, several isolates from F2 fell outside of the *T. vulgaris* and *T. intermedius* clusters, though they shared a common ancestor with both of these two clusters: for F2TFHDT17 (PD = 0.00018), F2TPHDT3 (PD = 0.00204), F2TWHDT2 (PD = 0.00222) and F2MC65HDT15 (PD = 0.00633). This may have been caused by errors during sequencing, or the presence of heterologous copies of the 16S rRNA gene in the genome of some isolates, thereby increasing sequence uncertainty and thus divergence between the isolates in the phylogenetic trees. Therefore, to check for heterologous copies, 16S rRNA gene sequences were extracted from all genomes using the ContEst16S tool [EZbiocloud.net, (Lee et al., 2017b)], which identifies fragments and copies of the 16S rRNA gene in genome sequences as a mean of detecting contamination. In most genomes, (n = 9), the 16S rRNA gene was monomorphic, and complete as one or multiple fragments (i.e., 1548 bp or more). The remaining three isolates had incomplete 16S rDNA fragments (1300 – 1430 bp). Allelic polymorphism was observed in two isolates, and with low divergence between the different copies (F2TWHDT2: 99.70% similarity between the two fragments; and F2MC65HDT15: 99.74% similarity). The 16S rRNA gene sequences of the F2 isolates F2TFHDT17, F2TPHDT3, F2TWHDT2 and F2MC65HDT15, which previously clustered outside of the *T. vulgaris* and the *T. intermedius* clusters, were either polymorphic (F2MC65MDT15 and F2TWHDT2) or incomplete (F2TFHDT17 and F2TPHDT3).

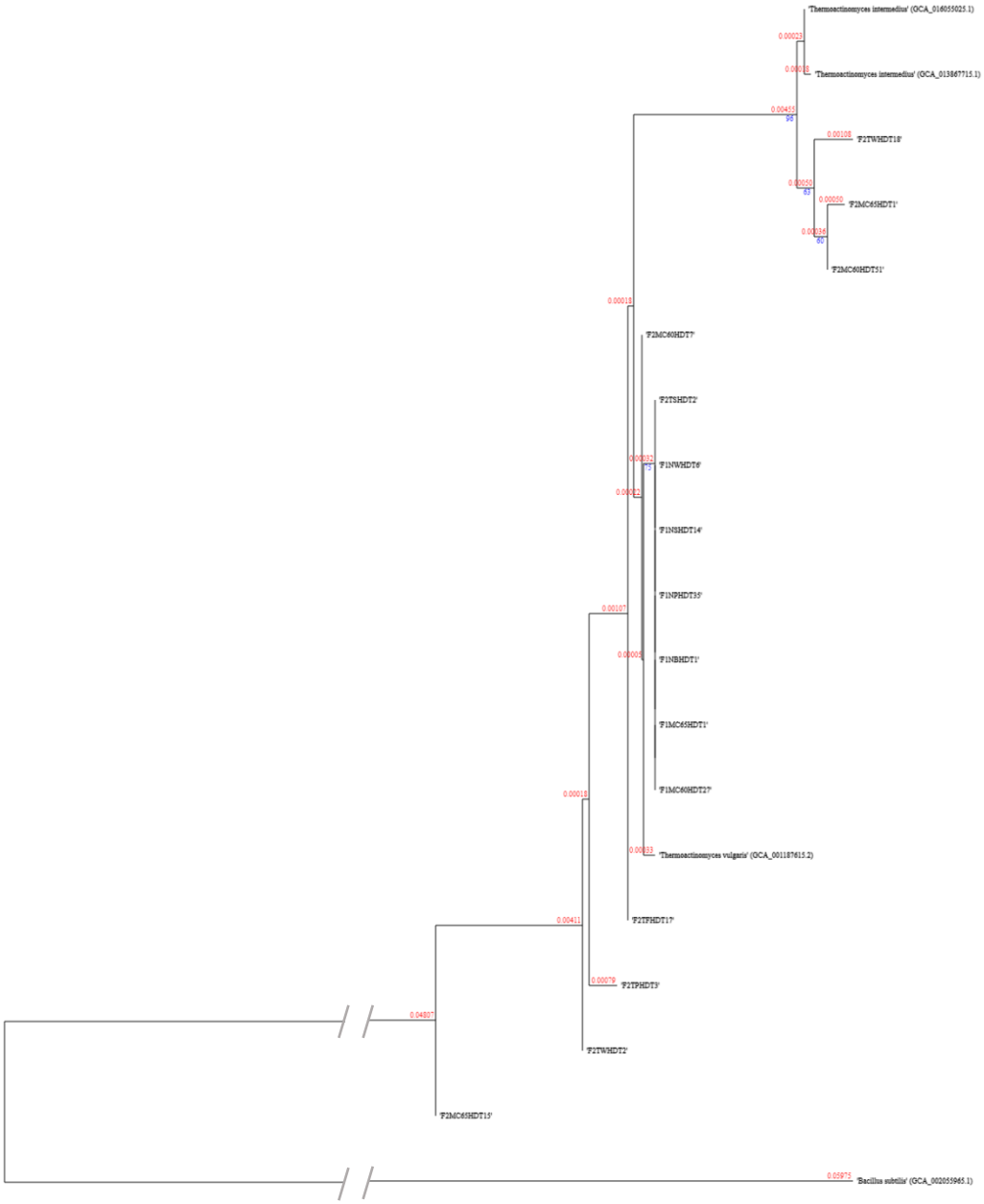


Figure 6.13. Tree inferred with FastME 2.1.6.1 (Lefort et al., 2015) from GBDP distances calculated from 16S rDNA gene sequences of *Thermoactinomyces* genomes

The branch lengths are scaled in terms of GBDP distance formula d5. The tree was rooted at the midpoint (Farris, 1972)**.** Analysis of 16S rDNA phylogeny was conducted as part of the TYGS analysis pipeline (https://tygs.dsmz.de/). Phylogenetic distances between the different nodes are visible in red. Isolate denominations can be accessed in Table 6.1. *Bacillus subtilis* 168^{T} is included for reference.

This suggested that scattering of these isolates from the remaining isolates on the phylogenetic tree may have been the result of sequencing artefacts. None of the genomes were found to be contaminated according to the ContEst16S tool, though this was often because there was a single unique copy of the 16S rDNA detected in each isolate. Other publicly available genome sequences of *Thermoactinomyces* spp. had different numbers of copies of the 16S rRNA gene: one in the case of *T. intermedius* s-9 and *T. intermedius* DSM43846^T, seven for *T. vulgaris* 2H and *T. vulgaris* CDF (including four and one different alleles, respectively) while surprisingly, no 16S rRNA gene was detected by the ContEst16S software in the genome of *T. vulgaris* DSM43016^T. These results suggest that monomorphism of the 16S rRNA gene may be common in *Thermoactinomyces* spp., and/or that the coverage of this gene is low during genome sequencing of these bacteria. In comparison, *B. subtilis* 168^T possessed 10 fragments of the 16S rRNA gene, including eight different copies.

In parallel to 16S rDNA-based phylogeny, the TYGS tool was used to perform a Genome BLAST Distance Phylogeny analysis [GBDP, see section 3.2.6.2 (Henz et al., 2005, Meier-Kolthoff and Göker, 2019)]. For each pair of genomes, pairwise digital DNA-DNA hybridisation (dDDH) values were calculated based on three formulas (d_0, d_4 and d_6), each considering high-scoring segment pairs (HSPs) and similarities between HSPs, as well as the absolute or relative lengths of the genomes (Appendix 6.35). Formula d_4 (the sum of all identities found in HSPs divided by total HSPs length) is widely used to study the phylogeny of bacterial isolates for which draft genome sequences are available, as it is less negatively impacted by incomplete genome sequences as compared to the other two formula (Meier-Kolthoff et al., 2013). The isolates that belonged to a same species [i.e., *T. vulgaris* or *T. intermedius*, dDDH > 70% (Meier-Kolthoff et al., 2013)] were also part of the same subspecies [dDDH > 80% as defined by Meier-Kolthoff et al. (2014) and possibly the same strains, as suggested by high dDDH values (*T. vulgaris* d_4 > 93.7%; *T, intermedius* d_4 > 95%). On the genome-based phylogenetic tree obtained with the GBDP analysis and using the d5 distance formula (Figure 6.14), *T. vulgaris* and *T. intermedius* isolates each formed a monophyletic cluster (PD = 0.04615). The trough water and milking cups isolates of *T. intermedius* were particularly similar one to another (PD < 0.00068) and they clustered apart from the type strain (PD > 0.00616). *Thermoactinomyces vulgaris* isolates formed a monophyletic clade (PD < 0.00776), with *T. vulgaris* CDF as an outlier. The F2 isolates F2MC60HDT7 and F2TSHDT2 were scattered from other strains of *T. vulgaris* (PD > 0.00228 & PD > 0.00187, respectively) while the remaining isolates formed four clusters with similar genome profiles. The first cluster included *T. vulgaris* DSM43016^T and the trough water isolate from F1. The second cluster comprised a set of very closely related strains isolated from trough water and pasture on F2, as well as from silage and faeces on F1. The genomes of these four isolates

were virtually identical, as shown by the remarkably high dDDH values recorded between pairs of these bacteria (d_0 > 98.2%, d_4 > 99.9% 100, and d_6 > 99.2, Appendix 6.35). The third cluster of *T. vulgaris* included two isolates from the milking cups of F1 (PD = 0.00402), while the fourth cluster comprised two related strains (PD = 0.00260) isolated from the bedding materials of F1 and the

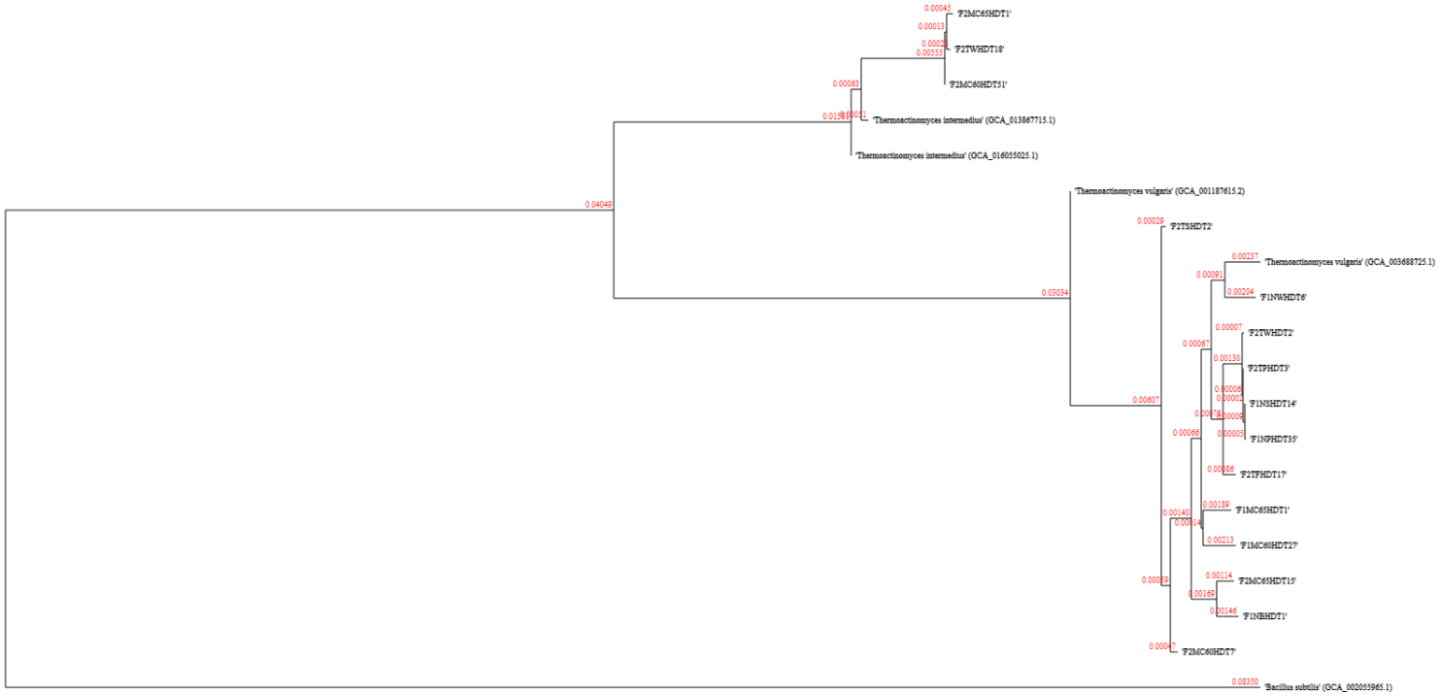


Figure 6.14. Tree inferred with FastME 2.1.6.1 (Lefort et al., 2015) from GBDP distances calculated from *Thermoactinomyces* spp. genome sequences

The branch lengths are scaled in terms of GBDP distance formula d5 ($d_5 = \frac{5 \times d}{L}$), d is the number of shared k-mers between the two genomes and L is the length of the shorter genome. The numbers above branches are GBDP pseudo-bootstrap support values > 60 % from 100 replications, with an average branch support of 9.9 %. The tree was rooted at the midpoint (Farris, 1972). The Genome BLAST Distance Phylogeny approach (GBDP) was conducted as part of the TYGS analysis pipeline (https://tygs.dsmz.de/). Phylogenetic distances between the different nodes are visible in red. Isolate denominations can be accessed in Table 6.1. *Bacillus subtilis* 168^T is included for reference.

Ribosomal multi-locus sequence typing (rMLST, section 3.2.7) was used on the *T. vulgaris* genomes to provide a comparison with the results of the genome-scale phylogeny. There were 53 different ribosomal gene loci that were present but not identical across the genomes of the isolates, all of which were included in the analysis. The rMLST analysis revealed the presence of 12 unique strains of *Thermoactinomyces* spp. across the 17 genomes (Table 6.3), and with only two strains containing two or more farm isolates. The isolates identified as *T. intermedius* or *T. vulgaris* formed two distinct monophyletic clades (PD = 0.018, Figure 6.15). The *T. intermedius* dairy farm isolates shared an

identical rMLST profile, which was also highly similar to that of the *T. intermedius* type strain (PD < 0.00020, Figure 6.16). Across the *T. vulgaris* isolates, PD < 0.00055 were observed and four different clusters could be distinguished (Figure 6.17). The first and second clusters included three farm isolates and the type strain of *T. vulgaris*, all of which were relatively distant to one another. The third cluster comprised two isolates with an identical rMLST profile (F1NBHDT1 and F2MC65HDT15) also closely related to F1MC65HDT1 (PD > 0.00013). The fourth cluster contained seven isolates, including *T. vulgaris* 2H, four of which shared an identical rMLST profile: F1NPHDT35, F1NSHDT14, F2TPHDT3 and F2TWHDT2. This phylotype was also similar to that of the faecal isolate from F1 (PD > 0.00016) and to the trough water isolate from the same farm (PD > 0.00026).

Table 6.3. Strain-level clustering of *Thermoactinomyces* spp. isolates based on the rMLST analysis

Strain 1	Strain 2	Strain 3	Strain 4	Strain 5	Strain 6
F1NPHDT35	F2MC60HDT51	F1MC65HDT1	F2MC60HDT7	Thermoactinomyces intermedius^T	F2TFHDT17
F1NSHDT14	F2MC65HDT1				
F2TPHDT3	F2TWHDT18				
F2TWHDT2					
Strain 7	**Strain 8**	**Strain 9**	**Strain 10**	**Strain 11**	**Strain 12**
Thermoactinomyces vulgaris^T	F1MC60HDT27	F1NWHDT6	F1NBHDT1	F2MC65HDT15	F2TSHDT2

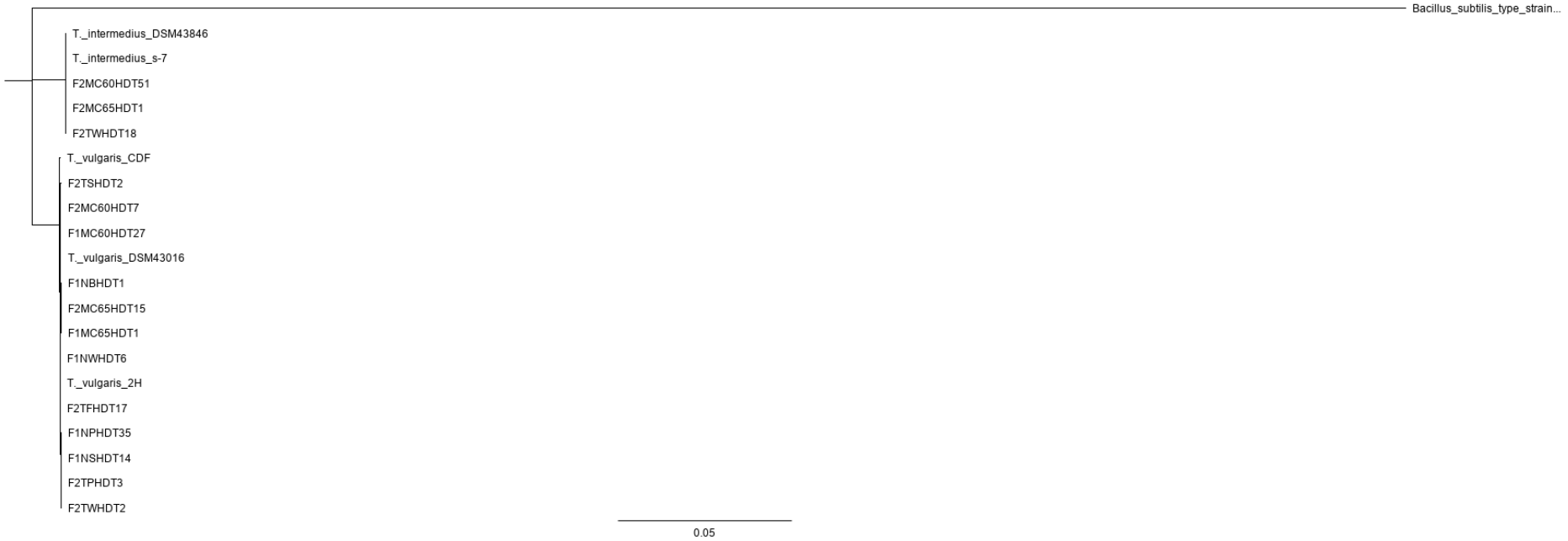


Figure 6.15. rMLST-based phylogeny of farm isolates and references strains of *Thermoactinomyces vulgaris* and *T. intermedius*

The phylogeny of the isolates was investigated using a rMLST analysis which was performed with a pubMLST scratch database (https://pubmlst.org/). The tree was created with the software Geneious version 2019.1 (Biomatters. https://www.geneious.com) using neighbour-joining clustering and the similarity matrices generated after alignment of 53 gene loci in each isolate. A scale for the phylogenetic distances is provided on the bottom of the tree. Isolate denominations can be accessed in Table 6.1. *Bacillus subtilis* 168^T is included for reference.

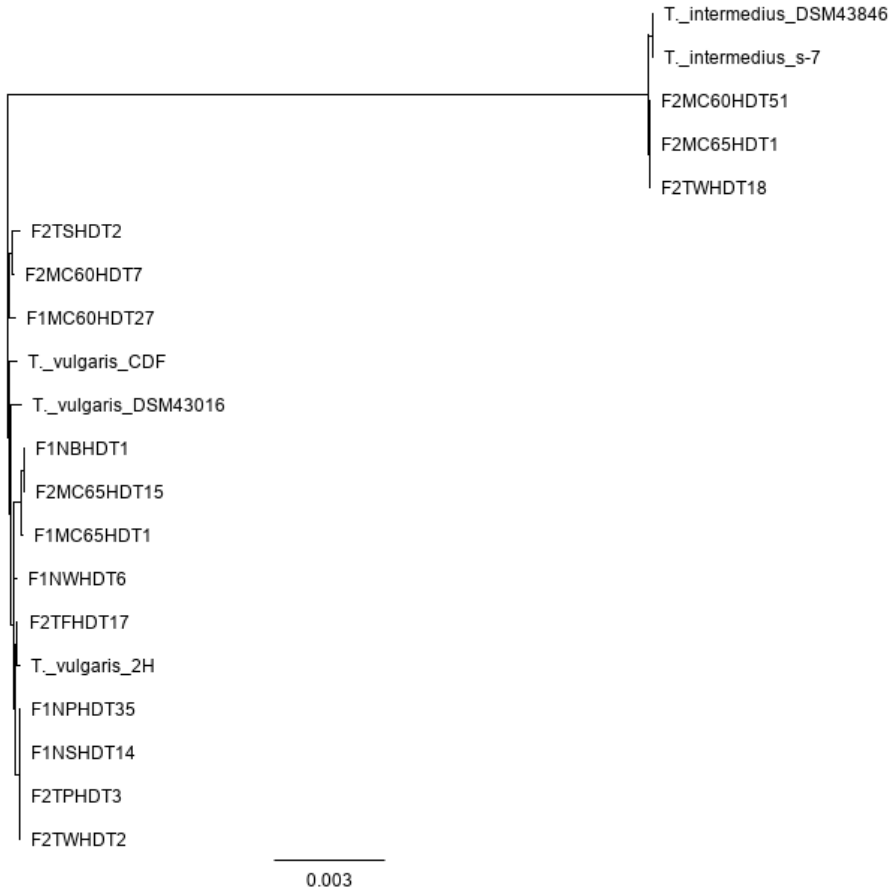


Figure 6.16. rMLST-based phylogeny of farm isolates and references strains of *Thermoactinomyces vulgaris* and *T. intermedius*

The phylogeny of the isolates was investigated using a rMLST analysis which was performed with a pubMLST scratch database (https://pubmlst.org/). The tree was created with the software Geneious version 2019.1 (Biomatters. https://www.geneious.com) using neighbour-joining clustering and the similarity matrices generated after alignment of 53 gene loci in each isolate. A scale for the phylogenetic distances is provided on the bottom of the tree. Isolate denominations can be accessed in Table 6.1.

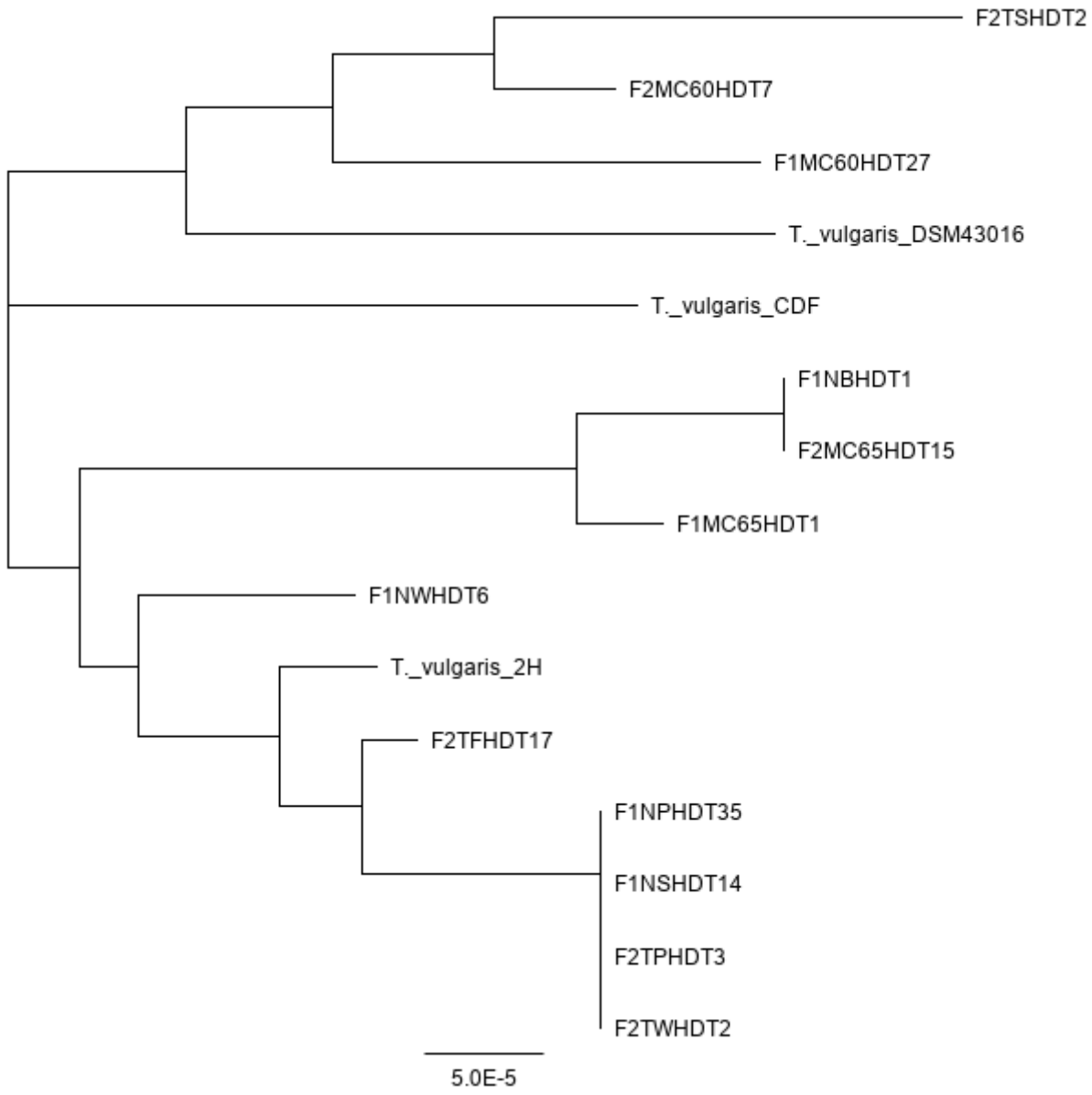


Figure 6.17. rMLST-based phylogeny of farm isolates and references strains of *Thermoactinomyces vulgaris*

The phylogeny of *Thermoactinomyces vulgaris* isolates was investigated using a rMLST analysis which was performed with a pubMLST scratch database (https://pubmlst.org/). The tree was created with the software Geneious version 2019.1 (Biomatters. https://www.geneious.com) using neighbour-joining clustering and the similarity matrices generated after alignment of 53 gene loci in each isolate. A scale for the phylogenetic distances is provided on the bottom of the tree. Isolate denominations can be accessed in Table 6.1.

During this work, I developed a MLST-like approach using protein instead of DNA sequences to probe the phylogeny of *Thermoactinomyces* spp. farm isolates from a more functional perspective (see section 3.2.7.2). Using the protein identification data from the OmicsBox analysis (section 3.2.5), I identified a set of genes coding for enzymes with protein sequences corresponding to putative exoproteases and exolipases that were conserved across *Thermoactinomyces* genomes. Proteins were selected based on their potential involvement in dairy spoilage (i.e., either documented or potential to be secreted as exoenzymes), if allelically monomorphic and present in a unique copy, and if they were found across all the considered genomes. Ultimately, nine proteins (seven proteases and two lipases, see Table 6.3) with lengths ranging 239-755 aa were selected for MPST analysis. Because some genes or protein sequences were not publicly available for each non-type *Thermoactinomyces* reference strains, only the type species were included in this analysis. The analysis revealed the presence of two well-defined clades containing isolates identified as *T. intermedius* and *T. vulgaris*, respectively (PD > 0.04, Figure 6.18). The three farm isolates of *T. intermedius* and the two reference strains each respectively shared the same MPST profile, which was also similar across these groups of bacteria (PD < 0.0085). The MPST profiles of the *T. vulgaris* isolates were particularly similar (PD < 0.0042) but five different clusters could be differentiated (Figure 6.19). The first two clusters each contained one isolate, *T. vulgaris*^{T} and F1NWHDT6, respectively. The third cluster included six isolates: F1MC65HDT1, F2TFHDT17, F1MC60HDT27, F2TWHDT2, F1NPHDT35 and F1NSHDT14, with the latter two sharing an identical profile, also highly similar to that of F2TWHDT2 (PD < 0.0003), as well as F1MC60HDT27 and F2TFHDT17 (each, PD < 0.0006). The fourth cluster included three isolates from soil, pasture and milking cups on F2 which had an identical MPST profile. The last cluster was made up of the bedding isolate from F1 and a milking cup isolate from F2 which shared high similarity (PD < 0.0005) in their MSPT profile.

Table 6.4. Gene loci and coding protein features of the proteins included in the MPST phylogenetic analysis

Gene	InterProScan protein description	Protein length (aa)	EggNOG Description
		Proteases	
vanY	M15 family metallopeptidase	248	D-alanyl-D-alanine carboxypeptidase
N/A	serine protease	309	Trypsin
ampS5	aminopeptidase	371	Thermophilic metalloprotease (M29)
ypwA	carboxypeptidase M32	500	Carboxypeptidase Taq (M32) metallopeptidase
pepF	oligoendopeptidase F	604	Oligopeptidase F
N/A	S8 family serine peptidase	643-665	Subtilase family
N/A	immune inhibitor A	755	Immune inhibitor A peptidase M6
		Lipases	
lipA1	triacylglycerol esterase/lipase EstA (alpha/beta hydrolase family)	535	Putative esterase
yjcH3	esterase family protein	239	acetyltransferases and hydrolases with the alpha beta hydrolase fold

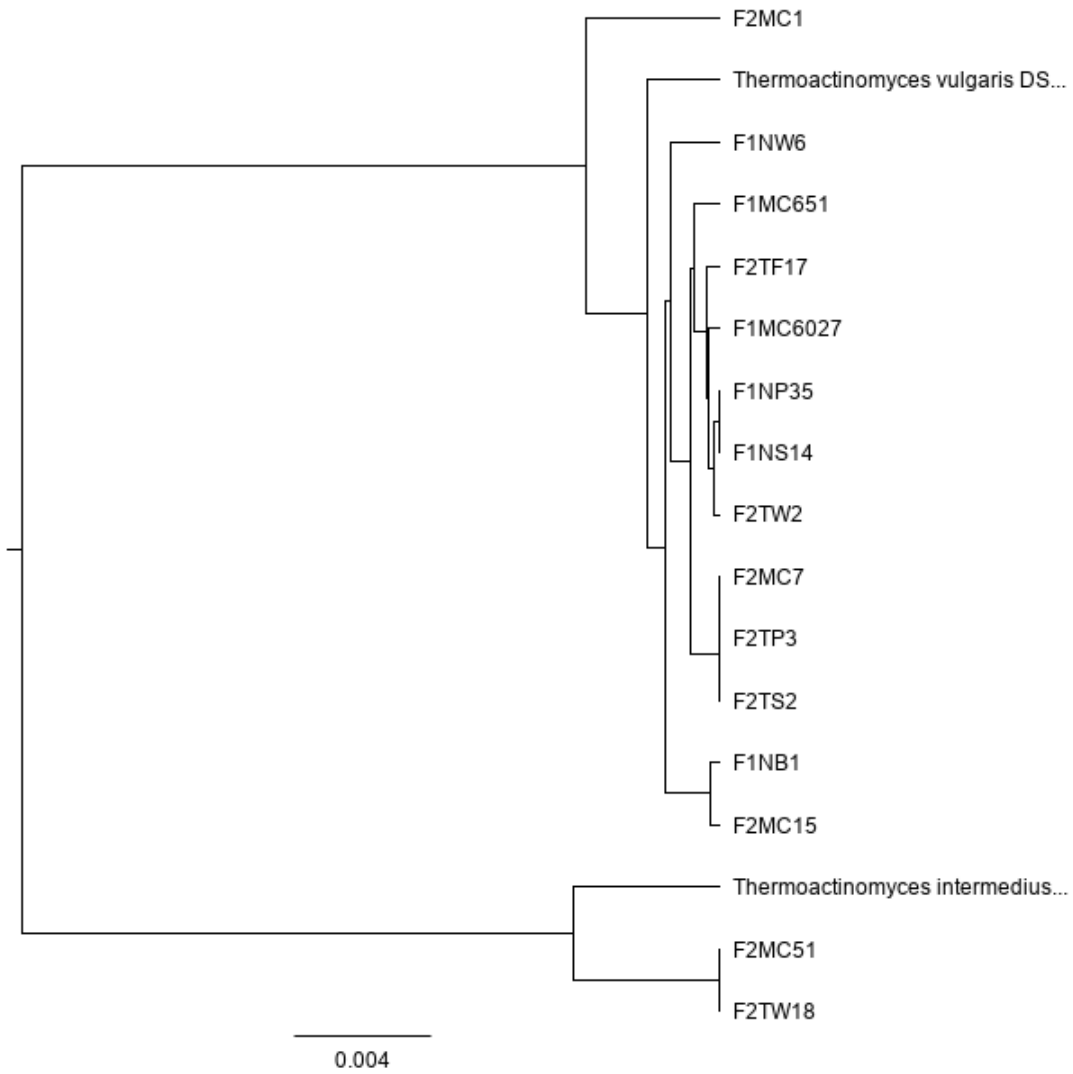


Figure 6.18. MPST-based phylogeny of farm isolates and references strains of *Thermoactinomyces*

The phylogeny of the isolates was investigated using a multi-protein sequence analysis approach (MPST) which was performed through the concatenation and alignment of 9 proteins present in the *Thermoactinomyces* genomes coding for putative exoprotease and exolipases. The tree was created with the software Geneious version 2019.1 (Biomatters. https://www.geneious.com) using neighbour-joining clustering and the similarity matrices generated after alignment of the 9 proteins sequences in each isolate. A scale for the phylogenetic distances is provided on the bottom of the tree. Isolate denominations can be accessed in Table 6.1, the type strains of *T. vulgaris* and *T. intermedius* were also included in the analysis.

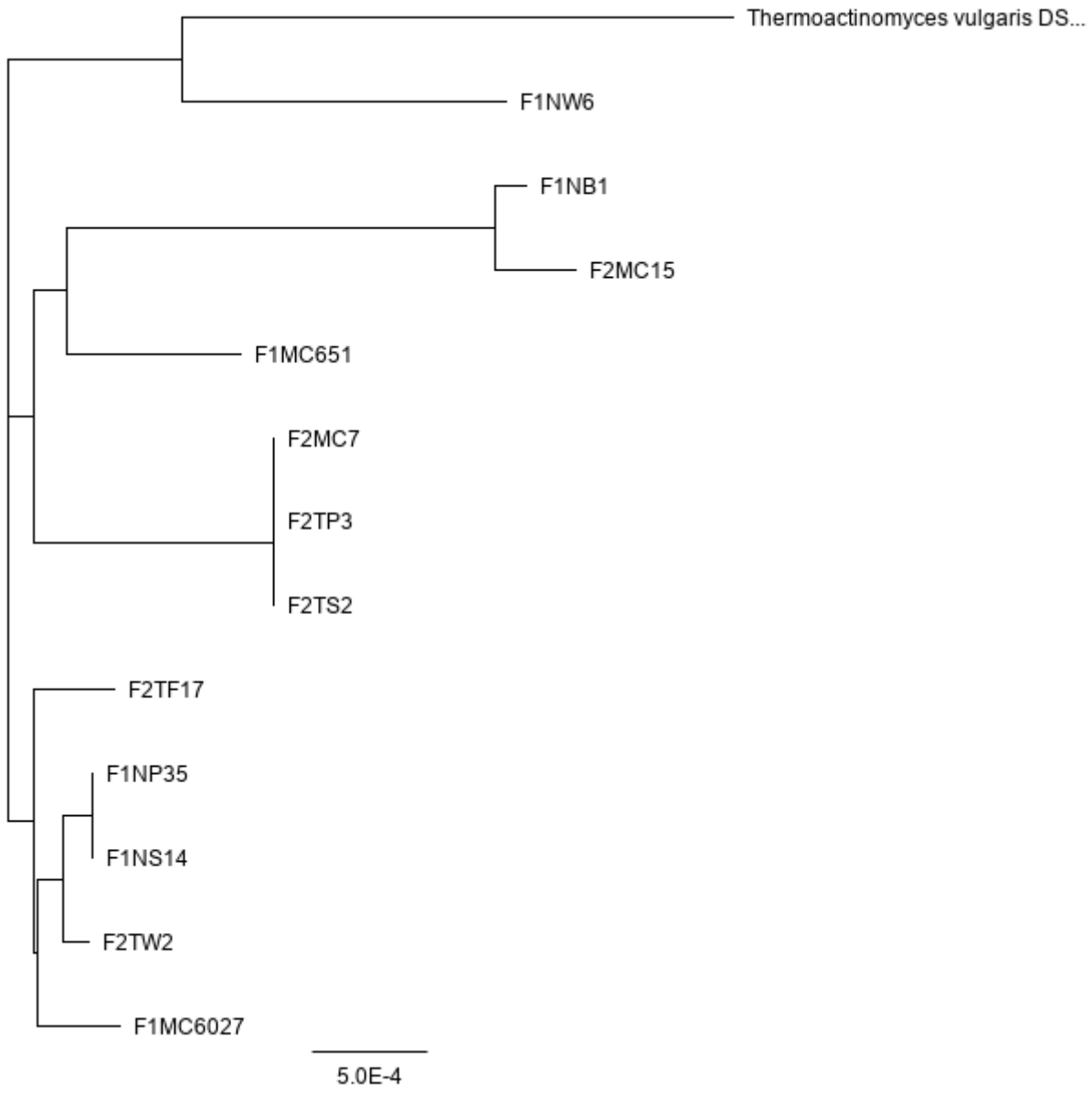


Figure 6.19. MPST-based phylogeny of farm isolates and reference strain of *Thermoactinomyces vulgaris*

The phylogeny of the isolates was investigated using a multi-protein sequence analysis approach (MPST) which was performed through the concatenation and alignment of nine proteins present in the *Thermoactinomyces vulgaris* genomes and coding for putative exoprotease and exolipases. The tree was created with the software Geneious version 2019.1 (Biomatters. https://www.geneious.com) using neighbour-joining clustering and the similarity matrices generated after alignment of the nine proteins sequences in each isolate. A scale for the phylogenetic distances is provided on the bottom of the tree. Isolate denominations can be accessed in Table 6.1, the type strain of *T. vulgaris* was also included in the analysis.

6.3 Discussion

6.3.1 Fingerprinting as a powerful and accessible tool for source-tracking of environmental bacteria

The use of molecular subtyping methods, including rep-PCR fingerprinting, pulsed-field gel electrophoresis, and other banding pattern-based methods, has been critical for gaining a better understanding of the transmission of spoilage bacteria and foodborne pathogens and eventually designing mitigation strategies to reduce the bacterial contamination (Swaminathan et al., 2001). These methods were partially superseded, first by DNA sequencing-based approaches [e.g., *rpoB* typing (Huck et al., 2008)], and subsequently by genome-based phylogenies (Garrido-Sanz et al., 2016), which provide improved resolution and reproducibility, albeit at a greater financial cost. Nonetheless, DNA fingerprinting remains widely used for preliminary phylogenetic surveys, with benefits including wide range of application across different species of bacteria, low cost, ease of implementation and execution, high throughput, and a resolution enabling strain-level discrimination in some instances (Ranjbar et al., 2017).

My results showed that ERIC-PCR is an approach that is suitable for the subtyping of aerobic spore-formers isolated from the sheep dairy farm environment, including for several species of dairy-associated spoilage bacteria such as *B. licheniformis*, *B. pumilus s.l.*, *B. subtilis*, *A. clausii* and *Aer. pallidus*. Overall, gel electrophoreses of ERIC-PCR products generated fingerprinting profiles with a numbers of DNA fragments consistent with previous work across different bacterial species [5-10 fragments (Weingart and Völksch, 1997, Waturangi et al., 2012, Ranjbar et al., 2017)]. Amplification of DNA during ERIC-PCR may not be necessarily directed at ERIC elements, as the primers can also bind to other partially matching sequences (Gillings and Holley, 1997). This may lead to variations in fingerprinting patterns across different bacteria, but also across experimental conditions. However, in this work I placed particular focus on optimising quality and consistency of the analysis, which was achieved through the exclusion of non-specific DNA fragments by performing each PCR experiment in duplicates for each species, in order to only select fragments that were consistently amplified.

A particularly high phylotype diversity was recorded with ERIC-PCR. For example, there were 54 unique profiles recorded for *Thermoactinomyces* spp. and 27 for *B. pumilus s.l.* on F1, as well as 50 unique profiles for *B. cereus s.l.* and 49 for *Pr. megaterium s.l.* on F2. Overall, isolates belonging to the same bacterial species shared > 70% similarity in their ERIC-PCR profile. This is consistent with

other studies reporting a similar threshold for species discrimination using ERIC-PCR (Asgarani et al., 2015), although high variations have also been reported amongst isolates identified as the same bacteria, such as *B. thuringiensis* [20-100% similarity (Katara et al., 2012)]. In my study, there were several bacteria for which the phylogenetic study revealed the presence of two phylogenetic clusters with < 70% similarity, including *B. licheniformis* (Appendix 6.2), *B. pumilus s.l.* (Appendices 6.5 and 6.6) and *Paenibacillus xylanexedens* on F1 (Appendix 6.10), as well as *B. cereus s.l.* (Appendix 6.22) and *Aeribacillus pallidus* on F2 (Appendix 6.24). On the phylogenetic trees corresponding to these bacteria, isolates clustering apart from the bulk of the isolates may have been misidentified and could instead represent closely related species of bacteria. For most of these bacteria, results provided by 16S rRNA gene sequencing and ERIC-PCR phylogeny did not enable identification of the putative different species, either because they were likely to represent novel, closely related bacteria not found in the RDP database (i.e., for *Aer. pallidus* and *Paenibacillus xylanexedens*, 16S rRNA gene RDP seqscore < 0.980), or because their 16S rRNA gene sequences were too similar, something seen across species such as *B. licheniformis*, *B. sonorensis* and *B. paralicheniformis* (Dunlap et al., 2015), or bacteria included in the *B. cereus s.l.* group (Okinaka and Keim, 2016).

The bacteria members of *B. pumilus s.l.* cannot be reliably identified at the species level using 16S rRNA sequencing (Liu et al., 2016). Here, combination of the results from ERIC-PCR phylotyping and 16S rRNA gene sequencing enabled improved identification of *B. pumilus s.l.* isolates, as members of the subgroups *B. safensis s.l.* (i.e., *B. pumilus* or *B. safensis*), *B. zhangzhouensis s.l.* (i.e., *B. zhanghouensis* or *B. australimaris*) and *B. altitudinis s.l.* (i.e., *B. altitudinis*, *B. aerophilus* or *B. stratosphericus*). Liu et al. (2013) were able to differentiate between members of the *B. pumilus* group (i.e., *B. pumilus*, *B. altitudinis* and *B. safensis*) by comparing the sequences of seven housekeeping genes and using this data to perform a MLST. My work suggests that ERIC-PCR may be used as a fast and affordable method to provide subgroup-level identification across members of the *B. pumilus* group. Additional work will be required to assess the potential application of ERIC-PCR to discriminate between other species of bacteria, including members of *B. cereus s.l.* which isolates were, in my work, scattered into 14 different clusters sharing 70% of their ERIC-PCR profile, suggesting the presence of multiple species (Appendix 6.22). Other authors have reported using ERIC-PCR for the subtyping of *B. cereus* group isolates, but few could provide accurate demarcation between *B. cereus s.s.* and other *B. cereus s.l.* species apart from *B. anthracis* (Shangkuan et al., 2000, Li et al., 2011).

Limited comparison could be performed between the results generated using ERIC-PCR and those obtained with the whole-genome approaches, because only a small number of genomes were

sequenced (n = 15) as compared to the number of isolates that was subjected to ERIC-PCR (n = 97). Genome-wide phylogenies provide the best phylogenetic resolution currently available, however sequencing of hundreds of individual genomes can quickly become expensive. Some of the findings obtained with genome-wide phylogeny or with ERIC-PCR were found to vary. For example, F1NSHDT14 (silage) and F1NPDT35 (faeces) *T. vulgaris* isolates shared only 88% similarity in their ERIC-PCR profile, though these isolates could be considered clonal from a whole-genome perspective (dDDH = 100%) and according to the results of the rMLST and MPST analyses. This suggests that the phylogeny generated with WGS or with ERIC-PCR may differ. Some organisms may have particularly diverse ERIC-PCR fingerprinting profiles as a result of rapidly changing variations in their genome (Katara et al., 2012, Ram Mohan et al., 2014). Ram Mohan et al. (2014) showed that populations of the archaea *Halorubrum* and *Haloarcula* could be subtyped with improved depth using random priming PCR amplification, which was capable of capturing local and more subtle genetic variations at the population level, as compared to a MLST approach. As little as single nucleotide polymorphisms may cause modifications in the ERIC-PCR profile of isolates if they are located in the vicinity of the binding region of the ERIC-PCR primers, or if they create new binding sites for primers. Conversely, the targets of MLST are housekeeping genes, and mutations of these genes occur more rarely as they need to be viable and produce functional proteins to ensure normal cell functions. Variations in the conformation of the template DNA (e.g., supercoiling, epigenetics) may also affect the kinetics of the PCR (Kiselev et al., 2015). There is little known about the roles and mechanisms of epigenetics in the genomes of prokaryotic cells, but these changes are often triggered by environmental factors and may not be hereditary (Hale et al., 1994, Blow et al., 2016). Genes coding for DNA methyltransferases (i.e., the enzymes involved in methylation of DNA in the epigenetic context), including D12 class N6 adenine-specific DNA methyltransferases (InterPro entry: IPR012327, MeTrfase_D12), could be identified in the genomes of the *Thermoactinomyces* isolates from my study, as well as in *T. vulgaris* strain 2H which was specifically studied for its epigenetic markers (Mankai et al., 2019). These enzymes are also involved in the methylation of host DNA which is required for protection against foreign DNA and restriction enzymes (Hagemann et al., 2018). Several soil bacteria are also naturally transformable and may acquire changes in their ERIC-PCR profile as a result of horizontal DNA transfer, which occurs at high frequency in soil bacteria such as *B. subtilis* and *T. vulgaris* (Nielsen et al., 2009), including amongst members of a same species. Future work would require complete assessment and comparison of the ERIC-PCR profile and whole-genome sequence of a large set of isolates to assess if the former method is able to provide a more accurate phylogeny which also takes into account environmentally driven, and perhaps non-hereditary, changes in the genome of these bacteria.

In my study, when *Clostridium s.l.* isolates were subjected to ERIC-PCR, only few DNA fragments of weak intensity were visualised on the electrophoresis gel, indicating poor amplification. Rahmati et al. (2005) also found that ERIC-PCR could not be used to differentiate between isolates of *Clostridioides difficile* (formerly *Clostridium difficile*) but more recently Xiaoting et al. (2021) reported using ERIC-PCR to successfully type *C. perfringens* isolates, although the DNA fragments of the ERIC-PCR profiles presented in their study were particularly faint on the gel electrophoresis.

As compared to ERIC-PCR, the phylogenies obtained using ARDRA had lower resolution. ARDRA fingerprinting generated a suitable number of DNA fragments (n > 7) in each species of *Clostridium s.l.* but variations of the fingerprinting profiles across the isolates were low. The first step of ARDRA consists of a PCR targeting a highly conserved region of the DNA which covers the 16S and 23S rRNA gene sequences (Pourshafie et al., 2005). Mutations in this region that would be capable of altering the ARDRA profile of the isolates would need to occur in the vicinity of the restriction sites targeted by the enzymes (here, HhaI or AluI), or create new restrictions sites, in order to be detected by the assay. ARDRA profile can also vary depending on the restriction enzyme used, as seen for the *C. butyricum* isolates which clustered somewhat differently depending on the restriction enzyme used for ARDRA, HhaI (Appendix 6.28) or AluI (Appendix 6.29). Therefore, whether ARDRA is particularly successful at discriminating between species, it does not perform well for strain-level identification of *Clostridium sensu lato*. Brightwell and Horváth (2018) typed a set of psychrophilic *Clostridium* spp. associated with the refrigerated meat spoilage environment and found that isolates identified as the same species could show distinct ARDRA profiles, including for *C. gasigenes, C. estertheticum* and *C. botulinum*. Here, I found that isolates of *C. perfringens, Paracl. benzoelyticum* and *C. butyricum* could be assigned to putative strains based on differing ARDRA profiles. Despite high similarities across the profiles of isolates identified as a same species, this analysis enabled the identification of potential links between bacteria isolated across different samples. Assessment of other subtyping methods should be considered to improve the resolution of the phylogenetic study of *Clostridium* isolates, including WGS, or other fingerprinting approaches such as pulse field gel electrophoresis, RAPD and rep-PCR, which have been used to type *C. perfringens* and *C. botulinum* (Maslanka et al., 1999, Lúquez et al., 2015, Afshari et al., 2016).

6.3.2 Environmental sources of spoilage bacteria on sheep dairy farms

On bovine dairy farms, sources contributing to the contamination of milk have been identified as multiple and variable across farms as a result of different farming systems and farming practices.

Here, I used DNA fingerprinting to study the phylogeny of different species of bacteria and to perform source-tracking of the contaminants recovered from milking cups, back to the farm environment. On the two farms, possible sources of milking cup contamination were identified for anaerobic mesophilic, as well as aerobic mesophilic and thermophilic spore-formers. My results indicate that the contamination of milking cups by spoilage bacteria follows similar overarching principles on bovine and ovine dairy farms, with sources such as soil, feed, faeces, trough water and bedding materials being identified as the origin of milking cups contaminants. Farm sources that were implicated in the transmission of mesophilic and thermophilic spore-forming bacteria to milking cups were found to vary across the different species of bacteria considered, as well as across farms with different farming systems. However, it was not possible to establish clear phylogenetic relationships between environmental isolates of spoilage-associated psychrotrophic spore-formers, or *Pseudomonas,* and those recovered from milking cups. This discrepancy may be attributed to the temporal gap in sample collection, with milking cup samples being collected three weeks subsequent to the environmental samples. The bacterial populations in the milking cups could have undergone significant changes during this period, leading to difficulties in linking them phylogenetically with other environmental isolates. Another plausible explanation for the observed disparity in phylogenetic relationships is the potential contamination of milking cups by *Pseudomonas* across different milking runs. Biofilm formation on milking equipment surfaces can facilitate the persistence of microbial populations, including *Pseudomonas* species. This biofilm-mediated persistence may result in carry-over effects, where bacteria from previous milking sessions contaminate subsequent samples. The resilient nature of *Pseudomonas*, coupled with the ability to form biofilms, could contribute to the observed incongruities in phylogenetic relationships between environmental isolates and those retrieved from milking cups. Understanding the dynamics of bacterial colonisation and persistence in the milking environment is crucial for elucidating the sources and transmission pathways of spoilage bacteria, particularly *Pseudomonas*, in dairy production settings.

On the barn-based sheep dairy farm (F1), several phylotypes of aerobic mesophilic, thermophilic, and anaerobic mesophilic spore-formers were recovered from multiple sources, including in milking cups, thereby pointing to the transfer of these bacteria across the barn environment. This agreed with the results obtained in Chapter 4 which suggested that the bacterial diversity recovered across sources of the barn environment was particularly homogeneous, and many bacteria were ubiquitous or found in multiple sample types, and at similar concentrations. This finding provided further support to the hypothesis that spore-formers may be transmitted at a high rate across sources associated with the

barn environment as a result of close proximity between the different farm matrices harbouring these bacterial populations.

In contrast, ERIC-PCR fingerprinting revealed the presence of a remarkably high diversity of bacterial phylotypes in the environment of the pasture-based farm (F2). In most cases, the origin of the bacterial phylotypes found in milking cups could be attributed to a single environmental source, and the transmission of bacteria across the environment could not be clearly evidenced. The sampling plan on the pasture-based farm did not cover the entirety of the living areas of the ewes, which included several other paddocks on which the animals were rotated for grazing (two paddocks sampled ≈ 10-20% of total living area), meaning the sampling was not able to capture the entire microbial diversity present on F2. Varying soil properties may impact local macro- and microflora, which can lead to variations in the microbiological composition of soils and pastures (Wollum, 1994) and may contribute to explain the wider diversity of bacterial phylotypes found on the pasture-based farm. In comparison, sampling on the barn-based farm covered most of the living area of the ewes (two large pens sampled ≈ 70% of total living area). These results corroborate my previous findings (see Chapter 5), specifically that, as a result of their wider surface area and varied living environment of the animals, the microbiota of pasture-based dairy farms may be particularly diverse and heterogeneous as the transmission of bacteria across the environment is limited.

In the barn environment, silage was identified as the main source of milking cups contaminants, including *A. clausii, B. zhangzhouensis s.l.*, *T. vulgaris*, *Aer. pallidus* and *C. perfringens*. These bacteria were likely transferred from silage to faeces, but also to other components such as bedding and trough water by faecal cross-contamination, and ultimately winded up on the udders of the animals and milking cups. There were other bacteria possibly originating from silage, though they were not transferred to milking cups but instead exclusively to bedding materials (*B. licheniformis*), faeces (*B. safensis s.l.*) or both (*Paracl. benzoelyticum*). These results are consistent with previous work on bovine dairy farms identifying silage as an important potential source of spore-formers contamination in raw milk. te Giffel et al. (2002) isolated *B. licheniformis*, *B. pumilus*, *B. subtilis* and *B. cereus s.l.* from grass and maize silage on cattle dairy farms, and highlighted the transmission of phylotypes of these bacteria to cow milk using rep-PCR. Vissers et al. (2007b) and Borreani et al. (2019) stressed the contribution of silage containing high concentrations of spore-formers (*B. cereus s.l.*, *Paenibacillus* and *Clostridium* spp.) in increasing the aerobic and anaerobic spore content of dairy cow faeces, as well as of raw milk. Silage represents a key source of bacteria around the world because the manufacturing process of this type of feed may allow for the proliferation of undesirable

microorganisms (e.g., *Clostridium* spp., *Bacillus* spp., *Paenibacillus* spp., moulds, yeasts) if ensiling conditions are not suitable [i.e., quick pH decrease, anaerobiosis maintained throughout and after ensiling (Driehuis, 2013)]. Zheng et al. (2018) isolated *C. sporogenes* and *C. perfringens* in clostridial silage and later showed that *C. perfringens* contributed to initiate clostridial fermentation while remaining at low relative abundance amongst clostridia [< 5%, (Zheng et al., 2020)]. My results also highlight silage as an important source of *C. perfringens* on sheep dairy farms, considering most ARDRA phylotypes of *C. perfringens* were recovered from silage. In the case of *Paracl. benzoelyticum*, there was also a cluster of isolates exclusively associated with milking cups and faeces, indicating that these bacteria may also thrive as part of the anaerobic gut microbiota of sheep. The transfer of silage organisms to raw milk takes place after ingestion by dairy livestock, via passage through the gastrointestinal tract (GIT), shedding in faeces and possible attachment of bacteria to the udders before milking (Vissers et al., 2007c). On ovine dairy operations, feeding of silage has been associated with the production of milk containing higher anaerobic spore counts, and as a result is often restricted in the production of certain dairy products in Europe, mainly cheeses (Pirisi et al., 2007, French Ministry of Agriculture and Food, 2017). My results expand our current understanding on the contribution of silage in the microbiological quality of ewe milk by demonstrating that it may represent a possible source of not only anaerobic spore-formers (in this case proteolytic *C. perfringens* and *Paracl. benzoelyticum*) but also of several species of mesophilic and thermophilic aerobic spore-formers associated with the spoilage of heat processed dairy products, including dairy powders. Silages that are farm-made may be of poorer microbiological quality than commercially produced silages that are monitored for adequate ensiling conditions (Arias et al., 2013). This may be partly owing to the fact that farmers are not always aware of the entire implications of silage manufacturing, and in particular of the relationships between ensiling conditions or practices and the microbiological quality of silage. New Zealand is a wet climate, and adequate ensiling conditions can be particularly challenging to achieve during the wet season without the use of additional preventive actions such as increased herbage chopping and wilting, use of a silage inoculant (probiotics and/or prebiotics), or adequate oxygen barriers (Driehuis, 2013, Zheng et al., 2017). My findings place emphasis on the necessity to monitor silage quality and ensure feeding high-quality silage to dairy ewes to help reduce the transmission of dairy spoilage bacteria in the environment and eventually to raw milk.

The main source of milking cups contaminants in the grazing environment of dairy ewes was identified as the natural environment, i.e., soils and pastures. According to ERIC-PCR fingerprinting, bacteria found to originate from soil included *Lysinibacillus macroides* and *Paenibacillus anaericanus*, while those found to originate from pasture included *Pr. megaterium* and *Aer. pallidus*.

Despite their ability to produce lipases and/or proteases, these bacteria have not yet been linked to dairy spoilage (Lucking et al., 2013), though they have been isolated from farms and natural environments as a result of their involvement in natural processes including bioremediation and interactions with plants. *Lysinibacillus macroides* was originally isolated from cow dung and soil (Pringsheim and Robinow, 1947, Karmakar et al., 2022), *Paenibacillus anaericanus* was recovered from the gut of earthworms (Horn et al., 2005), while *Aer. pallidus* and *Pr. megaterium* are frequently associated with green forage, pastures, and soil, including on dairy farms (Scheldeman et al., 2005, Zhou et al., 2008, Dobrzanski et al., 2018). The microbiota found in the phyllosphere of plants species is similar to the microbiota present in their rhizosphere as a result of the proximity and close association between these matrices, both of which facilitate the exchange of bacteria (Saito et al., 2007). Thus, it is not surprising that these bacteria are frequently isolated from both soil and pastures. However, few milking cups contaminants were found to originate from faeces, particularly as compared to what was observed on F1, suggesting that bacteria from the natural environment may not be excessively shed in faeces but may instead be transmitted to milking cups by adhering to the udders of the animals via particles from soil or pasture. This is consistent with the findings of Christiansson et al. (1999) identifying soil contamination of the udders as the main source of *B. cereus s.l.* in milk from pasture-based cattle dairy farms. In the same study, the wet season was identified as a risk factor increasing the attachment of soil on the udders of the animals, including due to high soil water content, low water evaporation, muddy walkways and increased direct contact between soil and the animals as a result of physical damage on pastures. During wet weather or with high stocking density, cows can inflict significant strain and damage on pastures. This phenomenon can be particularly common during New Zealand wet winter climate, and it can cause deformation and packing of the soil, leading to reduced soil porosity and breathability, waterlogging, and a decrease of up to 40% in pasture growth for the next season (Nie et al., 2001). Although sheep are known to cause less damage to pastures due to their weight being roughly seven times less than that of a cow (Drewry et al., 2000), sheep dairy farmers should ensure that the pasture rotation plan is well managed, including with low stocking density, so that grazing will not place unnecessary strain on the pasture and increase soiling of the udders of dairy ewes.

Soil and pastures were also identified as key sources of *Clostridium s.l.* contamination in milking cups on the pasture-based farm, including *C. perfringens*, *C. butyricum*, and *Paracl. benzoelyticum*. *Clostridium perfringens* and *Paracl. benzoelyticum* were associated with both pasture and soil, but they were transferred to different matrices: faeces and trough water, respectively. According to the ARDRA, *Paracl. benzoelyticum* isolates were highly clonal, underlining the low genetic diversity of

these bacteria in the pasture environment. *Clostridium butyricum* originated from soil and was likely transferred to water by cross-contamination of the troughs with soil particles. My findings provide the identification of sources present in the grazing environment of dairy sheep that are implicated in the contamination of milking cups by *Clostridium s.l.* capable of initiating, or aiding the development of late-blowing defects in cheeses (Arias et al., 2013, Bermudez et al., 2016). *Clostridium s.l.* are obligate anaerobes for which oxygen is toxic, as a result they are rarely active in aerobic environments and instead remain as dormant spores. The ability of these bacteria to colonise aerobic environments may also be linked to their varying ability to resist oxygen stress (Palmer et al., 2019). *Clostridium butyricum*, *C. perfringens* and *Paracl. bifermentans* (which is closely related to *Paracl. benzoelyticum)* were amongst the clostridia with the highest O_2 tolerance in the study by Hill and Osterhout (1972), which may contribute to explaining how they may travel unscathed throughout the farm. Soil is a key source of clostridial cells because it may act as both a reservoir and incubator of these bacteria. One of the consequences of poor soil structure and livestock overstocking can be the accumulation and stagnation of rainwater on water-saturated paddocks after heavy precipitations, also called soil waterlogging. The normal oxygen content of the upper soil layer is at around 1.5 to 20% depending on factors such as soil structure and irrigation (Ioannou et al., 1977), but during waterlogging, telluric oxygen levels decrease to < 2 % as oxygen is consumed by aerobic bacteria and replenished at a low rate due to the limited gas exchange taking place with the atmosphere (Ioannou et al., 1977, Palmer et al., 2019). Obligate anaerobic bacteria, and particularly *Clostridium s.l.* which are highly abundant as spores in soil, can resume vegetative growth upon the establishment of anaerobic conditions in soil. High abundance of clostridia and clostridia-like lineages have been noted in saturated paddy soils and in sporadically waterlogged pasture and arable land (Weber et al., 2001, Spigaglia et al., 2020). This indicates that farmers should place particular emphasis on the prevention of waterlogging in order to limit the outgrowth of clostridial populations in soil, which may include several species associated with dairy spoilage such as *C. butyricum*, *C. sporogenes* and *C. perfringens*, and which represents the main source of clostridia contamination in milk during the grazing period (Vissers et al., 2007e, Barash et al., 2010).

The faecal spore microbiota of dairy livestock reflects their intestinal contents, and is mainly influenced by their diet (Wu et al., 2007), but also potentially by the water they consume (Van Eenige et al., 2013). On both farms, I was able to identify a set of milking cups contaminants potentially originating from trough water, with faeces likely serving as an intermediary facilitating their passage to milking cups. These bacteria were either thermotolerant [*B. licheniformis* on F2, *B. subtilis* on F1 (Meng et al., 2014)], or thermophilic (*Cald. kokeshiiformis* on F1, *T. vulgaris* on F1 and F2 and *T.*

intermedius on F2*)*. Trough water can represent a reservoir of bacteria in the farm environment resulting from the accumulation of cells in the troughs after cross-contamination by other components, including bedding materials, pastures, soil, faeces and feed (LeJeune et al., 2001). Biofilms may form on the surface of the troughs and help maintain stable populations of bacteria in the drinking water of the animals over time. Several studies have highlighted the contribution of trough water in the environmental colonisation and transmission of the bovine commensal and human pathogen Shiga toxin-producing *Escherichia coli* on cattle farms (Beauvais et al., 2018, Hayer et al., 2022). The species of bacteria most likely to accumulate in water troughs may include those capable of forming spores particularly resistant to stresses (e.g., starvation, UV light), or cells suited to life in aquatic environments (i.e., planktonic or as biofilm). Highly heat-resistant spore-formers and thermophilic spore-formers, in particular, are known to be able to remain in the environment for particularly long periods of time as endospores (Nilsson and Renberg, 1990), due to their unique stress resistance features (Sadiq et al., 2016b). My findings indicate that trough water may be implicated in the transmission of bacteria, particularly thermotolerant or thermophilic dairy spoilage bacteria, to milking cups and to raw milk. Factors found to be deleterious for the microbiological quality of trough water on livestock farms include the use of high-volume troughs, trough materials other than stainless steel, infrequent water replenishing, lack of sun exposure, and high environmental temperatures (LeJeune et al., 2001). Some of these represent actionable aspects that may be considered by farmers to limit the potential contribution of trough water bacteria to the contamination of milk.

The discovery that *Bacillus*, long thought to represent transient members of the gut microbiota of mammals, were in fact integral members of the healthy gut microbiota of several animal species has prompted their successful use as gut probiotics to improve animal health and production (Kritas et al., 2006, Souza et al., 2017). This has highlighted the potential for these bacteria to proliferate in and colonise ecological niches found in the gut of dairy livestock. Gut bacteria can then be shed in faeces as the contents of the faecal flora are assumed to reflect those of the gut, though intestinal contents are generally more dilute (Seeliger and Werner, 1963). On both farms, several *A. clausii* and *B. licheniformis* phylotypes were exclusive to milking cups and faeces, which suggested that they may originate from the GIT of the animals. Wu et al. (2007) isolated *B. licheniformis* and *A. clausii* as the predominant spore-formers in the faeces of feedlot dairy cattle but the authors could not isolate *A. clausii* from the feed of the animals, suggesting that the abundance of these bacteria increased in the GIT, including through accumulation or proliferation. In my study, some of the phylotypes of *A. clausii* and *B. licheniformis* isolated from both faeces and milking cups could also not be isolated

from feed (i.e., silage or pasture). Both *B. licheniformis* and *A. clausii* have been extensively used as sheep probiotics (Kritas et al., 2006, Lopetuso et al., 2016), therefore it is plausible that these bacteria are capable of colonising the GIT of dairy ewes, from which they could be shed in faeces. Thus, my results indicate that the GIT of dairy ewes may represent a source of spoilage bacteria in the farm environment and possibly in raw milk, with faeces functioning as the vector of this contamination. These findings also stress that particular caution should be taken before considering using spore-forming bacteria as livestock probiotics in dairy production, as this may increase the shedding of these bacteria in faeces, their transfer to milking cups and thus possibly in raw milk.

When penned, either housed or outdoors, sheep tend to defecate at higher density in the vicinity of their night lying areas (White and Hall, 1998), which can promote contamination of the surface of their udders by faecal bacteria. However, faecal density across the general living area of dairy ewes is lower in a grazing set-up compared to when animals are housed, as a result of shifting locations, also with larger surface area, during grazing. In my study, the faeces of dairy ewes represented a more substantial source of contamination on the farm where the animal were housed (i.e., source of *A. clausii*, *B. licheniformis*, *B. zhangzhouensis s.l.*, *C. perfringens* and *T. vulgaris*) than on the farm where they were grazed (i.e., source of *C. perfringens*, *B. licheniformis* and *Parag. caldoxylosilyticus*). These findings are in agreement with the hypothesis that housing may contribute to increase faecal contamination of the udders of dairy livestock. During housing, bedding materials represent a matrix with nutrients, adequate temperatures and an influx of bacteria from faeces that was shown to sustain bacterial growth (Godden et al., 2008), and to promote the transfer of bacteria to the udders of dairy cows and to raw milk (Murphy et al., 2019). Indeed, bacteria found in bedding materials can easily adhere to the udders of dairy livestock when the animals lay down (Rowbotham and Ruegg, 2015). Barned dairy ewes spend significantly more time lying down, and in a smaller area, than grazed animals (Williams et al., 2021), highlighting the potential for increased udder contamination on dairy farms housing dairy ewes. On bovine dairy farms, bedding materials were shown to be indirectly associated with the contamination of raw milk (Martin et al., 2019, Murphy et al., 2019), but few studies have identified the spoilage bacteria contaminating raw milk and originating from bedding materials, apart from *B. cereus s.l.* (Vissers et al., 2007d). In my study, there were no bacteria for which a milking cups phylotype could be exclusively traced back to bedding materials, however, several bacteria were associated with bedding materials, and silage or faeces, as well as milking cups, including *B. licheniformis*, *A. clausii*, *B. zhangzhouensis s.l.*, *B. safensis s.l.*, *C. perfringens* and *Paracl. benzoelyticum.* These bacteria were likely to have contaminated bedding materials after cross-contamination by silage or faeces, though it is also possible that some were able

to grow in the bedding, including during spontaneous composting of the woodchips (Driehuis et al., 2014, Leso et al., 2020). This is plausible considering bedding materials contained high concentrations of mesophilic and psychrotrophic spores (> 6 $\log_{10}$ cfu/g, see Chapter 4), as well as thermophilic spores (> 5 $\log_{10}$ cfu/g). My results suggest that bedding materials may be implicated in the transmission of several species of mesophilic spoilage bacteria to milking cups on sheep dairy farms, possibly by acting as a microbial hub and interface between the udders of the animals and the environment. Nonetheless, there were no spoilage bacteria found to exclusively originate from bedding materials, suggesting that the bedding management practices in place on F1 prevented extensive proliferation of spoilage bacteria in the bedding.

6.3.3 First insights into the transmission and genetic diversity of *Thermoactinomyces* in the sheep dairy farm environment

Thermoactinomyces spp., and particularly *T. vulgaris*, are thermophilic spore-forming bacteria that have recently been increasingly detected in dairy powders around the world (Yuan et al., 2012, VanderKelen et al., 2016, Delaunay et al., 2021). Upon proliferating, these bacteria can produce an array of potent hydrolytic enzymes including proteases and lipases (Falkowski, 1979, Kleine, 1982), which suggests that they may be capable of altering the quality of dairy products if provided with suitable, high-temperature growth conditions, such as those found during milk powder manufacturing (Murphy et al., 1999). In the dairy production, the ecology and characteristics of *Thermoactinomyces* spp. have been largely ignored in favour of that of other thermophilic bacteria such as thermotolerant *B. licheniformis*, *Anoxybacillus*, and *Geobacillus*, which often constitute the predominant contaminants of dairy powders (Burgess et al., 2010). Sources of *Thermoactinomyces* in dairy powders remain unclear, but they have been found in the bovine dairy farm environment and in raw milk (Buehner et al., 2014), and more generally in association with agricultural environments (Jackson et al., 1997). My previous findings indicated that *T. vulgaris* was prevalent and highly abundant in the environment of New Zealand sheep dairy farms, which suggested that these bacteria may be transmitted to ewe milk, and perhaps subsequently passed on to dairy powders thus contributing to increase their thermophilic spore counts (Sadiq et al., 2016b).

The fingerprinting profiles generated across *Thermoactinomyces* isolates using ERIC-PCR were diverse on both farms, with 54 different profiles out of 72 isolates recorded on F1, and 24 different profiles out of 25 isolates recorded on F2 (Table 6.1). There was a clear demarcation between the

ERIC-PCR profiles of the species *T. vulgaris* and *T. intermedius* on the dendrogram as the isolates shared < 70% similarity in their ERIC-PCR profiles (Appendix 6.26). In addition, three isolates from F1 that had been identified as *T. vulgaris* by 16S rRNA sequencing with moderate confidence (0.970 < RDP seqscore < 0.980) were found to cluster away from the remaining *T. vulgaris* isolates (similarity < 73%), indicating they may represent novel species of *Thermoactinomyces* closely related to *T. vulgaris*, or perhaps a subspecies of this bacterium. This is plausible considering the genus *Thermoactinomyces* contains only five validly published species, some of which were only recently described (Mokrane et al., 2016, Mutschlechner et al., 2021). At the species level, the ERIC-PCR profile of the isolates was found to be similar across the *T. intermedius* (75 – 100% similarity) or the *T. vulgaris* isolates (80 – 100% similarity). Large variations have been recorded across the ERIC-PCR profile of bacterial isolates members of a same species, including for *B. thuringiensis* [20 to 100% similarity (Katara et al., 2012) and *Aeromonas popofii* [30 to 100% similarity (Soler, 2003)]. Dorneles et al. (2014) showed that ERIC-PCR could be used for the subtyping of *Corynebacterium pseudotuberculosis* isolates from multiple sample types and countries, with fingerprinting profiles sharing 50 to 100% similarity. The same authors reported that the use of 80% similarity threshold for clustering of these bacteria provided adequate grouping of the isolates according to their epidemiological background (Dorneles et al., 2014). My results suggest that the fingerprinting patterns obtained by ERIC-PCR are sufficiently conserved across the *Thermoactinomyces* genus, and simultaneously variable enough at the species and subspecies levels, to establish phylogenetic relationships and perform source-tracking of environmental isolates. In my study, the comparison of ERIC-PCR profiles was limited to isolates from the same farm location (i.e., F1 or F2). Further comparison of the ERIC-PCR profiles of the farm isolates to those of type strains and of isolates from other geographical locations will need to be performed to assess the applicability of ERIC-PCR for studying the phylogeny of *Thermoactinomyces* in a wider geographical context.

A total of 15 *Thermoactinomyces* isolates, which represented the sample-wise predominant ERIC-PCR phylotypes of *T. vulgaris* and *T. intermedius* recovered from the two farms, were subjected to whole-genome sequencing. The draft genome sequences generated by this analysis help complement the particularly limited currently available pool of *Thermoactinomyces* genomes. The GenBank database contains 29 genomes of *Thermoactinomyces* spp. (accessed 10/2022), with only half of them being currently assigned to a species: *T. vulgaris* (n = 9), *T. intermedius* (n = 3), *T. daqus* (n = 2), and *T. mirandus* (n = 1), while there was no publicly available whole-genome sequence for *T. khenchelensis* at the time of access. Genetic diversity of the *Thermoactinomyces* isolates was particularly low at the species level, including between farm isolates and the reference strains, with

genome dDDH values (phylogenetic distance formulae d4) ranging between 94 to 100% across pairs of *T. vulgaris*, or *T. intermedius* isolates. Genome-extracted 16S rRNA gene sequences of the *T. intermedius* and *T. vulgaris* isolates were highly conserved and comparison of this gene did not enable meaningful phylogenetic clustering of the isolates, demonstrating that sequencing of the 16S rRNA gene cannot be used to provide accurate subtyping of these bacteria. The genome sequences of the reference strains were highly similar to that of the farm isolates, which pointed to a low genetic diversity at the world scale for *T. vulgaris* and *T. intermedius*, considering the reference strains had been isolated from locations including Tunisia, the USA, and China (Tsilinsky, 1899, Li et al., 2019, Mankai et al., 2019). In comparison, dDDH values between the genomes of *T. vulgaris* and *T. intermedius* were > 63%, which is consistent with the threshold of 70% used for the delimitation of species (Meier-Kolthoff et al., 2013). Meier-Kolthoff et al. (2014) proposed dDDH < 80% as a threshold for the delimitation of subspecies after analysing a set of 250 genomes of *E. coli* and related bacteria. The same authors also pointed out that strains sharing dDDH values greater than 90% indicated particularly close relationship between the genomes, while values greater than 95% were particularly likely to point towards their clonality. However, dDDH values lower than the subspecies threshold have been reported in other bacteria, including across isolates identified as the same subspecies of *Azotobacter chroococcum* [e.g., 63.4% < dDDH < 89.6% (Jin et al., 2020)]. Low genetic divergence was observed across the *T. vulgaris* genomes, including across that of isolates from different locations and time periods (i.e., 100+ years (Tsilinsky, 1899)]. In prokaryotes, low genetic diversity may result from specialisation of a population leading to the loss of redundant genes and of genes that are not required by the organisms to achieve their life cycle, such as during the establishment of symbiotic relationships with higher eukaryotes (Bennett and Moran, 2015), or upon prolonged exposure to non-variable environments (Van Waasbergen et al., 2000). The low genetic diversity of *Thermoactinomyces* spp. may be explained by the fact that these thermophilic bacteria are rarely capable of growth, and instead mostly persist as endospores, including in soil and sedimental layers (Cross and Johnston, 1971), and become dispersed as a result of agricultural activity (farming of crops or animals). However, the formation of spores requires sporulation of vegetative cells, which indicate that these bacteria must have been proliferating at some stage and location in the environment, from which their spores could become dispersed. It would be interesting to sequence the genome of isolates recovered from historical sediment samples [e.g., ca. 1100 AD (Nilsson and Renberg, 1990)] for comparison with the genome of modern isolates, in order to assess the degree of genetic divergence found in these bacteria over a longer period of time. This may help further our understanding of the ecology and the populations dynamics of *Thermoactinomyces* spp., and perhaps of other thermophilic spore-forming bacteria that may behave ecologically similarly in the farm

environment. Locations containing populations of *T. vulgaris* with high genetic variability may indicate sources in which these bacteria could proliferate and accumulate mutations at a higher rate than during dormancy as spores. According to my results and the literature, these sources may include self-heating plant matter such as silage or spontaneously composting plant cuttings.

Together, the results of ERIC-PCR phylotyping and genome sequencing of selected representative isolates provided insights on the transmission of *Thermoactinomyces* spp. in sheep dairy farm environments and on the potential source of these bacteria in milking cups, and thus possibly raw milk. According to ERIC-PCR, my findings suggest that the transmission of *T. vulgaris*, the predominant *Thermoactinomyces* spp. isolated on both farms, involves different sources when dairy sheep are housed (i.e., faeces, silage) or grazed (i.e., trough water, soil), which may result from differing farming systems. These findings are also supported by the genome phylogeny, which demonstrated that representatives of the predominant strains of *T. vulgaris* from silage (F1NSHDT14) and faeces (F1NPHDT35) were virtually identical at the genomic level according to each of the different analyses performed in this study, including a $dDDH_{d4}$ value of 100% (Appendix 6.35). These clones were also virtually identical to the predominant *T. vulgaris* found on pasture in F2 ($dDDH_{d4}$ = 100%), suggesting that the predominant populations of *Thermoactinomyces* present on farm-grown herbage may be conserved across farms, as well as throughout and after the ensiling process. This is consistent with several other studies highlighting the survival of spore-forming bacteria present on herbage throughout ensiling and in the resulting silage (Coblentz et al., 2014, Zheng et al., 2020), but this had not been shown for thermophilic spore-formers. It is also important to note that significantly lower concentrations of thermophilic spores were recorded in grass pasture (3.34 $\log_{10}$ cfu/g, Chapter 5.2.1) as compared to grass silage (5.41 $\log_{10}$ cfu/g, Chapter 4.2.1), which may suggest that the spores of *Thermoactinomyces* spp. were concentrated, or that these bacteria proliferated, during ensiling. *Thermoactinomyces* spp. are aerobic and would require high temperature and oxygen to proliferate during ensiling, both of which are conditions that may be found during aerobic deterioration of silage, a form of spontaneous composting (Borreani et al., 2013). The growth of mesophilic and thermotolerant *Paenibacillus macerans* (and its heterotypic synonym *P. thermophilus* (Kobayashi et al., 2019)] have been demonstrated in silage (Borreani et al., 2019). Small pockets with unique microenvironments (i.e., high temperature and oxygen (Vissers et al., 2007b)) could potentially be able to trigger germination and growth of *Thermoactinomyces* and other thermophilic bacteria. This will need to be tested, for example by measuring spore counts pre-, during and post-ensiling, as well as by artificially initiating aerobic deterioration and monitoring populations of thermophilic spore-formers in the areas of silage that are spoiling.

In the pasture-based environment, the only source with an isolate matching the ERIC-PCR phylotype of a milking cups isolate was trough water, which was also the only sample alongside milking cups in which *T. intermedius* was detected. The phylotype of *T. vulgaris* that was detected in both trough water and milking cups was also somewhat related to that of a set of isolates from pasture, soil, trough water, faeces and milking cups sharing relatively high ERIC-PCR profile similarity (> 90%), indicating the presence of a ubiquitous and somewhat related cluster of *T. vulgaris* subtypes in the pasture environment. The possible source of *T. intermedius* contamination in milking cups was also identified as trough water, as suggested by ERIC-PCR, and further confirmed by WGS [genome dDDH = 99-100% (Appendix 6.35)]. *Thermoactinomyces intermedius*, like many thermophilic spore-formers, is a telluric bacterium that is also associated with artificial and spontaneous composting processes (Kurup et al., 1980, Liu et al., 2022). *Thermoactinomyces* spp. produce spores at the tip of the aerial hyphae that can be aerosolised, resulting in the frequent isolation of these bacteria from air systems and the respiratory system of animals (Kurup et al., 1980, Van Den Bogart et al., 1993). My results suggest that airborne *Thermoactinomyces* spores may be particularly prone to attach to the surface of trough water, for example during windy weather, or from contamination by soil particles. If the troughs are not washed regularly, the accumulation or growth of bacteria and spores in water may subsequently increase their uptake by the animals as they drink. According to the genome-based phylogeny, representatives of the predominant phylotypes of *T. vulgaris* in pasture and trough water were identical (dDDH = 100%) which suggested that pasture may represent the main source of trough water contamination. This is consistent with other studies identifying feed and the buccal contents of livestock as key sources of trough water contamination, although faeces were also found to contribute significantly, particularly in housing conditions (LeJeune et al., 2001, Van Eenige et al., 2013). Although *Thermoactinomyces* spp. have been associated with the faeces of livestock (Jackson et al., 1997), including on F1 (see above), my results suggest that faeces were mostly not implicated in the transmission of predominant strains of *T. vulgaris* and *T. intermedius* to milking cups and other farm sources on the pasture-based farm. Instead, my results suggest that the main source of *Thermoactinomyces* spp. on pasture-based dairy farms is the natural environment, with trough water potentially acting as an interface facilitating accumulation and transmission of environmental bacteria in the vicinity of dairy ewes.

The findings from my study represent the first attempt at identifying sources of the novel dairy powder-associated bacteria *Thermoactinomyces* spp. on sheep dairy farms. The putative sources and transmission patterns established here should be further investigated, including on additional sheep dairy farms but also perhaps in the bovine dairy farm environment, in order to improve our

understanding of the ecology of *Thermoactinomyces* spp. on dairy farms. Such data may be utilised to work towards limiting the transmission of *Thermoactinomyces* spp. to raw milk, but also simultaneously to limit their abundance on farm and the exposure of farm workers to high concentrations of these bacteria, which has been associated with farmer's lung (immunological pneumonitis).

6.4 Conclusions

In this chapter, I set out to explore two fundamental aspects of microbial dynamics in sheep dairy farming environments. Firstly, I aimed to identify the main sources of raw milk contamination on two sheep dairy farms. Through comprehensive analyses, I uncovered key reservoirs contributing to milking cup contamination, specifically emphasising feed as an important source of spoilage-associated bacterial contaminants on both farms. Concurrently, I explored whether different farming systems (i.e., housing vs grazing) may exert an impact on the transmission of bacteria within the environment. By meticulously investigating both a barn-based and a pasture-based sheep dairy farms, my findings shed light on the varying degrees of bacterial transmission and contamination pathways associated with each system. This research, in addressing these two objectives, provides critical insights and empirical evidence contributing to a more comprehensive understanding of microbial dynamics in sheep dairy farming environments. These findings also pave the way for future investigations, offering a foundation for focused inquiries into specific aspects unveiled in this work.

Genome sequencing and genome-based phylogenetic analyses currently have the highest resolution for accurate subtyping and tracking of environmental bacteria. However, these approaches quickly become expensive and labour-intensive when applied to a large set of isolates. In the present study, I show that affordable DNA fingerprinting techniques such as ERIC-PCR and ARDRA can be used to subtype hundreds of environmental bacteria isolated from sheep dairy farm environments. In particular, ERIC-PCR was successfully applied to more than 20 different mesophilic, psychrotrophic and thermophilic bacteria, including key dairy spoilage bacteria such as *B. licheniformis, B. pumilus s.l., B. cereus s.l., Paenibacillus*, and *Pseudomonas* species. Because DNA fingerprinting approaches are easily implemented, they can provide a rapid overview of the transmission of environmental bacteria and help screen for routes and hotspots of contamination that can be subsequently targeted and investigated in more depths using methods with higher resolution.

Subtyping of the isolates recovered from the sheep dairy farm where the animals were housed revealed a high frequency of identical bacterial phylotypes across multiple samples, including silage, faeces, bedding, trough water and milking cups, which pointed to the transmission of these bacteria across the barn environment. On that farm, the main source of spoilage bacteria in both the farm environment and milking cups was identified as the feed-faeces axis, with silage representing the possible initial source of key spoilage bacteria such as *B. licheniformis*, *A. clausii*, *B. zhangzhouensis s.l.*, *T. vulgaris*, *Aer. pallidus* and *C. perfringens*. This finding highlights the contribution of silage in the contamination of sheep milk, and it suggests that farmers should consider placing particular emphasis on manufacturing or purchasing high-quality silage containing low counts of spore-forming bacteria. Trough water and bedding materials were not found to contribute hugely to the contamination of milking cups. Yet, these sources may instead serve as intermediaries from which silage bacteria could gain access to the udders of the dairy ewes. On bovine dairy farms, bedding materials have been identified as an important source of milk contamination by spoilage bacteria, but this had not been shown for dairy sheep farms, nor could it be clearly evidenced in the present study. Differences between the physiology and behaviour of housed dairy sheep and cows, including in relation to the rate of bedding contamination (e.g., quantity, rate, and type of defecation), may contribute to explain this phenomenon. Further assessment of the contribution of different types of bedding materials in the contamination of raw milk during housing of dairy ewes will need to be carried out to help probe this hypothesis.

On the pasture-based farm, a high diversity of bacterial phylotypes was recorded and only few phylotypes were simultaneously detected in multiple samples, suggesting low transmission of bacteria across the pasture-based farm environment. Possible sources of milking cups contamination were multiple and included soil, pasture, trough water and faeces. The natural environment (i.e., soil and pastures), but surprisingly not faeces, represented a key source of *Clostridium* spp. contamination, including of the cheese spoiling bacteria *C. butyricum* and *C. perfringens*. When kept on pasture, exposure of the animals to high concentrations of clostridial spores should be limited, particularly when the dairy production is intended for the manufacturing of sheep milk cheeses. The impact of practices and risk factors that have been previously associated with high concentrations of clostridial spores in soil and on the pastures of bovine dairy farms should be assessed on sheep dairy farms, including the use of biological amendments with high spore counts and inadequate land management, structure, or composition. Trough water and the faeces of grazed dairy ewes represented potential sources of mesophilic and thermophilic spore-formers contamination in milking cups, including *B. licheniformis*, *A. clausii*, *T. intermedius* and *T. vulgaris*. The fact that several bacterial phylotypes

were exclusively found in trough water and milking cups suggest that the drinking water of the animals may represent a source of milk contaminants that will require further assessment. When not adequately upkept, outdoor water troughs could become bacterial hotspots as they become contaminated by the natural environment (pasture, soil, weather) and as a result of the high animal density around the troughs.

Genome-based phylogenetic analysis of selected *Thermoactinomyces* isolates revealed that their genomes were highly similar (dDDH > 94%) including between isolates from different farms and reference strains, which highlighted the remarkably low genetic diversity of *T. vulgaris* and *T. intermedius*. Because of this, and the fact that the genomes of only few isolates were sequenced out of the total of isolates initially recovered, the tracking of *Thermoactinomyces* spp. in the environment using their genomic data was limited. However, the genome sequences that were generated during this work more than double the number of currently available genome sequences for *T. vulgaris* and *T. intermedius*. In addition to the search for genes associated with dairy spoilage, such as spoilage enzymes, sporulation genes and biofilm formation genes, these genomes will facilitate further study of the broader enzymatic capability of these bacteria. Thermophilic spore-formers, including *Thermoactinomyces* spp. are a key source of thermoactive enzymes that are sought after by several industries, such as cellulases, thermitases and amylases.

Chapter 7 - Microbiological quality of ewe milk and sheep dairy products

7.1 Introduction

In this research chapter, I explore the microbiological quality of ewe milk and sheep dairy products manufactured in New Zealand, addressing two primary research questions: First, what is the microbiological quality of ewe milk and sheep dairy products? Second, are these food commodities at risk of microbial spoilage or quality decrease? This investigation builds upon the three preceding chapters, which uncovered the diverse bacterial communities within sheep dairy farm environments and subsequently shed light on potential milk contamination sources and transmission pathways. As the focus shifts to the final products derived from these environments, my aim is to determine the microbial composition and quality of ewe milk and sheep dairy products, with particular emphasis on powder-based goods that play a pivotal role in New Zealand's dairy industry. Importantly, this last research chapter serves as a critical link, providing a cohesive narrative that bridges the preceding chapters and offers insights into the parallels between the microbiota of the farm environment and that of raw milk and dairy products. This holistic approach aims to identify the microbes most crucial for sheep milk quality in New Zealand, thereby guiding future efforts through empirical evidence.

The manufacturing of powdered products is a key part of the dairy industry because of their long shelf life, convenience of use and complete nutritional profile. New Zealand is the leading exporter of dairy powders in the world, with 1.4 million tons exported between 2015 and 2020, well above the Netherlands, second at 180,000 tons, and Uruguay, third at 120,000 tons (and and Agriculture Organization of the United Nations, 1997). The bulk of these figures is accounted for by bovine dairy powders, but the developing New Zealand sheep dairy industry has followed the example of the local cow dairy production and set its focus on the manufacturing of sheep milk powders in majority intended for export to Asian markets (Peterson and Prichard, 2015).

Milk powders are an important food commodity that represent an easily accessible source of nutrition throughout the world, particularly in regions where fresh milk is not locally produced or available.

Ewe milk has been shown to contain higher levels of macronutrients (i.e., proteins and fat) and micronutrients (i.e., trace elements: vitamin B12, Ca and P) than cow milk (Park et al., 2007, Fantuz et al., 2016, Moatsou and Sakkas, 2019). Furthermore, evidence from recent in vitro (Roy et al., 2021), animal (Roy et al., 2022), and clinical trials (Shrestha et al., 2021), has brought further support to previous anecdotal observations reporting better digestibility of ewe milk as compared to cow milk, including in populations with specific nutritional requirements such as infants or the elderly (Anderson et al., 2010, Vojdani et al., 2018). These nutritional features make ewe milk a premium dairy product and thus, protecting the nutritional profile of sheep dairy products is crucial to preserve the quality and stability of these products and maintain their premium branding.

Powdered milk is considered microbiologically inert due to its low water activity which prevents the growth of microbes. Nonetheless, it contains contaminants, in majority spore-forming bacteria, that may originate from raw materials (i.e., raw milk) or through handling and processing. Bacterial spores can survive an array of environmental stresses, and in some instances, proliferate during processing or colonise the processing pipeline after attaching on its surface and forming biofilms, leading to contamination across processing runs (Murphy et al., 1999, Burgess et al., 2010). Although non-pathogenic, spore-forming bacteria, and more specifically thermophilic aerobic species, have become an important parameter for monitoring production hygiene in dairy powders (Murphy et al., 2021). As a result, raw materials and end-products are being increasingly monitored and subject to stringent microbial thresholds (McHugh et al., 2017).

Although a minority, other types of sheep dairy products are also produced and sold locally in New Zealand (e.g., pasteurised milk, specialty cheeses, yoghurt). These dairy products are routinely monitored for their aerobic plate count (APC), but their spoilage-associated microbiota remains largely undocumented, which may place them at some risk of microbial spoilage. An improved understanding of the microbiota of ewe milk, and sheep dairy products is required to assess the microbiological quality of the New Zealand sheep dairy production and to inform preventive actions aiming at safeguarding their quality from bacterial contamination and alteration.

There is a clear paucity of information pertaining to the microbiology of sheep milk products worldwide, possibly as they only represent a sizeable share of the dairy market in few countries (e.g., France, Spain, Italy, Greece). Special emphasis has been placed on the monitoring of *Clostridium* spp. in ewe milk and sheep milk cheeses to prevent late-blowing and other defects in the latter (Arias et al., 2013), while bacterial counts have also been incidentally reported for several groups of dairy

spoilage microbes such as psychrotrophic or thermoduric bacteria, without identification of the isolates (Jiménez-Sobrino et al., 2018, Tonamo et al., 2020).

Therefore, the objectives of this work were to study the microbiological quality of sheep milk and powder-based dairy products produced in New Zealand. Organisms of interest, including spore-forming bacteria capable of growing at several temperatures (mesophiles, psychrotrophs and thermophiles) and with or without oxygen, but also *Pseudomonas* species, were enumerated and isolated from the samples and were further characterised.

7.2 Results and discussion

This chapter describes the results of the microbiological analysis of samples consisting of milking cups (n = 1), ewe raw milk (n = 4) and powder-based sheep dairy products (n = 2). Although initially included in the original research plan, the analysis of these sample types was abandoned after the scope of the project was revisited. Thus, the analysis of some samples remains partially incomplete in some instances (i.e., raw milks). Nonetheless, these results provide a snapshot of the cultivable microbiota of selected sheep milk and dairy products, which provides a basis for comparison with the bovine and ovine dairy production of other countries, and the previously described New Zealand sheep dairy farm microbiota.

Milking cups and raw milk samples originated from four sheep dairy farms, for which a more comprehensive description is available in Chapter 3 (section 3.1.1). Briefly, Farm 2 (F2, pasture-based) and Farm 3 (F3, mixed housing/grazing) were under the same management, although they were operating with different farming systems. Similarly, Farm 4 (F4, mixed housing/grazing) and Farm 5 (F5, pasture-based) operated under the same management, but with different farming systems. For each farm, raw milk samples were collected simultaneously to the milking cups samples. A culturomics approach similar to that used in Chapters 4 and 5 (described in section 3.1.3) was applied for the isolation of bacteria from the different samples, although there were slight variations according to the sample type that was analysed (see Table 7.1).

Table 7.1. Culture conditions used for the bacterial isolation

Target bacteria	Pre-treatment	Incubation conditions	Milking cups	Raw milk				Dairy product	
			F5	F2	F3	F4	F5	A	B
Mesophilic spores	80 ± 1°C 10 min	35°C SBA Atmospheric conditions 24-48 h	×	×	×	×	×	×	×
Thermophilic spores		60°C SBA and MA Atmospheric conditions 48 h	×	×	×	×	×	×	×
		65°C SBA and MA Atmospheric conditions 48 h	×						
Psychrotrophic spores		7°C SBA Atmospheric conditions 10 days	×	×	×	×	×		×
Clostridium s.l.		CMG 35°C 48h AnO_2 EYA 35°C 24 h AnO_2	×	×	×	×	×	×	×
Mesophiles	None	35°C SBA Atmospheric conditions 24 h		×	×	×	×	×	×
Thermophiles		65°C SBA Atmospheric conditions 48 h						×	×
Pseudomonas spp.		20°C CFC Atmospheric conditions 48 h	×	×	×	×	×		

F2-F5: Farm 2 to 5; SBA: Columbia sheep blood agar; MA: Milk agar; CMG: Cooked meat glucose broth; EYA: Egg yolk agar; CFC: Cetrimide Fucidin Cephalosporin agar; AnO_2: Anaerobic atmosphere. Crosses indicate that a given culture condition was employed across selected samples.

7.2.1 Milking cups: possible point of entry of environmental bacteria into raw milk

Milking cups represent the interface where most farm-borne contaminants cross into raw milk. Analysis of this sample provides an estimate of the environmental contamination occurring during milking, whilst overlooking the bacteria entering raw milk through the milking pipeline, or during its storage and transport (Tonamo et al., 2020).

The spore counts recorded in the milking cups of F5 were relatively low across the different detection methods (Table 7.2). Mesophilic and psychrotrophic spore counts were similar (~ 2.6 $\log_{10}$ cfu/swab), and 1 to 2 $\log_{10}$ lower than those recorded in the milking cups of F1 and F2 (see Chapter 4 & 5). Surprisingly, thermophilic spores were not detected in the milking cups of F5, after incubation at both 60 and 65°C (detection limit < 0.78 $\log_{10}$ cfu/swab). Previously, I recorded low thermophilic spore counts in the milking cups of F2, as well as in samples associated with the grazing environment of dairy ewes collected from the same farm (< 4 $\log_{10}$/unit, see Chapter 5). In contrast, samples collected on the indoor F1 had high thermophilic spore counts in the environment and milking cups (> 5 $\log_{10}$/unit, see Chapter 4). It is plausible that components associated with the farm environment of F5 (i.e., pasture, feed, faeces, etc) harboured, like those of the other outdoor farm, low spore counts. Few studies have reported lower bacterial and bacterial spore counts in ovine and bovine milk produced from pasture-based, so-called organic dairy farms, as compared to farms relying predominantly on housing, or so-called conventional dairy farms (Coorevits et al., 2008, Malissiova et al., 2017). This effect may be caused by varying hygienic practices during milking, as mentioned by other authors (Malissiova et al., 2017). However, my results have suggested that the environment of pasture-based dairy farms could harbour particularly low spore counts, which may in turn lead to the transfer of fewer spores to milking cups, and possibly to raw milk, on these farms. The analysis of environmental samples and milk from additional outdoors and indoors sheep dairy farms will be required to further probe this hypothesis. Should the milk produced on pasture-based sheep dairy farms be associated with lower spore counts, milk production from these farms may be particularly adapted for use in the manufacturing of dairy products typically at risk of contamination and spoilage by spore-forming bacteria, i.e., most heat-treated products. However, *Pseudomonas* counts in the miking cups of F5 were lower than that of the other 2 farms (Table 7.2), which suggests that the general contamination rate of the milking cups on F5 was lower than on F1 and F2 at the time considered.

Table 7.2 Bacterial counts in milking cups and raw milk from sheep dairy farms, as well as in ovine dairy products

Bacteria type	Raw milk[1]				Dairy products[2]		Milking cups[3]		
	Farm 2	Farm 3	Farm 4	Farm 5	A	B	Farm 1	Farm 2	Farm 5
Mesophilic spores	3.2	5.53	3.48	3.68	2.06	2	4.53	3.4	2.66
Thermophilic spores 65°C (SBA)	-	-	-	-	-	-	3.22	2.83	< 0.78
Thermophilic spores 65°C (MA)	-	-	-	-	-	-	-	2.26	< 0.78
Thermophilic spores 60°C (MA)	< 3.20	< 3.45	<2.74	<2.78	-	-	4.15	2.97	< 0.78
Thermophilic spores 60°C (SBA)	3.2	4.45	<2.74	2.96	< 0.52	< 0.81	4.12	2.46	< 0.78
Psychrotrophic spores	3.51	4.94	2.74	3.32	-	1.85	3.61	3.05	2.66
Pseudomonas	9.12	8.99	6.6	7.66	-	-	7.44	8.51	6.94
Total bacteria count	9.87	9.04	6.37	7.6	2.15	-	-	-	-
Thermophilic counts	-	-	-	-	0.52	1.63	-	-	-

[1] $\log_{10}$ CFU/L
[2] $\log_{10}$ CFU/g
[3] $\log_{10}$ CFU/swab

Bacterial counts in the milking cups samples of Farm 1 (see Chapter 4) and Farm 2 (see Chapter 5) are also presented for comparison. Cells with a "-" indicate culture conditions that were not used on a given sample. Cells containing "<" indicate that the bacterial count was below the detection limit, which is stated for each sample.

A total of 96 isolates were recovered from the milking cups swabs, and the majority (n = 78) were screened for spoilage potential. The identification approach was similar to that described in Chapters 4 & 5 and mainly focused on bacteria with spoilage potential. In total, 66 isolates were identified, they were distributed across 12 different genera and 24 species of bacteria. The predominant bacteria in the milking cups of F5 consisted of the mesophilic spore-formers *Bacillus cereus s.l.*, *Psychrobacillus* spp. and *Solibacillus* spp. (Figure 7.1A), the psychrotrophic spore-formers *Psychrobacillus* spp. and *Peribacillus psychrosaccharolyticus* (Figure 7.1B), *Pseudomonas libanensis*, *P. protegens* and *P. fragi* (Figure 7.1C), as well as the anaerobic spore-formers *Clostridium moniliforme* and *C. perfringens* (Figure 7.2). Isolates of mesophilic spore-formers and *Pseudomonas* spp. had the strongest spoilage potential, while psychrotrophic spore-formers and *Clostridium* spp. demonstrated weak spoilage potential (Figure 7.3). There were many bacterial species found to exhibit strong spoilage potential (Figure 7.2), or which had been previously

documented as important dairy spoilers (De Jonghe et al., 2010, Sadiq et al., 2016b, Yuan et al., 2018b), including *B. cereus s.l.*, *B. licheniformis*, *B. pumilus s.l.*, *Alkalihalobacillus clausii*, *Paenibacillus* spp., *Pseudomonas* spp. and *C. perfringens*.

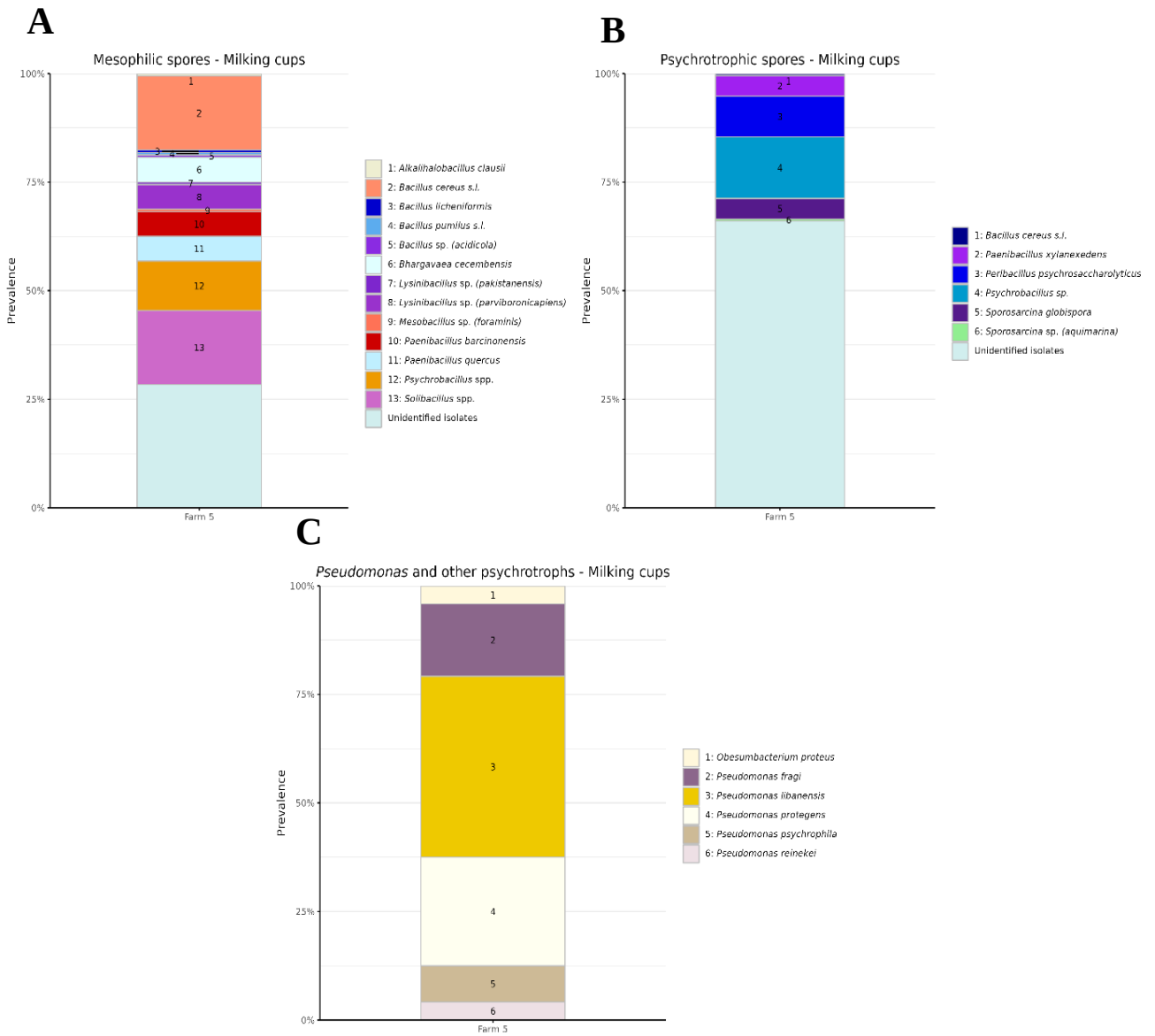


Figure 7.1. Relative abundance of mesophilic, psychrotrophic spores, and *Pseudomonas* spp. in milking cups of F5

Coloured rectangles with a number represent the relative abundance of a bacteria in a given sample, the numbers referring to bacterial species in the legend. The proportion of unidentified isolates, if any, is visible as pale turquoise rectangles on the bottom of each bar graph. The isolates referred to as "sp." could not be confidently attributed to a type strain species based on their partial 16S rRNA gene sequence and are instead associated with the most closely related species.

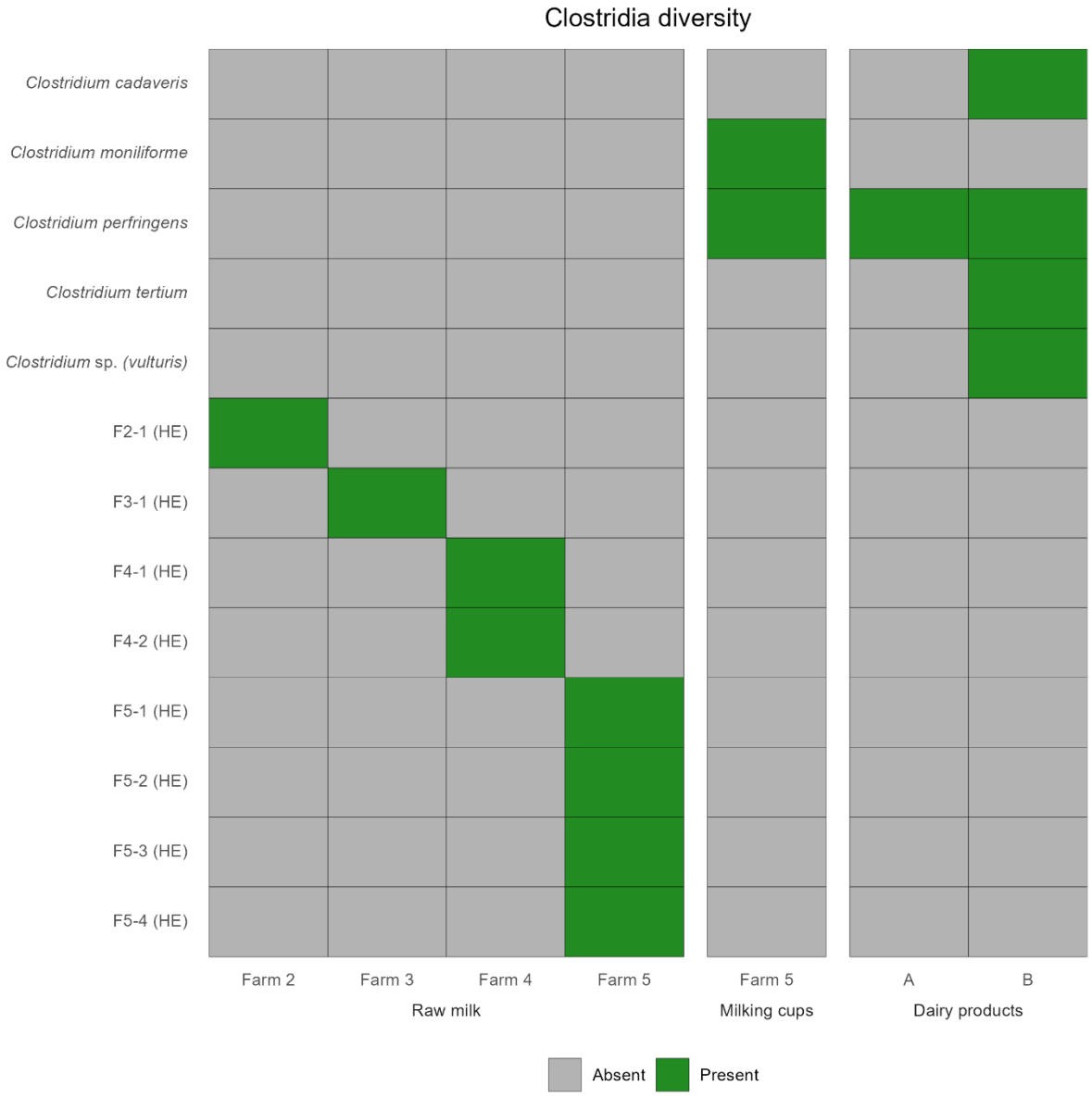


Figure 7.2. Clostridial diversity in milking cups and raw milk collected on sheep dairy farms, as well as in ovine dairy products, following cooked meat glucose starch broth (CMG) enrichment

The detection of at least one isolate of a bacteria in a given sample is pictured as green squares while grey squares indicate the absence of detection of the organism. For milking cups and dairy products, representative isolates identified by partial 16S rRNA gene sequencing and compared with sequences in the RDP depository. For raw milk samples, isolates remained unidentified and were given generic designations (e.g., F3-1).

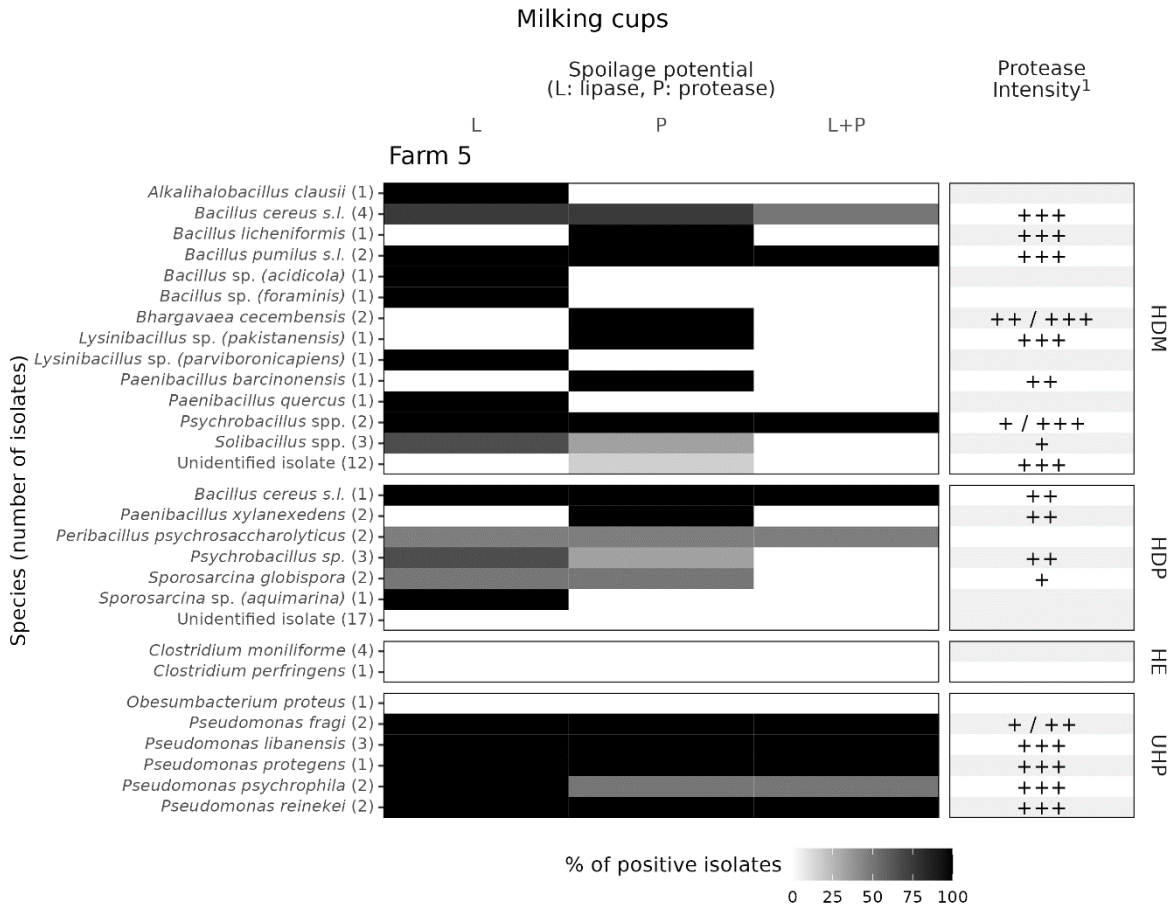


Figure 7.3. Spoilage potential of the bacteria isolated from the milking cups of F5

For a given species or cluster, the proportion of isolates positive for lipase and/or protease activity is visible in shades of grey. HDM = mesophilic spore-formers; HDP = psychrotrophic spore-formers; HE = anaerobic spore-formers; UHP = *Pseudomonas* and other psychrotrophs.

[1]The intensity of the protease activity was ranked as weak (+, weak casein hydrolysis), medium (++, casein hydrolysis only directly under the colony) or strong (+++, diffused casein hydrolysis around the colony). The protease intensity displayed for each species or cluster corresponds to the most common protease intensity.

These results bring further support to some of the hypotheses mentioned in the previous chapters and in the literature. Specifically, these findings suggest that detection of *B. cereus s.l.* may be more likely to occur in samples collected in association with the grazing environment of dairy ewes, which aligns with findings on bovine dairy farms (Christiansson et al., 1999). Other bacteria such as *B. licheniformis*, *B. pumilus s.l.* and *Alkalihalobacillus clausii* were also detected but they were present at low relative abundance. Their detection aligns with previous detection of these bacteria on bovine dairy farms (Buehner et al., 2014, Miller et al., 2015b), and specifically highlights sheep dairy farms as a possible source of entry of these ubiquitous contaminants into raw milk and dairy products. *Clostridium perfringens* and *C. moniliforme* were the only anaerobic spore-formers that were detected

in the milking cups of F5 after CMG enrichment (Figure 7.2). Previously, I found *C. perfringens* to be particularly prevalent in samples associated with both indoor and outdoor sheep dairy farms, including milking cups (see Chapter 4 & 5). This corroborates the findings of the Drean et al. (2015) who found these bacteria to be highly prevalent in the farm environment of Australian bovine, ovine and caprine dairy farms. *Pseudomonas* spp. were successfully isolated using CFC agar and there was only one isolate that did not belong to this genus (*Obesumbacterium proteus*) and was likely detected because of natural antibiotic resistance (Farmer and Brenner, 2015). The two *Pseudomonas* spp. found to be predominant in the milking cups of F5 were *P. libanensis* and *P. fragi* (Figure 7.1C). These bacteria, as well as all other *Pseudomonas* spp., demonstrated strong spoilage potential producing both lipases and proteases (Figure 7.3). *Pseudomonas libanensis* and *P. fragi* were previously isolated on both F1 and F2 (see Chapter 4 & 5), indicating they are likely to represent some of the predominant *Pseudomonas* present on sheep dairy farms, and perhaps in ewe milk. *Pseudomonas libanensis* was particularly abundant in soil collected from F2 as well as in the milking cups collected on F1. Although reports of these bacteria are rare in the dairy context, they belong to the *P. fluorescens* subgroup and can produce biofilm, pigments, and potent hydrolytic enzymes, indicating strong spoilage potential (Cantoni et al., 2006, Yuan et al., 2018a). Other contaminants included members of the *P. fragi* subgroup such as *P. fragi*, *P. weihenstephanensis* and *P. psychrophila*, which have been repeatedly associated with dairy spoilage (Marchand et al., 2009, Marchand et al., 2017) and were also abundant in samples collected from F1 and F2, with faeces identified as a potential source of these bacteria.

7.2.2 Microbiological quality of sheep raw milk produced in New Zealand

After entering raw milk, the removal of bacterial contaminants, or of their metabolites and enzymes, becomes particularly challenging. *Pseudomonas* present in raw milk are efficiently removed by processing, but it is in the bulk tank milk maintained at low temperature on farm that these bacteria may proliferate, secrete compounds associated with dairy spoilage (e.g. spoilage enzymes including thermostable enzymes, pigments), and thereby impact the quality of raw milk before it leaves the farmgate (Capodifoglio et al., 2016, Tribst et al., 2019). As both vegetative cells and endospores in raw milk, spore-formers can colonise dairy manufacturing pipelines, with the latter form allowing them to withstand stringent conditions during processing (Flint et al., 1997). Previous work in the bovine dairy production has identified key differences across the predominant bacterial species found

in the microbiota of raw milk and in that of dairy products (Miller et al., 2015b). This may be partially explained by the fact that additional contamination can occur during processing, but also by the fact that the microbiota of raw milk shifts throughout the manufacturing of dairy products as it is shaped by the conditions of processing (Postollec et al., 2012, McHugh et al., 2020). Thus, the microbial quality of raw milk contributes hugely to that of dairy products because some of the contaminants or the compounds they produce, may be carried over from raw milk. Monitoring the spoilage microbiota of ewe raw milk may help predict potential microbial threats to the quality of sheep dairy products.

In this study, the analysis of raw milk samples provided, for the first time, insights into the microbiological profile of ewe milk produced in New Zealand. The raw milk bulk tank from F2 and F3 contained a mix of raw milk from the same day as well as from the day before, while the raw milk bulk tank from F4 and F5 contained only fresh milk from the day. On all farms, target cooling temperature of the bulk tank was 4°C, to be reached within 2h from the completion of milking. The microbiological quality of the different raw milk samples was found to vary according to the type of bacteria considered (i.e., *Pseudomonas* spp., mesophilic, thermophilic or psychrotrophic spore-formers), but also across four different farms. According to their aerobic plate count (APC) at 35°C, raw milk samples were grouped in two categories, which corresponded to raw milk produced on farms under the same management, namely from F4 and F5 (6.34 and 7.60 $\log_{10}$ cfu/L, respectively, Table 7.2), or from F2 and F3 (9.87 and 8.99 $\log_{10}$ cfu/L, respectively). When comparing farms under the same management, the highest APC were recorded in milks produced from pasture-based farms, i.e., F2 and F5. In New Zealand, recent legislation has set the limit for APC in sheep raw milk at 8.00 $\log_{10}$ cfu/L (Ministry for Primary Industries, 2022). According to European ovine dairy processing guidelines, the APC of sheep raw milk should not exceed 9.18 $\log_{10}$ cfu/L when it is meant to be pasteurised, and 8.70 $\log_{10}$ cfu/L when its processing does not involve heat treatments, such as when consumed as such, or used for the manufacturing of raw milk cheeses (European Parliament, 2004). Milks from F4 and F5 had low APC as compared to previous studies, while those from F2 and F3 were similar to these reports (de Garnica et al., 2013, Jiménez-Sobrino et al., 2018, Tonamo et al., 2020). Worldwide, the dairy microbial thresholds set by food legislative bodies constitute an umbrella approach to encourage hygiene throughout farming and particularly during milking, but the bacteria that are isolated as part of the APC (i.e., vegetative cells of mesophilic bacteria) are not directly associated with milk quality. Under suitable conditions of transport, storage and processing, most of the mesophilic microbiota of raw milk should not be able to proliferate, and should, in majority, be subsequently inactivated by processing. Nonetheless, as shown by some studies, the APC of sheep

milk has been shown to increase during refrigerated storage, even at 4°C (Zweifel et al., 2005, de Garnica et al., 2011), indicating that some mesophilic bacteria may also be psychrotrophic. The main genus of bacteria capable of proliferating in raw milk stored at low temperature is *Pseudomonas*. In my study, the distribution of the *Pseudomonas* counts across the different milks was similar to that of the APC and they were separated in two groups according to the farm management, and with either high (F2 and F3; 9.12 and 9.02 $\log_{10}$ cfu/L, Table 7.2), or low CFC counts (F4 and F5; 6.61 and 7.66 $\log_{10}$ cfu/L). In the literature, reports on the concentrations of *Pseudomonas* in ewe milk are scarce and variable: 6.63 $\log_{10}$ cfu/L recorded by Bruzaroski et al. (2020) in Brazil, and 8.79 $\log_{10}$ cfu/L as reported by Salmerón et al. (2002) in Spain. In bovine raw milk, average *Pseudomonas* cell counts ranging between 5 to 8 $\log_{10}$ cfu/L have been reported (Leriche and Fayolle, 2012, von Neubeck et al., 2015, Skeie et al., 2019). Several other studies have recorded average psychrotrophic vegetative cell counts in ewe milk, amongst which *Pseudomonas* are often predominant, ranging between 8.25 to 9.40 $\log_{10}$ cfu/L (Zweifel et al., 2005, de Garnica et al., 2011, de Garnica et al., 2013, Jiménez-Sobrino et al., 2018). Based on these reports, my results suggest that the levels of *Pseudomonas* spp. in milk produced on F4 and F5 were on the lower end of other reports from the literature. However, milks collected from F2 and F3 contained particularly high concentrations of these bacteria, which may place them at risk of psychrotrophic spoilage. In the study by Zweifel et al. (2005), the median for psychrotrophic cell counts in sheep milk was particularly lower than the average (7.79 $\log_{10}$ cfu/L vs 9.05 $\log_{10}$ cfu/L), indicating the presence of individual milk samples with high bacterial concentrations (up to 10.26 $\log_{10}$ cfu/L in that same study). The values I report in the study represent samples from a unique timepoint and only few farms, whereas other authors generally report these counts as the average of a larger number of individual samples with variations in their microbial content [e.g. 5.30 to 10.26 $\log_{10}$ cfu/L (Zweifel et al., 2005)]. However due to the timing of sampling and the lack of access to sheep dairy farms and ewe milk, additional milk samples could not be analysed in my work, but they would have allowed for comparison across additional farms and seasons. With the growing number of sheep dairy farms in New Zealand it would be interesting, and will become possible, to monitor the microbiological quality of the milk production from different farms. This data may be linked with individual farm metadata to identify potential associations between farm practices or farming systems, and different counts of bacteria in sheep milk, similar to the work of Martin et al. (2019) on bovine dairy farms.

Markedly high APC and *Pseudomonas* counts were recorded in milks from F2 and F3, as compared to those from F4 and F5. The bulk tank on F2 and F3 contained both fresh milk as well as milk from the previous day, a practice that is common on sheep dairy farms and which aims at pooling the farm

production for collection (Tribst et al., 2019). In comparison, bulk tank from F4 and F5 contained only fresh milk from the day at the time of sampling. This suggests that bacteria were able to proliferate during storage of raw milk on F2 and F3. Storage of raw milk, even at low temperatures, can increase its bacterial content as some bacteria proliferate during refrigeration (de Garnica et al., 2011, Tribst et al., 2019). Bruzaroski et al. (2020) found that sheep raw milk stored at 4 and 9°C for 24 h had comparable concentrations of *Pseudomonas* (3.14 to 3.73 $\log_{10}$ cfu/L), but that after 72 h, the *Pseudomonas* counts in the milk stored at 9°C had increased a thousandfold, whereas those in the milk stored at 4°C remained similar (3.91 $\log_{10}$). In contrast, several other works have reported that *Pseudomonas* populations present in bovine raw milk could grow by 3.5 $\log_{10}$ cfu/L in 24 h at 4°C (De Jonghe et al., 2011, Giacometti et al., 2016, Lin et al., 2016). A similar increase (3.5 $\log_{10}$ cfu/L of *Pseudomonas* in raw milk in 24 h at 4°C) during storage of ewe raw milk would indicate that initial *Pseudomonas* counts in fresh raw milk on F2 and F3 may have been in the vicinity of 5.50 to 7.00 $\log_{10}$ cfu/L, which is consistent with the concentrations of *Pseudomonas* recorded in fresh raw milk produced on F4 and F5 (6.61 and 7.66 $\log_{10}$ cfu/L, respectively). These results indicate that the storage of sheep raw milk at 4°C for extended periods of time may represent a quality risk by favouring the growth of psychrotrophic organisms, specifically *Pseudomonas*, which can subsequently impact the quality of sheep dairy by degrading some of its nutrients. However, different species of bacteria can exhibit heterogenous growth capabilities despite being members of the same genus [e.g., *Pseudomonas* (Moreno and Rojo, 2014)]. In addition, milk composition varies across animal species (Roy et al., 2020), and the higher nutrient content of sheep milk may also affect the dynamics of microbial growth. Therefore, the psychrotrophic growth capabilities of the individual contaminants found in New Zealand sheep milk should be evaluated to identify the local bacterial species that are most likely to proliferate during refrigeration of ewe raw milk on farm.

The APC is the technique most commonly employed to monitor milk microbiological quality, but the bacteria that are targeted by this detection method are often not implicated in dairy spoilage and are rather more reflective of the overall hygiene of the farm production. Further, the monitoring of psychrotrophic cell counts in raw milk provides information about the (pre-processing) stability of milk at refrigeration temperatures, but it does not provide information on the thermoduric microbiota of raw milk that may remain after heat processing, e.g., spore-forming bacteria.

Spore counts in raw milk from F2, F4 and F5 were similar, while those recorded in raw milk from F3 were higher. Specifically, raw milk spore counts were similar across both farms, and detection methods, for F2, F4 and F5, including for mesophilic (3.20 to 3.68 $\log_{10}$ cfu/L, Table 7.2),

thermophilic (2.70 to 3.20 $\log_{10}$ cfu/L), and psychrotrophic spore counts (2.74 to 3.93 $\log_{10}$ cfu/L). Thermophilic spores were not detected at levels above the detection limit in raw milk from F4 (< 2.74 $\log_{10}$ cfu/l). Spore counts in the milk collected from F3 were higher by a factor 1 to 2 $\log_{10}$ cfu/L across all three spore detection methods: 5.54, 4.43 and 4.68 $\log_{10}$ cfu/L for mesophilic, thermophilic and psychrotrophic spores, respectively. To the best of my knowledge, this work represents the first study that specifically enumerated aerobic bacterial spores in sheep raw milk. Few other authors have enumerated thermoduric bacteria (which include spores but also other thermoduric bacteria such as some *Micrococcus* spp.) in ewe milk, with a reported average of around 6 $\log_{10}$ cfu/L, although counts of individual farm milk samples were particularly variable [range < 3 $\log_{10}$ cfu/L to 8 $\log_{10}$ cfu/L (de Garnica et al., 2013, Jiménez-Sobrino et al., 2018)]. The spore content of bovine raw milk has been far more documented than that of ewe milk, and a high variability in spore counts has also been reported worldwide as a result of varying farming practices and systems. Over a calendar year, McGuiggan et al. (2002) performed a weekly assessment of spore counts in bovine milk produced on Irish dairy farms and recorded concentrations ranging between 3.15 to 8.38 $\log_{10}$ cfu/L (average: 6.88 $\log_{10}$ cfu/L) for mesophilic spores, 0.90 to 4.70 $\log_{10}$ cfu/L (average: 3.60 $\log_{10}$ cfu/L) for thermophilic spores, and 1.30 to 3.54 $\log_{10}$ cfu/L (average: 2.90 $\log_{10}$ cfu/L) for psychrotrophic spores. In the four milks sample I analysed, mesophilic spores counts were lower by up to a 3-$\log_{10}$ factor than those reported by McGuiggan et al. (2002). On bovine dairy farms, Martin et al. (2019) recorded low average bulk tank milk spore counts that were similar to my findings: 3.50, 3.36 and 2.76 $\log_{10}$ cfu/L for mesophilic, thermophilic and psychrotrophic spores, respectively. My results suggested that the concentration of spores in ewe raw milk from F2, F4 and F5 was on the lower end of the range reported in the literature for bovine raw milk, and particularly for mesophilic spores. Higher spore counts in raw milk collected from F3 suggest that increased spore contamination of milk may be taking place on this farm. Contamination of raw milk by spore-formers can occur in the milking pipeline, however, with adequate monitoring and washing of the milking pipeline, the farm environment represents the main source of raw milk contaminants. Although F2 and F3 were under the same management and thus shared common milking practices, the rate of entry of bacteria into raw milk may have differed as a result of varying farming systems and practices. Specifically, F3 represented the sheep dairy operation on which the animals were housed the most throughout the year, across all farms (45% of the year spent housed, as compared to 10% on F4). These results complement my previous findings by suggesting that the concentration of bacterial spores in the farm environment but also in raw milk may be higher on sheep dairy farms with a housing component. This hypothesis aligns well with the findings of several studies in the bovine farm environment that

have reported higher spore contamination in milk produced by barned animals (Vissers et al., 2007e, Coorevits et al., 2008). Further assessment of this hypothesis will be key to providing guidance to farmers on the general effect of farming systems on ewe milk quality. This will require the sampling and microbial analysis of milk and environmental samples from additional pasture-based sheep dairy farms and from farms with various housing frequency.

Bacteria were isolated from the raw milk enumeration plates for aerobic mesophilic, thermophilic, psychrotrophic and anaerobic mesophilic spore-formers, as well as *Pseudomonas* species. Although I was not permitted to formally identify the isolates recovered from raw milk, due to stakeholder sensitivities, I used morphological features of the bacterial colonies and their ERIC-PCR profiles to assign the different isolates to putative bacterial species in order to produce relative abundance charts. The populations of spore-formers in raw milk samples showed high diversity. Mesophilic spore-formers were particularly diverse (average species richness = 11.5, Figure 7.4) as compared to thermophilic spore-formers (average species richness = 1.3, Figure 7.5), psychrotrophic spore-formers (average species richness = 5.5, Figure 7.6), *Pseudomonas* spp. (average species richness = 6.3, Figure 7.7) and anaerobic spore-formers (average species richness = 2, Figure 7.2).

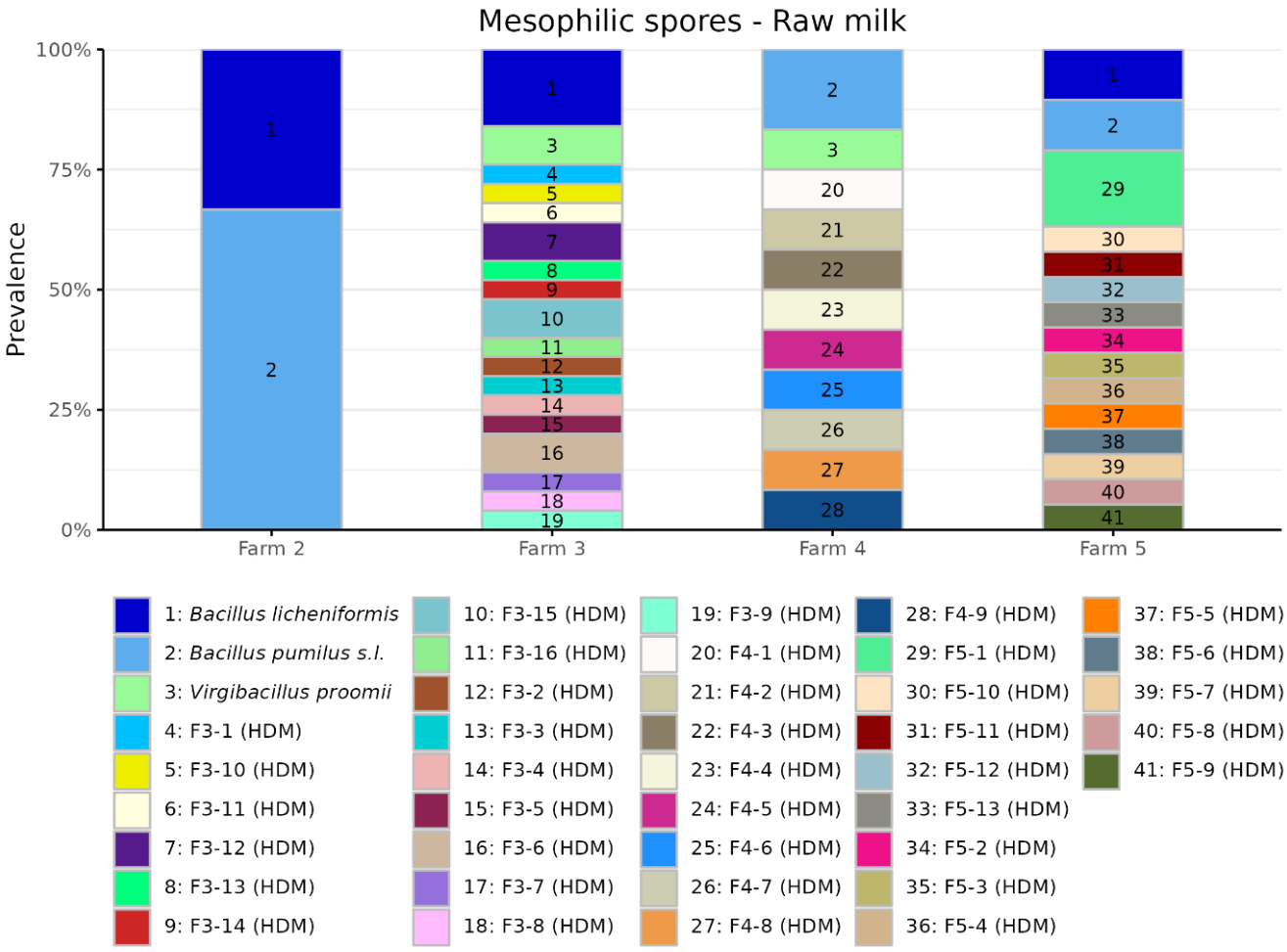


Figure 7.4. Diversity of mesophilic spores in ewe raw milk.

Coloured boxplots with a number represent the relative prevalence of a bacteria in a given sample. Isolates corresponding *to Bacillus licheniformis, B. pumilus s.l.* and *Virgibacillus proomii* could be identified based on morphological features, while other isolates remained unidentified and were given generic designations [e.g., F3-1].

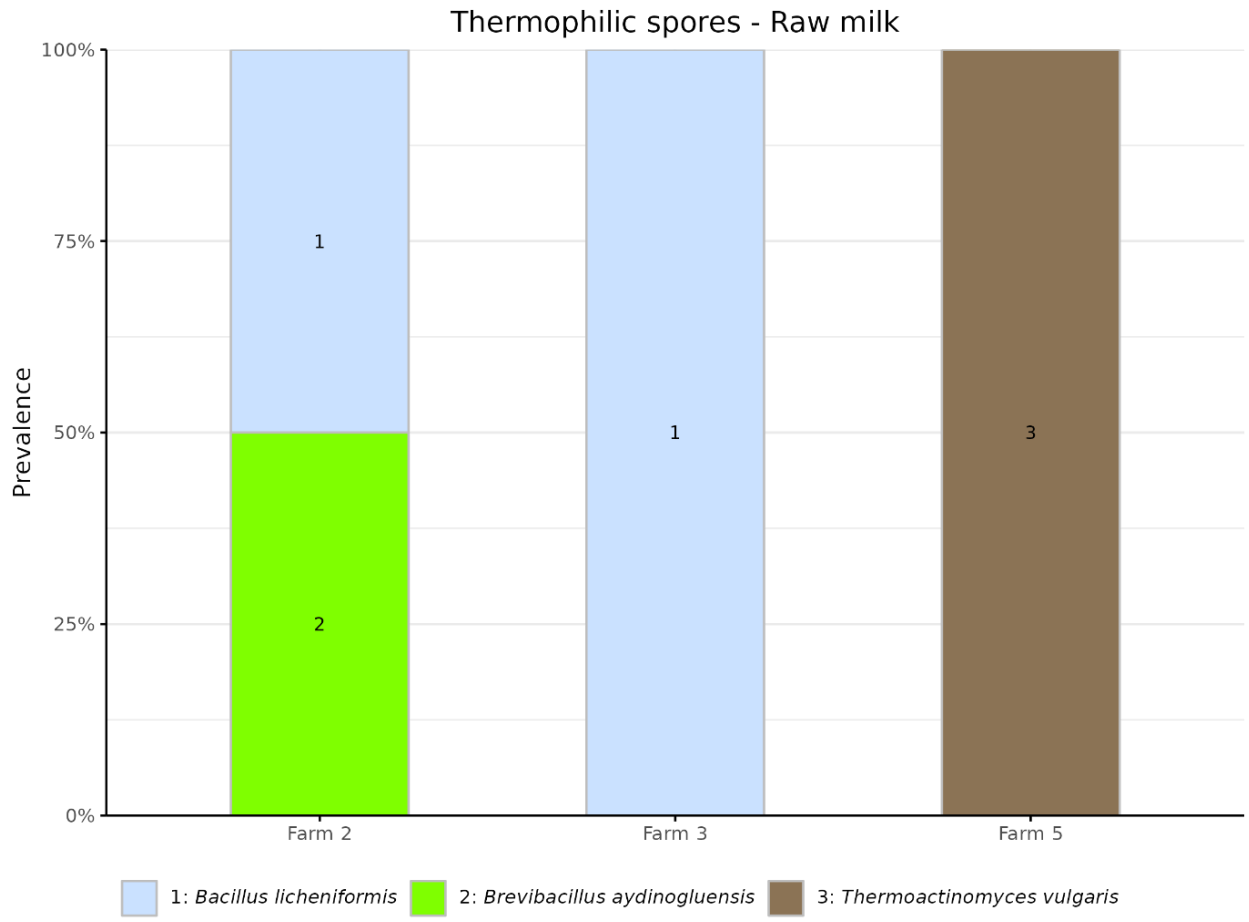


Figure 7.5. Diversity of thermophilic spores in ewe raw milk.

Coloured boxplots with a number represent the relative prevalence of a bacteria in a given sample. *Bacillus licheniformis*, *Thermoactinomyces vulgaris* and *Brevibacillus aydinogluensis* could be identified based on morphological features. There were no thermophilic bacteria detected in raw milk from F4.

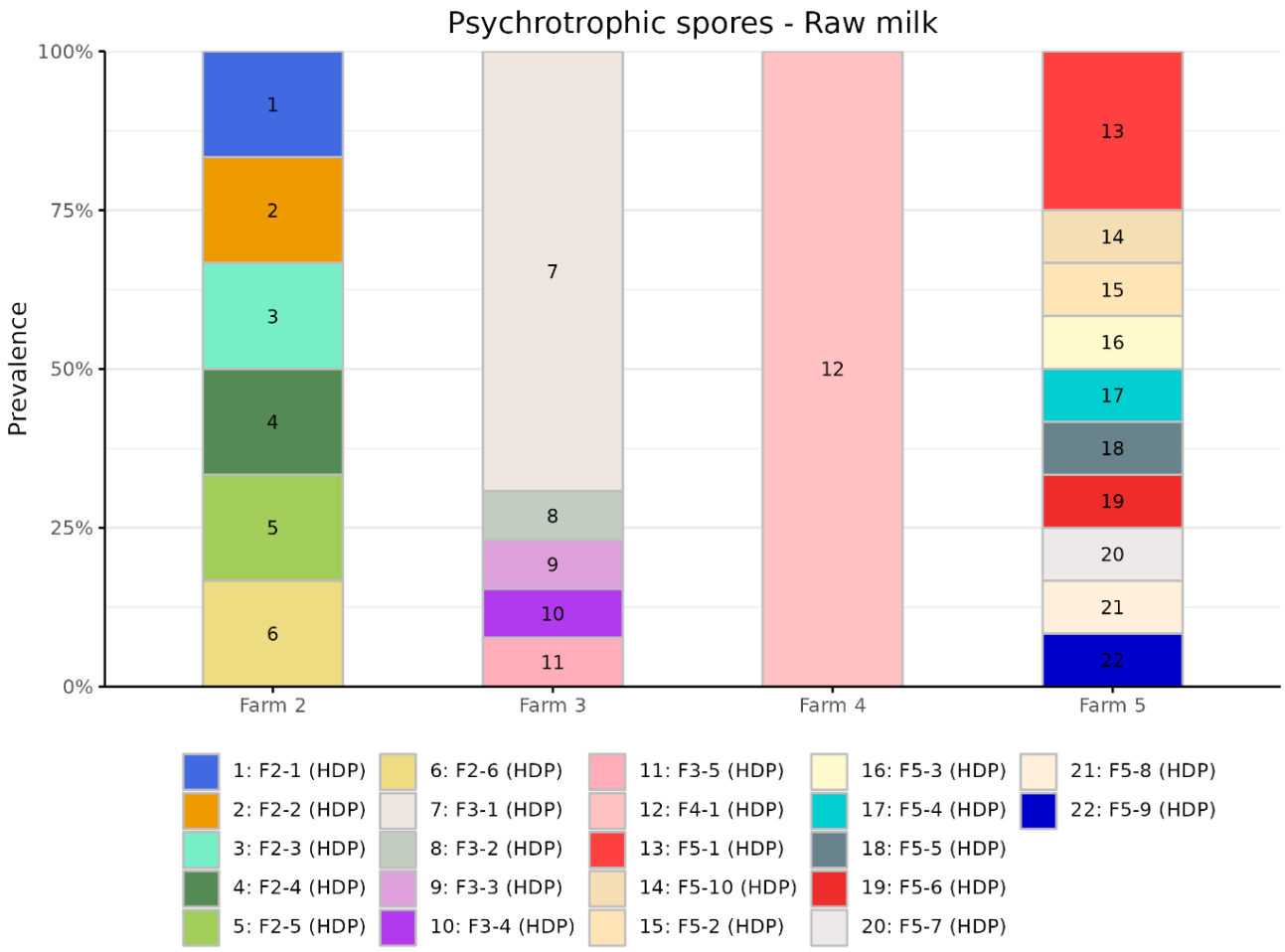


Figure 7.6 Diversity of psychrotrophic spores in ewe raw milk.

Coloured boxplots with a number represent the relative prevalence of a bacteria in a given sample. The isolates could not be identified based on their morphology and were given generic designations [e.g., F2-1(HDP)].

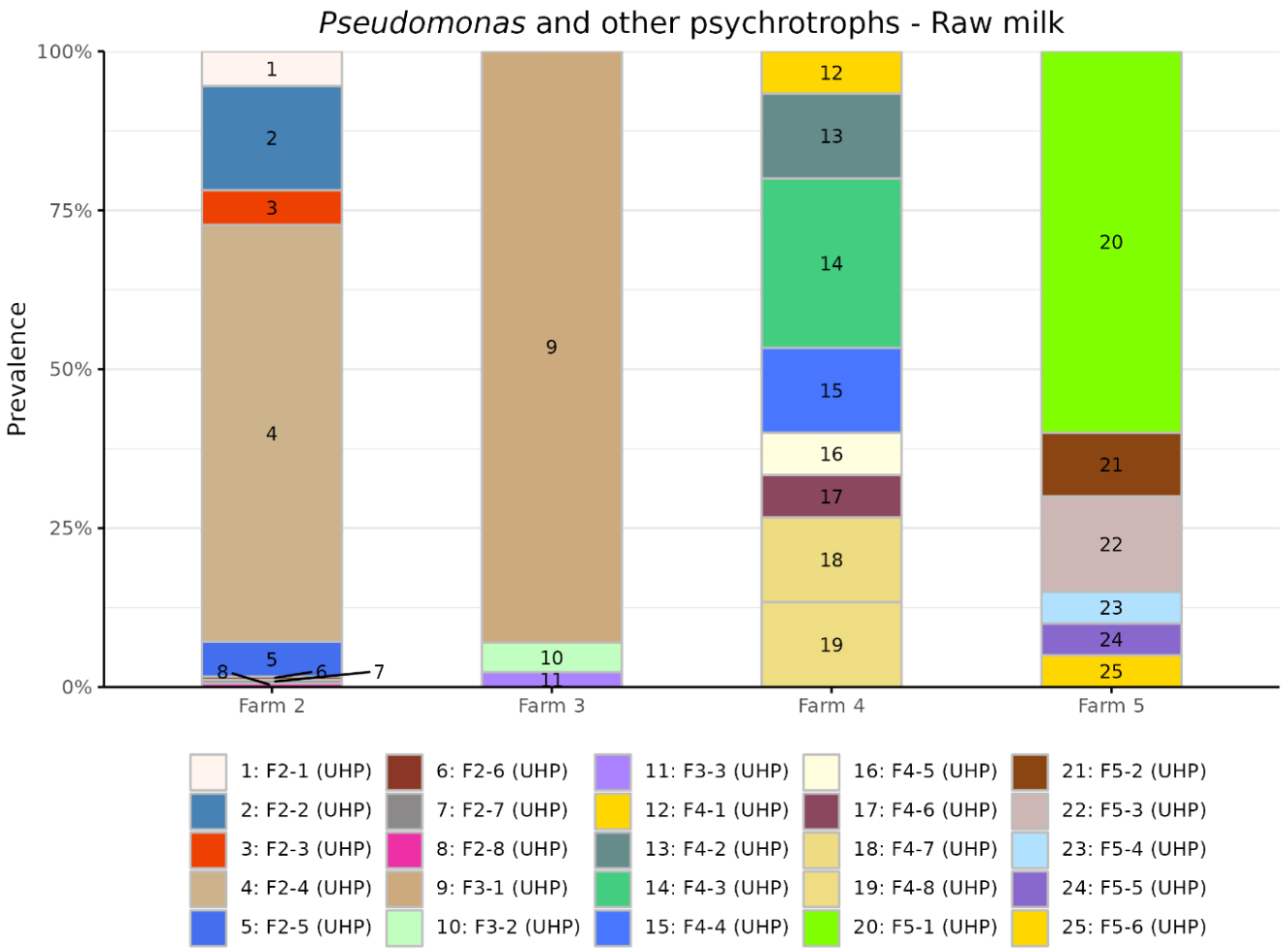


Figure 7.7 Diversity of *Pseudomonas* spp. and other psychrotrophs in ewe raw milk.

Coloured boxplots with a number represent the relative prevalence of a bacteria in a given sample. The isolates could not be confidently identified based on their morphology and were given generic designations (e.g., F2-1)

Plate-based assays were used to screen 269 selected representative raw milk isolates for protease and lipase production. The results showed that spoilage potential was particularly common across the raw milk isolates (Figure 7.8). Overall, 73% of the bacteria produced proteases, 51% produced lipases, and 48% produced these enzymes simultaneously. However, spoilage potential varied across the bacteria obtained from different detection methods. Mesophilic and thermophilic spore-formers were often proteolytic. In contrast, psychrotrophic spore-formers frequently lacked any spoilage potential. These results are consistent with my previous findings which showed that proteolytic activity is often more common than lipolytic activity in spore-forming bacteria.

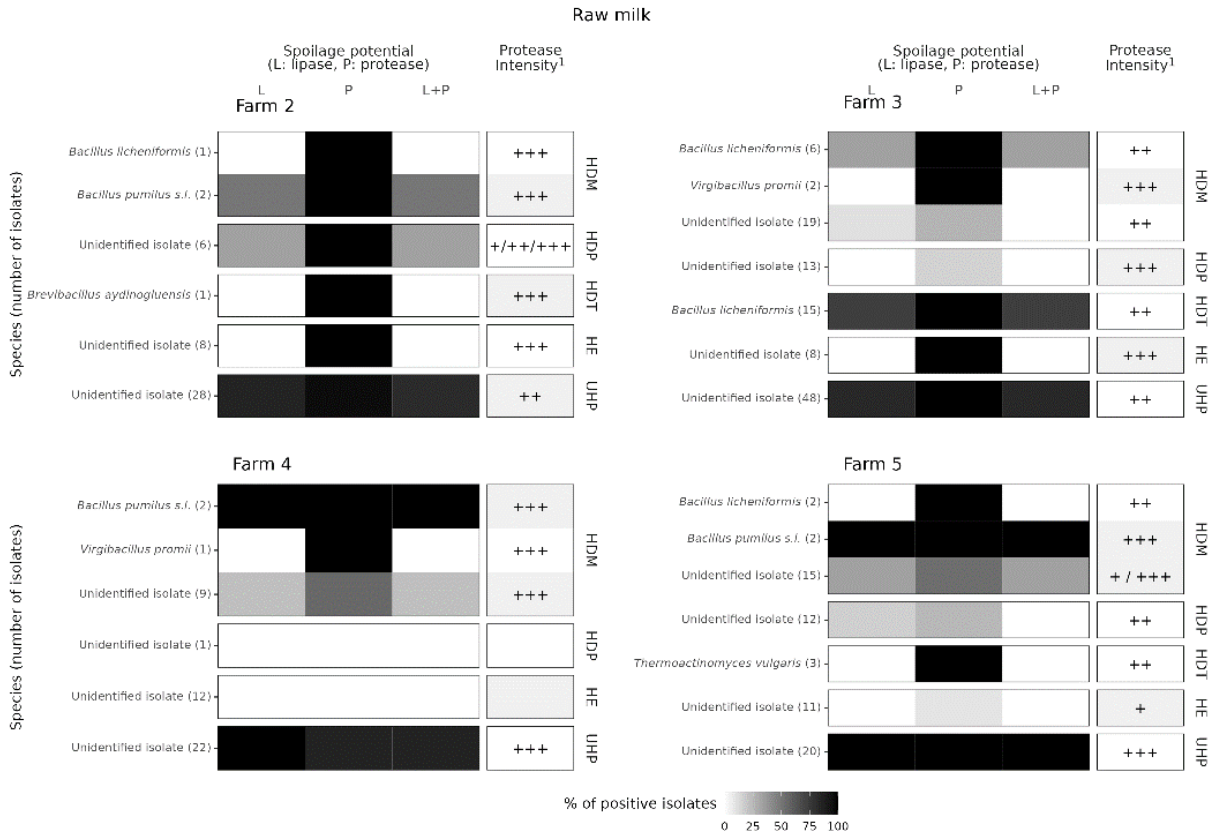


Figure 7.8 Spoilage potential of the bacteria isolated from ewe raw milk

For a given species or cluster, the proportion of isolates positive for lipase and/or protease activity is visible in shades of grey. HDM = mesophilic spore-formers; HDT = thermophilic spore-formers; HDP = psychrotrophic spore-formers; HE = anaerobic spore-formers; UHP = *Pseudomonas* and other psychrotrophs.

[1]The intensity of the protease activity was ranked as weak (+, weak casein hydrolysis), medium (++, casein hydrolysis only directly under the colony) or strong (+++, diffused casein hydrolysis around the colony). The protease intensity displayed for each species or cluster corresponds to the most common protease intensity for that species.

Most of the *Pseudomonas* isolates produced both proteases and lipases (86% of the isolates). Further, of the 118 *Pseudomonas* isolates screened for spoilage potential, there were only 4 and 12 isolates lacking protease or lipase activity, respectively. These results show that the populations of *Pseudomonas* present in sheep milk possess strong spoilage potential, indicating that they may impact the quality of raw milk during refrigerated storage. According to my previous hypothesis, *Pseudomonas* may have proliferated in raw milk from F2 and F3 due to extended refrigerated storage. In raw milk from these two farms, there was one bacterium that was particularly predominant after incubation on CFC agar (F2-4 and F3-1, Figure 7.7), in raw milk from F2 and F3, respectively). It is plausible that these bacteria, which produced both lipases and proteases, belong to *Pseudomonas* and that they represented a species with particularly high fitness, and able to proliferate in raw milk during

refrigeration. Although it was not possible to do so here, it would be interesting to identify these bacteria as they may be producing spoilage enzymes in raw milk during growth throughout refrigerated storage, with potential impacts on milk quality. *Pseudomonas* are efficiently removed by thermal processing, however the production of cold-active or thermostable enzymes by these bacteria during refrigeration, particularly in the context of extended on-farm refrigerated storage of ewe milk, may be deleterious for raw milk quality.

Although I was not able to formally identify the isolates recovered from raw milk, I could provide putative identification for several species of mesophilic and thermophilic spore-formers which colonies displayed distinctive morphological features. These bacteria were putatively identified as *B. licheniformis*, *B. pumilus s.l.*, *Virgibacillus proomii*, *Brevibacillus aydinogluensis*, *Thermoactinomyces vulgaris* and some *Clostridium* species. Mesophilic *B. pumilus s.l.* and *B. licheniformis* were particularly prevalent and were each detected in raw milk from three out of the four farms (Figure 7.4). In addition, thermophilic isolates of *B. licheniformis* were also isolated from F2 and F3 (Figure 7.5). *Virgibacillus proomii* was only isolated from milks collected on farms with a housing component in their farming system: F3 and F4 (Figure 7.4). Interestingly, there was no raw milk isolates that were identified as members of *B. cereus s.l.* according to their characteristic plate morphology (Soufiane and Côté, 2013). *Bacillus licheniformis*, *B. pumilus s.l.* and *V. proomii*, as well as others bacteria such as *Alkalihalobacillus clausii*, *B. subtilis* and *B. cereus s.l.* are part of a consortia of species of spore-formers that have been isolated from all stages of the dairy production continuum, from the dairy farm (te Giffel et al., 2002, Buehner et al., 2014), to raw milk transport and storage (Miller et al., 2015b), different processing environments (Huck et al., 2007a, Lucking et al., 2013), and ultimately to fork (Delaunay et al., 2021). Most of these species are also classified as potentially highly resistant spore-forming bacteria, with some strains being capable of surviving particularly stringent heat treatments [e.g., 110°C for 30 min (Scheldeman et al., 2005, Sadiq et al., 2018)]. In bovine raw milk, numerous authors have found *B. licheniformis* and *B. pumilus s.l.* to represent the two most abundant and prevalent species of mesophilic spore-formers (Coorevits et al., 2008, Buehner et al., 2014, Miller et al., 2015b). Miller et al. (2015b) isolated both *B. licheniformis* and *B. pumilus s.l.* from raw milk collected on 33 different cow dairy farms in the USA. By sequencing a portion of the *rpoB* gene, the same authors could also demonstrate that a phylotype of *B. licheniformis* was found in raw milk from all farms, while several other phylotypes of *B. pumilus s.l.* and *B. licheniformis* could also be isolated from one to two third of the farms, suggesting the presence of strains ubiquitous across farms. Gupta and Brightwell (2017) isolated bacteria identified as *B.*

licheniformis, *B. pumilus s.l.*, *A. clausii*, *B. cereus s.l.*, *V. proomii* and *B. subtilis* in dairy effluents collected on bovine dairy farms in New Zealand, demonstrating that these bacteria are prevalent in New Zealand dairy farm environments. My results further these observations and suggest that these key spoilage organisms may also be particularly prevalent in ewe milk as demonstrated here, and on sheep dairy farms in sources such as feed, soil and bedding materials (see Chapters 4 & 5), with milking representing the possible point of entry of these contaminants into raw milk.

All thermophilic spore-forming bacteria recovered from raw milk (n = 20) could be putatively identified based on their morphology. The vast majority consisted of thermotolerant *B. licheniformis* which were isolated from raw milk produced on F2 and F3 (n = 16, Figure 7.5). Other thermophilic bacteria included *T. vulgaris* and *Br. aydinogluensis* which were isolated in raw milk from F2 and F5, respectively. Though I was not able to analyse environmental samples from F3 and F5, I isolated *B. licheniformis*, *T. vulgaris* and *Br. aydinogluensis* in the farm environment of F1 and F2, which points to their environmental provenance. Buehner et al. (2014) isolated *T. vulgaris* from cow milk during the winter season, while Sadiq et al. (2016a) isolated *T. vulgaris* from bovine dairy powders, suggesting that the dairy farm environment and raw milk may represent sources of these bacteria in bovine dairy products. My work highlights the presence of thermophilic spore-formers with spoilage potential, and documented high heat resistance, in ewe milk. This suggests that ewe raw milk may represent a source of thermophilic spore-formers in the processing environment and in sheep milk powders.

Clostridium spp. were isolated from all raw milk samples. However, according to plate morphology, there was only one species of clostridia after CMG enrichment of raw milk from F2 and F3 (Figure 7.2), which was also strongly proteolytic (Figure 7.8). In raw milk from F4 and F5, there were two, and four different species of clostridia, respectively (Figure 7.2) but they lacked any spoilage potential (Figure 7.8). Plate morphology did not enable reliable identification of *Clostridium s.l.* isolates but it is plausible that they included some of the species I found to be prevalent in sheep dairy farm environments, including *Clostridium sporogenes*, *C. butyricum*, *C. perfringens*, *Terrisporobacter petrolearius*, or *Paraclostridium benzoelyticum*. In raw milk produced on European sheep dairy farms, Turchi et al. (2016) identified *C. perfringens* as the most prevalent species of *Clostridium s.l.*, being detected in 56% of the raw milk samples positive for clostridial spores, followed by *C. sporogenes* at 44% and *C. tyrobutyricum* at 8%. My results indicate that similar bacteria may be present in ewe milk produced in New Zealand.

7.2.3 Microbiological quality of sheep powdered dairy products

The microbiological profile of two New Zealand-produced powder-based dairy products destined for consumption by the general public was established to assess their microbiological quality. Dairy products were analysed after their expiration date (5 months after for dairy product A (DPA) and 11 months for dairy product B (DPB). The formulation of each dairy product was in majority milk-based (skimmed milk in the case of DPA, and whole milk in the case of DPB), but they also contained other ingredients from which some of the contaminants detected may have originated.

Overall, low concentrations of bacteria were recorded in the sheep dairy products. The APC of DPA was particularly low (2.15 $\log_{10}$ cfu/g, Table 7.2). According to the microbiological guidelines set by the New Zealand Ministry for Primary Industry, dairy powders should contain < 3.70 $\log_{10}$ cfu/g when intended for consumption by vulnerable individuals (i.e., infants, the elderly) and < 5 $\log_{10}$ cfu/g when intended for consumption by the general public (Ministry for Primary Industries, 2022). In New Zealand, the APC of dairy powders made from milk of different animal species was reported to range between < 1.00 to 2.86 $\log_{10}$ cfu/g (Ministry for Primary Industry Regulation and Assurance Branch, 2019, Pramularsih et al., 2022). In Ireland, Paludetti et al. (2019) recorded higher APC, between 2.26 and 3.69 $\log_{10}$ cfu/g, in bovine skim milk powders.

The quality of dairy powders is primarily monitored using the APC, but this metric has little to do with the quality and stability of dairy powders and is more reflective of the degree of hygienic practices during production. Spore-formers are the main bacteria that may proliferate during manufacturing or survive processing it and impact the quality of dairy powders. Mesophilic spore-formers are known for causing defects in a variety of shelf-stable milk and milk products, but it is the thermophilic spore-formers that are often the predominant contaminants of dairy powders (Burgess et al., 2010). These two groups of bacteria also contain some species of spore-formers displaying the highest levels of heat and stress resistance (Scheldeman et al., 2006, Setlow, 2006). In the powder manufacturing environment, spore-forming bacteria can cause spoilage of dairy powders and other food formulations comprising dairy powders (Sadiq et al., 2016b, Eijlander et al., 2019). In the present study, mesophilic spore counts were similar in both dairy products: 2.06 and 2.00 $\log_{10}$ cfu/g for DPA and DPB, respectively (Table 7.2). Thermophilic spores were not detected at levels above the detection limit for DPA (detection limit < 0.52 $\log_{10}$ cfu/g) and DPB (detection limit < 0.80 cfu/g), however vegetative cells of thermophilic bacteria could be enumerated, at levels of 0.52 and 1.63 $\log_{10}$ cfu/g, respectively. Psychrotrophic spores were only enumerated in DPB, and they were

particularly low (1.85 $\log_{10}$ cfu/g). After CMG enrichment, anaerobic spore-formers could be detected, albeit not enumerated, in both dairy powders.

A total of 95 isolates, the majority from DPB (n = 75), were recovered. Each isolate from DPA (n = 20), as well as most of the isolates from DPB (n = 66) were identified and screened for lipase and protease activities using plate-based assays. Predominant mesophilic spore-formers were similar in both dairy products and consisted of *B. licheniformis* and *B. pumilus s.l.*(Figure 7.9A). These two bacteria were particularly proteolytic, as well as lipolytic for the isolates from DPA only (Figure 7.10). Other abundant species included *A. clausii* in DPA, and *Solibacillus* spp., *Lederbergia galactosidilytica* and *B. subtilis* in DPB (Figure 7.9A). Most mesophilic spore-formers were strongly proteolytic, however, lipolytic activity was only present in those isolates from DPA (Figure 7.10). Several other genera of mesophilic spore-formers were isolated, including *Oceanobacillus*, *Terribacillus*, *Paenibacillus*, *Rummelibacillus* and *Cytobacillus*. Thermophilic bacteria were isolated without the use of a heat treatment, yet they were all identified as belonging to species of thermophilic spore-formers: *Anoxybacillus flavithermus* and *Aneurinibacillus thermoaerophilus* in DPB (Figure 7.9B), both of which lacked spoilage potential (Figure 7.10), and proteolytic *Thermoactinomyces vulgaris* in DPA. The psychrotrophic spore-formers in DPB had low diversity and the predominant species consisted of *Psychrobacillus* spp. and *Sporosarcina* spp., with *Peribacillus muralis*, *Paenibacillus* spp. and *Solibacillus* spp. being also detected (Figure 7.9C). Spoilage potential across psychrotrophic spore-formers was particularly low; only one isolate was found to be proteolytic (*Peribacillus muralis*, Figure 7.10) while lipase activity was detected in 35% of the isolates: *Peribacillus muralis*, *Psychrobacilus* spp., *Sporosarcina globispora* and *Sporosarcina psychrophila*. After CMG enrichment, four species of anaerobic spore-formers were isolated from DPB: *Clostridium perfringens*, *C. tertium*, *Clostridium* sp. (*vulturis*) and *C. cadaveris* (Figure 7.2), while *C. perfringens* was the only species of anaerobic spore-formers isolated from DPA.

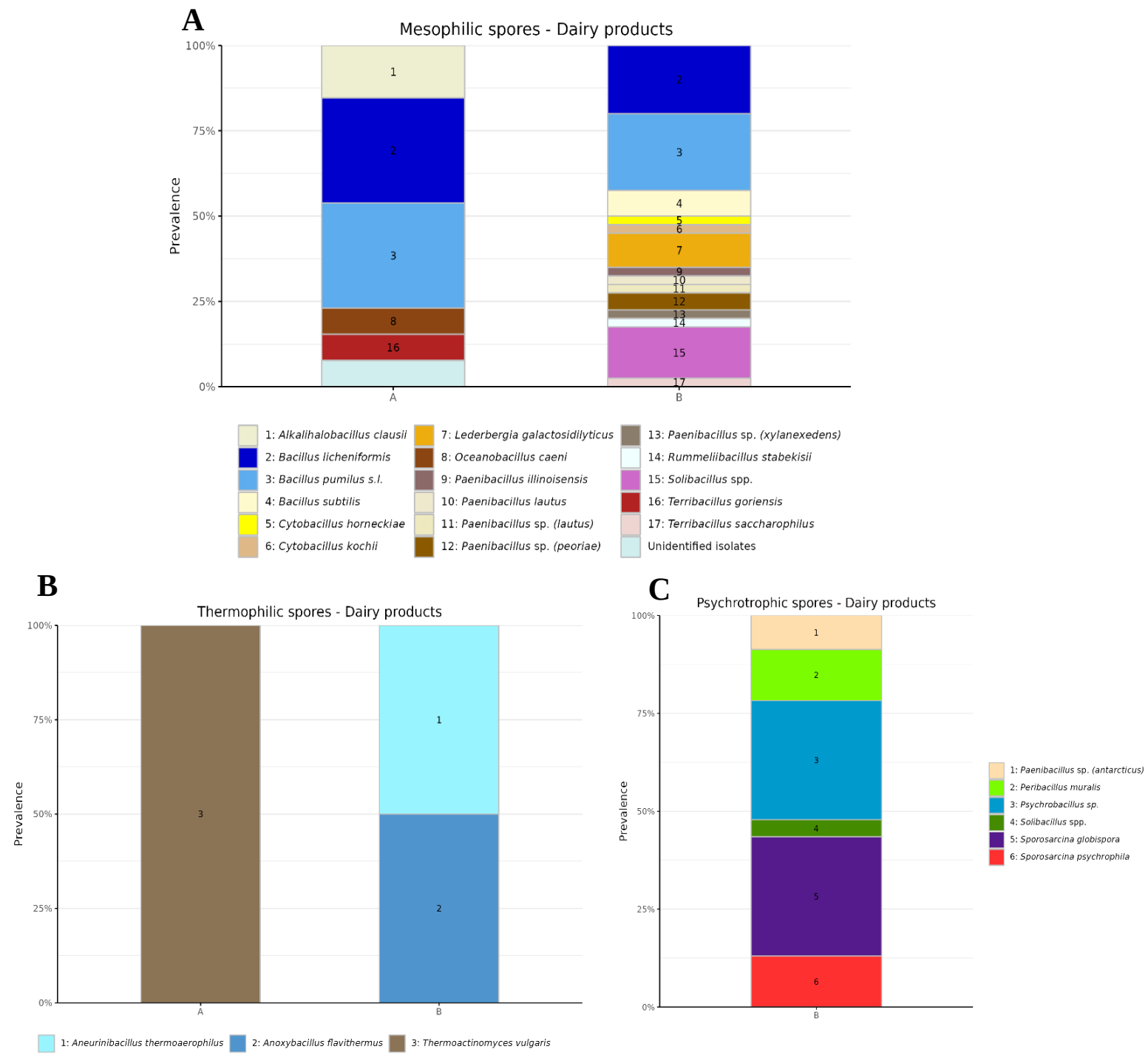


Figure 7.9 Relative abundance of mesophilic, thermophilic, and psychrotrophic spores in selected powder-based sheep dairy products

Coloured rectangles with a number represent the relative abundance of a bacteria in a given sample, the numbers referring to bacterial species in the legend. The proportion of unidentified isolates, if any, is visible as pale turquoise rectangles on the bottom of each bar graph. The isolates referred to as "sp." could not be confidently attributed to a type strain species based on their partial 16S rRNA gene sequence and are instead associated with the most closely related species.

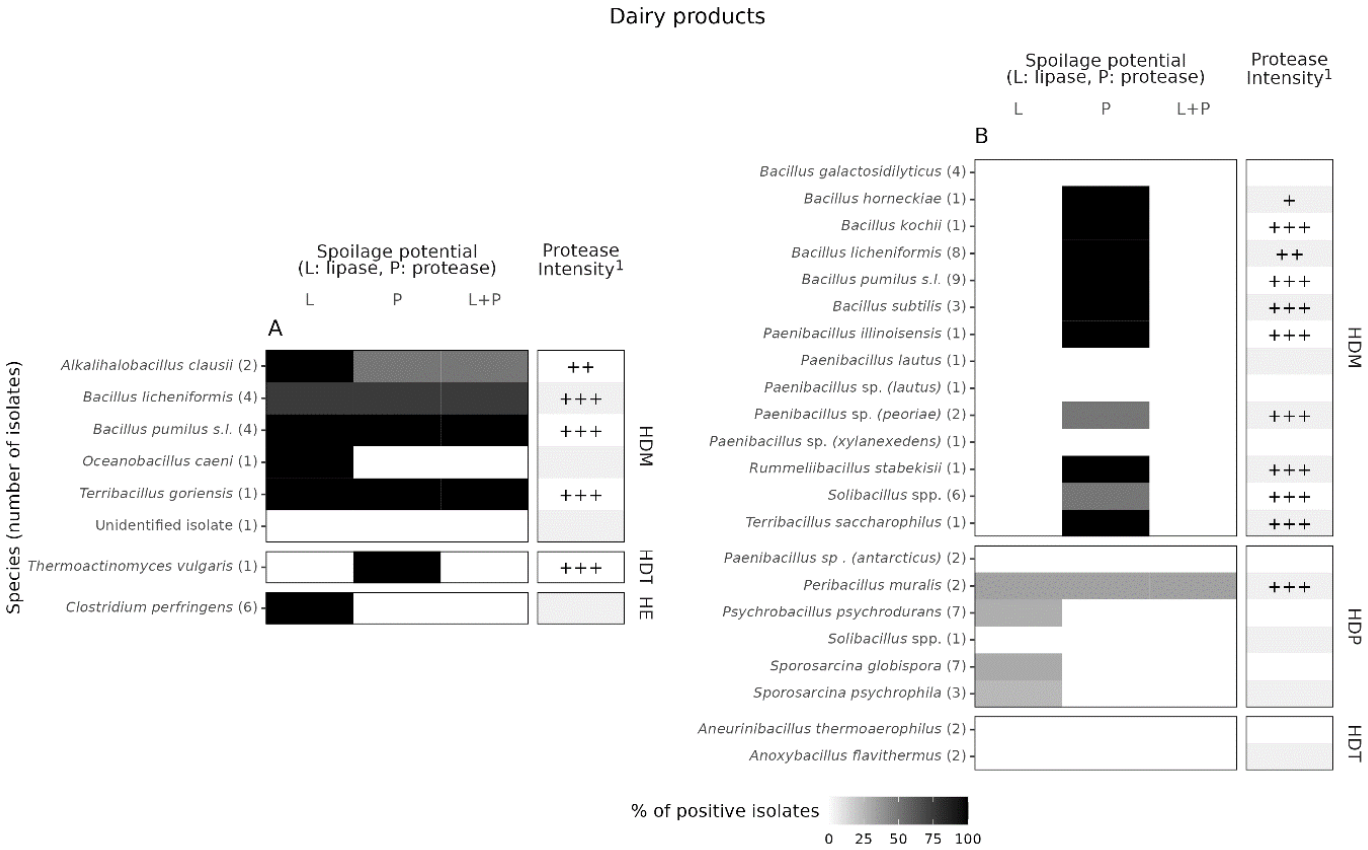


Figure 7.10 Spoilage potential of the bacteria isolated from powdered dairy products

For a given species or cluster, the proportion of isolates positive for lipase and/or protease activity is visible in shades of grey. The numbers in the brackets next to each species or cluster shows the number of isolates screened for spoilage potential for that species. HDM = mesophilic spore-formers; HDT = thermophilic spore-formers; HDP = psychrotrophic spore-formers; HE = anaerobic spore-formers.

[1]The intensity of the protease activity was ranked as weak (+, weak casein hydrolysis), medium (++, casein hydrolysis only directly under the colony) or strong (+++, diffused casein hydrolysis around the colony). The protease intensity displayed for each species or cluster corresponds to the most common protease intensity for that species.

Some isolates recovered from DPB belonged to species previously found to consistently produce lipases, although they did not in this instance, including *B. pumilus s.l.*, *B. subtilis*, and *Lederbergia galactosidilytica* [see Chapter 4 & 5, Figure 7.10, (Sadiq et al., 2016b)]. The assay used to test for the production of lipases was based on the detection of a fluorescent signal with varying intensity across bacterial species and the settings of the recording instrument [i.e., exposure time to UV, (Kouker and Jaeger, 1987)], and was considered difficult to interpret without experience and adequate controls. As DPB was analysed by another operator who was not fully proficient with this technique, some potential positives for lipase activity may not have been recorded adequately.

Thermophilic spore-formers are widely regarded as the predominant contaminants of dairy powders because these bacteria can proliferate during manufacturing, colonise the processing pipeline through biofilm formation, and form highly heat resistant spores (Murphy et al., 1999, Burgess et al., 2010, Sadiq et al., 2016b). Some species of mesophilic spore-formers, including *B. licheniformis*, share the same attributes and may also be particularly abundant in dairy powders (Pramularsih et al., 2022). In addition, numerous species of mesophilic, thermotolerant, or thermophilic spore-formers form highly heat resistant spore-formers, with a few examples including *Heyndrickxia sporothermodurans* (formerly *Bacillus sporothermodurans*), *B. licheniformis*, *Geobacillus stearothermophilus* and *Aeribacillus pallidus* (Scheldeman et al., 2006, Sadiq et al., 2016b). However, with the exception of strains of *B. cereus s.l.* (te Giffel et al., 2002, Sadiq et al., 2016b), few psychrotrophic spore-formers are considered to form highly heat resistant spores, and even fewer are capable of proliferating at both refrigeration temperatures and those found throughout powder manufacturing (Scheldeman et al., 2005, Miller et al., 2015b). The rare cases where psychrotrophic spore-formers may be relevant for the quality of dairy powders is when they are domestically reconstituted and subsequently stored for extended time, enabling germination and proliferation. At a concentrated milk manufacturing plant, Martinez et al. (2017) detected psychrotrophic spores mostly in the holding tanks of pasteurised and concentrated milk. After simulated refrigerated storage of concentrated milk aliquots, the same authors identified a set of psychrotrophic *Paenibacillus* spp. (*P. graminis*, *P. cookii* and *P. odorifer*) potentially capable of initiating spoilage and found at concentrations > 7.3 $\log_{10}$ cfu/L (Martinez et al., 2017). Because psychrotrophic spore-formers are not capable of proliferating throughout powder manufacturing, the populations found in dairy powders are likely to represent a particularly stress-resistant microbiota carried over from raw milk. Interestingly, *Psychrobacillus* spp. and *Sporosarcina* spp., which I found to be some of the predominant genera of psychrotrophic spore-formers isolated from sheep dairy farm environments and milking cups, were also predominant in DPB, although they showed weak spoilage potential in agreement with my previous assessments (see Chapter 4 & 5). This finding may be explained by a combination of the following processes: (1) these bacteria were particularly successful at surviving throughout dairy powder manufacturing and in dairy powders, (2) their presence at high levels in raw milk may have enabled the survival of a large number of their spores even after processing, or (3) they contaminated dairy powders at some stage during transport or manufacturing. Miller et al. (2015b) isolated *Sporosarcina luteola* and *Solibacillus* spp. from acid whey and non-fat dry milk in the USA but found that the rare psychrotrophic bacteria present in several types of dairy powdered products were in majority *Bacillus* and *Paenibacillus* species. My results contrast with this limited literature pertaining to psychrotrophic spore-formers in dairy

powders, and they indicate that the psychrotrophic bacterial species that were isolated from sheep dairy products are not capable of initiating spoilage.

Thermophilic and mesophilic spores represent the most abundant contaminants of dairy powders because their numbers can significantly increase as they are concentrated during powder manufacturing, sloughed off from biofilms present on the pipelines, or upon proliferation in the high-heat sections of the powder manufacturing line (Burgess et al., 2010). In my study, the microbiological quality of the two sheep powdered dairy products was particularly high with regards to their spore content. Specifically, spore counts (Table 7.2) were well below the requirements for export infant formula dairy powders in the USA: 2.70 and 3.00 $\log_{10}$ cfu/g for thermophilic and mesophilic spores, respectively. However, thermophilic counts in dairy powders are frequently recorded after incubation at 55°C, whereas I used an incubation of temperature of 60°C. In New Zealand, Pramularsih et al. (2022) tested the use of different incubation temperatures (30, 37, 55 and 65°C) to enumerate the contaminants present in bovine dairy powders. Higher counts of thermophilic bacteria were recorded at 55°C than at 65°C but the predominant species were similar at 30°C and 37°C (*B. licheniformis*), and at 55°C and 65°C [*G. stearothermophilus* and *Anoxybacillus flavithermus* (Pramularsih et al., 2022)]. Mesophilic and thermophilic spore counts in the dairy products considered in my study were also were similar to the range reported by Pramularsih et al. (2022) in New Zealand bovine dairy powders (1.56 to 2.86 and 1.00 to 3.18 $\log_{10}$ cfu/g, respectively) with the exception of thermophilic counts being particularly low in DPA (0.51 $\log_{10}$ cfu/g). In DPB, the diversity of the thermophilic microbiota was consistent with that of bovine dairy powders, as reported in the literature, and which includes *Geobacillus stearothermophilus*, *Anoxybacillus flavithermus*, *Aneurinibacillus thermophilus* and *Bacillus licheniformis* (Sadiq et al., 2016a, Delaunay et al., 2021). However, the thermophilic microbiota of DPA consisted exclusively of *Thermoactinomyces vulgaris*. My previous findings suggest that the farm environment (especially silage, faeces, soil and trough water, see Chapter 7) is likely to represent the source of these bacteria in milk and dairy products. *Thermoactinomyces vulgaris* has been previously isolated from dairy powders of bovine and ovine origin (Delaunay et al., 2021) but it is interesting to note that it was the only species of thermophilic bacteria detected in DPA. *Thermoactinomyces vulgaris* can form highly heat resistant spores, which could explain its presence post-processing (Sadiq et al., 2016b). *Thermoactinomyces vulgaris* has not been directly implicated in dairy quality, but these bacteria secrete potent proteases that may impact the quality of milk and dairy powders, particularly during long processing runs at high temperatures. The detection of these bacteria in powdered dairy products further suggests that some of the

contaminants found at the stage of primary production (i.e., dairy farm and raw milk) may persist throughout the entire production chain to end up in, and potentially impact the quality of, sheep powdered dairy products.

My results show *B. licheniformis* and *B. pumilus s.l.* were predominant in the two powdered dairy products analysed. This is consistent with my previous detection of these species as the predominant spore-formers in the sheep dairy farm environment and in milking cups (see Chapter 4 & 5), as well as in raw milk (see 7.1.3). *Bacillus licheniformis*, *B. cereus. s.l.*, *B. pumilus s.l.*, and to a lesser extent *A. clausii* and *B. subtilis* represent dairy-ubiquitous bacteria with unique survivability features (e.g., psychro- or thermotolerance, formation of highly heat resistant spores, biofilm formation) and spoilage potential, the former enabling them to thrive in a variety of different environments. My results suggest that the ubiquitous status of these bacteria in the bovine dairy production also applies to the ovine dairy production, further implying that they may also be capable of impacting the quality of sheep dairy products (Lucking et al., 2013). Other bacteria found in the sheep dairy products analysed here included the mesophilic spore-formers *Lederbergia galactosidilytica* and *Oceanobacillus caeni*, which are rarely encountered in bovine dairy products, though I isolated both from the environment (bedding, silage, faeces, and trough water), as well as the milking cups of F1. *Lederbergia galactosidilytica* was discovered by Heyndrickx et al. (2004) after heat-treating bovine raw milk for 30 min at 100°C, highlighting its highly thermoduric potential. Comparison of the DNA fingerprint, or the genome sequence, of isolates from dairy products and the sheep dairy farm environment will be required in order to ascertain the transmission and persistence of these bacteria from the dairy farm to the manufacturing environment.

In this study, *Clostridium s.l.* isolated from sheep dairy products included *C. perfringens*, which was detected in both DPA and DPB, as well as *C. tertium*, *C. cadaveris* and *Clostridium* sp. (*vulturis*). These bacteria have been documented to inhabit soil (Henry, 1918), but also the gastrointestinal tracts of many animals species where they are often found as commensals (Guo et al., 2020). I previously isolated *C. perfringens* and *C. cadaveris* from the farm environment of the sheep dairy farms studied in Chapter 4 and Chapter 5. In bovine milk infant formulas produced in the UK, Barash et al. (2010) isolated 12 different species of soil *Clostridium s.l.*, with the most prevalent consisting of *C. sporogenes* and *C. butyricum* but also including *C. perfringens*. Amongst these, the authors identified a set of species with documented pathogenic potential, including *Paracl. bifermentans*, *C. novyi*/*haemolyticum*, *C. perfringens*, *C. septicum*, and, to a lesser extent, *C. cochlearium*, *C. innocuum*, and *C. subterminale*. Of these, only *C. perfringens* was isolated from sheep dairy powders

in my study. Due to the nature of the processing, storage and consumption of dairy powders (i.e., aerobic conditions), the spores of *Clostridium* are not likely to germinate and/or be found at concentrations capable of causing illness, such as those that may occur in temperature-abused solid food products where anaerobic conditions can settle (Hatheway et al., 1980, Mahamat Abdelrahim et al., 2019). This means that *Clostridium* spores do not represent a health hazard for healthy individuals when present in dairy products and powders. A report available from MPI states that the risk of illness from dairy-associated *C. perfringens* is low and that "There is no epidemiological link between *C. perfringens* and illness arising from the consumption of dairy products." (Hill et al., 2003). Nonetheless, the report recommends limits on the concentrations of *C. perfringens* in food as product safety limits: 3 $\log_{10}$ cfu/g and 2 $\log_{10}$ cfu/g for dairy products intended for consumption by the general public, or higher risk populations, respectively. In my study, enrichment in CMG media of the powder samples did not enable enumeration of clostridial spores; however, this will need to be carried out to determine the contamination levels of sheep dairy powders produced in New Zealand.

7.3 Conclusions

In this chapter, the culture methodology previously applied to farm environments was extended to isolate bacteria from sheep raw milk and dairy products, offering a complementary perspective that completes the picture of the microbiota associated with the ovine dairy production. This study represents the first attempt at evaluating the microbiological quality of sheep dairy products in New Zealand, revealing encouraging findings as the quality of sheep raw milk and dairy products was deemed satisfactory. The analysis presented here also sheds light into the possible transfer of spoilage bacteria from the farm setting to raw milk and their persistence throughout the dairy processing chain. As a pioneering effort, my work establishes a valuable foundation for future investigations, providing insights into prospective areas of interest and paving the way for further advancements in microbiological quality and safety standards within the New Zealand sheep dairy industry.

The analysis presented here suggests that some of the bacteria found in the sheep dairy farm environment are likely to be transferred to raw milk and remain in dairy products. In addition, these findings provided further support to possible links between different farming systems or management practices and the concentration of bacteria in ewe milk (e.g., higher abundance of aerobic spores in a housing set-up, or of *Pseudomonas* spp. in the pasture environment), or their diversity and spoilage

potential (e.g., *B. cereus s.l.* associated with grazing, some spore-formers only isolated from barn environments).

The results of my work indicate that the quality of sheep milk and dairy products produced in New Zealand was satisfactory; most of the raw milk samples, and each dairy products, satisfied the newly set out microbiological guidelines. Spore counts in raw milk were low, with recorded values being similar (psychotrophic and thermophilic spores) or lower (mesophilic spores) than reported in the literature pertaining to bovine raw milk. Spore counts and bacterial counts in dairy powders were also low and well within the local and international limits set for exports, thus providing confidence that good manufacturing and hygienic practices are being applied under the New Zealand regulatory framework. However, the APC of two raw milk samples, which consisted of pooled fresh and day-old raw milk, was higher than the recommended limits. In those milks, concentrations of *Pseudomonas* were also markedly high, suggesting that some of these bacteria may have been able to proliferate during refrigerated storage. Further work in collaboration with the industry will be required to identify members of the psychrotrophic microbiota of sheep milk that may proliferate during refrigeration and monitor their growth kinetics during storage of ewe raw milk storage. Such work will provide a quality risk assessment for the practice of storing raw milk on-farm for bulk collection by processers.

Overall, the bacterial diversity recorded here was similar to the spoilage microbiota of bovine dairy products worldwide, which includes a set of ubiquitous bacteria that exhibited strong spoilage potential, such as *B. licheniformis*, *B. pumilus s.l.*, *T. vulgaris*, *Paenibacillus* spp., *Pseudomonas fragi*-like and *Pseudomonas fluorescens*-like bacteria. These findings contribute to address the lack of data pertaining to the ovine dairy production, which will help identify the spoilage bacteria implicated in the quality of ewe milk and sheep dairy products. The entry of these bacteria in raw milk cannot be entirely prevented due to their ubiquity, nor can they be efficiently removed by processing due to their high heat-resistance. However, concentrations of spores were low in milk and dairy products indicating that the current processes in place provide satisfactory safeguards against contamination by spore-forming bacteria.

It is important to acknowledge the inherent limitation imposed by the small sample size, which restricts the application of rigorous statistical analyses. The observed associations between farming practices and microbial composition in sheep milk, while indicative, will require validation through analysis of larger and more diverse sample sets over extended periods. Future research endeavours

should prioritise the expansion of sample sizes to explore and strengthen the statistical robustness of conclusions drawn from this study. Ongoing surveillance and monitoring within the industry, accompanied by the analysis of a more extensive dataset, will be essential in refining our understanding of the microbes associated with the ovine dairy production and in upholding the continued quality and safety of sheep milk and dairy products.

Chapter 8 - General discussion and conclusions

8.1 General discussion

In this thesis, I used culture methods and a polyphasic identification approach to survey the microbial communities associated with dairy spoilage, and present in the environment of an indoors (Chapter 4) or an outdoors (Chapter 5) sheep dairy farm, as well as in sheep raw milk and dairy products (Chapter 7). Bacteria that were targeted by this modest culturomics approach included anaerobic mesophilic, aerobic mesophilic, thermophilic and psychrotrophic spore-formers, as well as *Pseudomonas* spp., all of which have been implicated in the quality modulation or spoilage of dairy products. In Chapters 4 and 5, the bacterial diversity obtained from milking cups swab samples, which were used as proxies for raw milk, was compared with that of different farm components in order to identify possible farm-level sources of milk contamination. Subsequent use of DNA fingerprinting and whole genome sequencing as phylogenetic tools in Chapter 6 enabled source-tracking of 26 selected spoilage bacteria isolated from milking cups and the confirmation of environmental sources for some of these bacteria on each farm. An overview of the phylogenetic diversity of spoilage bacteria was also obtained, which further helped understand their ecology in the sheep dairy farm environment.

My work represents the first attempt to describe farm-level bacterial communities associated with dairy spoilage and their dynamics of transmission in the sheep dairy farm environment. The results of this thesis considerably advance our understanding of the ecology of spoilage bacteria in the ovine dairy farm environment while also complementing current knowledge pertaining to bovine dairy farms. The identification of spoilage bacteria and microbial hotspots, as well as potential risks factors and sources of raw milk contamination will help guide future research towards the development of farm practices and other practical applications targeting specific aspects of milk contamination to improve the microbiological quality of ewe milk.

8.1.1 Detection methods for dairy microbiology

The detection scope of the five culture conditions used for bacterial isolation in this study proved to be comprehensive and complementary, with 224 different species of bacteria recovered from the two farm environments, including only eight species that were detected across multiple detection methods. This culturomics approach highlighted, for the first time, the presence of several bacteria in dairy farm environments, including bacteria that had been previously isolated from the processing environment or dairy products, thereby suggesting that the farm environment may represent a source of these contaminants in milk processing and dairy products. A particularly diverse array of bacteria with spoilage potential and belonging to yet-undescribed species or taxa were also isolated and will require future characterisation to assess their potential to affect dairy quality. Though some of the culture conditions used in my study had been extensively applied before, albeit never in the ovine dairy farm environment, the isolation of thermophilic spore-formers and *Clostridium s.l.* was conducted using methods that differed from traditional approaches, but which improved the detection of these bacteria.

Thermophilic spore-formers were isolated at 65°C, permitting a focus on obligate thermophilic spore-formers (e.g., *Thermoactinomyces* spp., *Geobacillus s.l.* and *Anoxybacillus* spp.) which are the main contaminants of dairy powders, while the growth of normally interfering facultative thermophiles (e.g., *B. licheniformis*) was prevented. In addition, my work identified that different agar media could partially suppress interfering plate phenotypes (i.e., protruding mucoid colonies, swarming or spreading motilities) which are known to thwart accurate enumeration and isolation of thermophilic spore-forming bacteria associated with the early steps of the dairy production (Miller et al., 2015a, Murphy et al., 2021). The methods and media used in this study will serve as a basis for future development and consolidation of culture methods focusing on the isolation of obligate thermophilic spore-formers from dairy farm environments.

In accordance with Gupta and Brightwell (2017), I confirmed that the use of CMG enrichment circumvents one of the main issues associated with the detection of obligate anaerobic spore-formers in dairy microbiology, namely by preventing interfering growth of facultative anaerobic spore-forming bacteria such as those of the *Bacillus s.l.* and *Paenibacillus* taxa. The enrichment step may also contribute to improving the detection of lower abundance species that are typically dwarfed by highly abundant bacteria. However, neither enumeration nor study of the relative abundance of

clostridia were possible due to the enrichment. Novel methods that enable accurate enumeration of clostridia are now required to identify farm-level reservoirs of these bacteria.

In Chapter 6, source-tracking of milking cups contaminants was conducted using ERIC-PCR or ARDRA. ERIC-PCR was shown to be appropriate and have suitable resolution for the subtyping of a wide array of aerobic spore-forming bacteria and *Pseudomonas* species. Subtyping of *Clostridium s.l.* with ARDRA provided lower phylogenetic resolution but still permitted basic clustering of the isolates according to their fingerprinting profiles. Though the reproducibility of these approaches has been criticised in the past, my results suggest that they can be used reliably with adequate controls in place (i.e., uniform conditions for the PCR and the DNA migration, use of replicates). Subtyping with ERIC-PCR can be used on a wide range of bacterial species and is also easily implemented, as it only requires traditional laboratory equipment (thermocycler and gel electrophoresis station). Thus, during preliminary work aiming to identify microbial ecology trends, DNA fingerprinting may represent a reliable, more affordable and higher throughput alternative to existing, more expensive high-resolution subtyping techniques (WGS, MLST schemes or single-gene sequencing). In Chapters 4 and 5, ERIC-PCR was used for sample-wise clustering and triage of all bacterial isolates, which significantly reduced the number of isolates required to be identified using 16S rRNA gene sequencing. Previous studies have often relied on morphological features to select representative isolates for identification (Scheldeman et al., 2005, Miller et al., 2015b), but plate morphology is not a reliable indicator and only allows for limited discrimination across some taxa (e.g., *Pseudomonas* spp., *B. cereus s.l.*). My results demonstrate that ERIC-PCR allows for reliable and accurate species-level sorting of spore-forming bacteria and *Pseudomonas* species, thereby emphasising that it should be strongly considered as an additional, yet quick, step of polyphasic identification schemes in dairy microbiology.

8.1.2 Distribution and transmission of spoilage bacteria in sheep dairy farms environments

Diagrams picturing the ecology and transmission of spoilage bacteria during housing of dairy ewes (F1, Figure 8.1), or during grazing (F2, Figure 8.2) were constructed according to the results of my study. My findings suggest that the microbial communities present in the barn environment, or the grazing environment differ, both in abundance and diversity but also with regards to their dynamics

of transmission across the farm. On both farms, a core microbiota composed of species of bacteria with strong spoilage potential was detected. In addition, I was also able to highlight some of the possible implications of housing or grazing of dairy ewes on the microbiology of the farm and of raw milk.

Farm-level reservoirs of spoilage bacteria, i.e., sources that contain high concentrations of spoilage bacteria, were identified using the bacterial count and diversity data generated during Chapters 4 and 5. The potential transmission of spoilage bacteria throughout the farm and to milking cups was inferred from the phylogenetic data obtained in the source-tracking study reported in Chapter 7. On both farms, the transmission of bacteria throughout the environment and to milking cups was evidenced for anaerobic mesophilic spore-forming bacteria, as well as aerobic mesophilic and thermophilic bacteria. However, farm-level transmission of psychrotrophic spore-formers and *Pseudomonas* could not be ascertained, as there were never a pair of isolates from different samples that had an identical DNA fingerprinting profile, suggesting that they could be more genetically diverse, and therefore more challenging to track. Environmental communities of psychrotrophic bacteria are likely to proliferate in cold, ambient winter temperatures, during which samples were collected. It would be interesting to study the phylogeny of these farm populations during summer, when warmer environmental temperatures may confer higher fitness to mesophilic members of the farm microbiota, and possibly select for their growth over that of psychrotrophic bacteria.

8.1.2.1 Reservoirs and sources of spoilage bacteria in the barn environment

Bacterial spores were found to be particularly abundant in sources associated with the barn environment. A set of ubiquitous bacteria consisting of *Bacillus licheniformis*, *B. zhangzhouensis s.l.*, *Thermoactinomyces vulgaris*, *Psychrobacillus* spp., *Solibacillus* spp., *Pseudomonas fragi*, and *Ps. weihenstephanensis* were predominant across the different farm sources, which pointed to a high degree of bacterial cross-contamination in the barn environment. Reservoirs of mesophilic spore-formers were identified as animal faeces, bedding, and silage. Bedding materials and faeces were identified as the main reservoirs of psychrotrophic or thermophilic spore-formers, respectively. High concentrations of *Pseudomonas* were also recorded across the different samples, and particularly in milking cups and the set of environmental samples collected from pens with fresher bedding materials. Regarding thermophilic and mesophilic spore-formers, these results were in agreements with the findings of studies conducted on bovine dairy farms, which highlights parallels between the

microbiology of ovine and bovine dairy farms. The growth or accumulation of mesophilic and thermophilic bacteria in bedding materials has been demonstrated on bovine dairy farms (Murphy et al., 2019) but until now little was known about the ecology of psychrotrophic bacteria in bedding materials, and especially in the ovine dairy farm environment. Therefore, the fact that I identified bedding materials as a reservoir of both psychrotrophic spore-formers and psychrotrophic *Pseudomonas* warrants further research. As suggested by the higher abundance of *Pseudomonas* in fresher bedding materials, and by the results of previous work (Godden et al., 2008, Murphy et al., 2019), the addition of fresh bedding may promote bacterial growth directly after change as bedding becomes contaminated by bacteria, moisture and nutrients provided by animal faeces (catabolism waste) and unused bedding materials (e.g., lignocellulosic compounds). When transient outgrowth of the bedding microbiota occurs to an extent that may be deleterious for milk microbiological quality, it could be possible to consider implementing, in parallel, strategies to limit this growth (e.g., use of bedding inoculants or conditioners) or reduce the subsequent possible increased transfer of bacteria to the animals and to raw milk (e.g., implementation of a strategic pre-milking cleaning routine when bedding contains high concentrations of bacteria).

According to source-tracking of selected bacteria, the main source of milking cups contamination in the barn environment was identified as the silage-faeces axis. Several milking cup bacteria were found to originate exclusively from animal faeces (E arrow on Figure 8.1), indicating that the gastrointestinal tract of dairy ewes may represent a shedding reservoir and source of bacteria such as *Alkalihalobacillus clausii, B. licheniformis, B. zhangzhouensis s.l.*, *Clostridium perfringens*, and *Thermoactinomyces vulgaris*, but also possibly of *Paraclostridium benzoelyticum* and *Pseudomonas koreensis*. The five former bacteria were also found to originate from silage, and to be transmitted to milking cups via multiple routes of contamination: mainly after being ingested and shed in faeces (B arrow on Figure 8.1), or after contaminating bedding materials (C arrow). There was also evidence suggesting transfer of silage bacteria directly to milking cups (A arrow on Figure 8.1) or via trough water (D arrow).

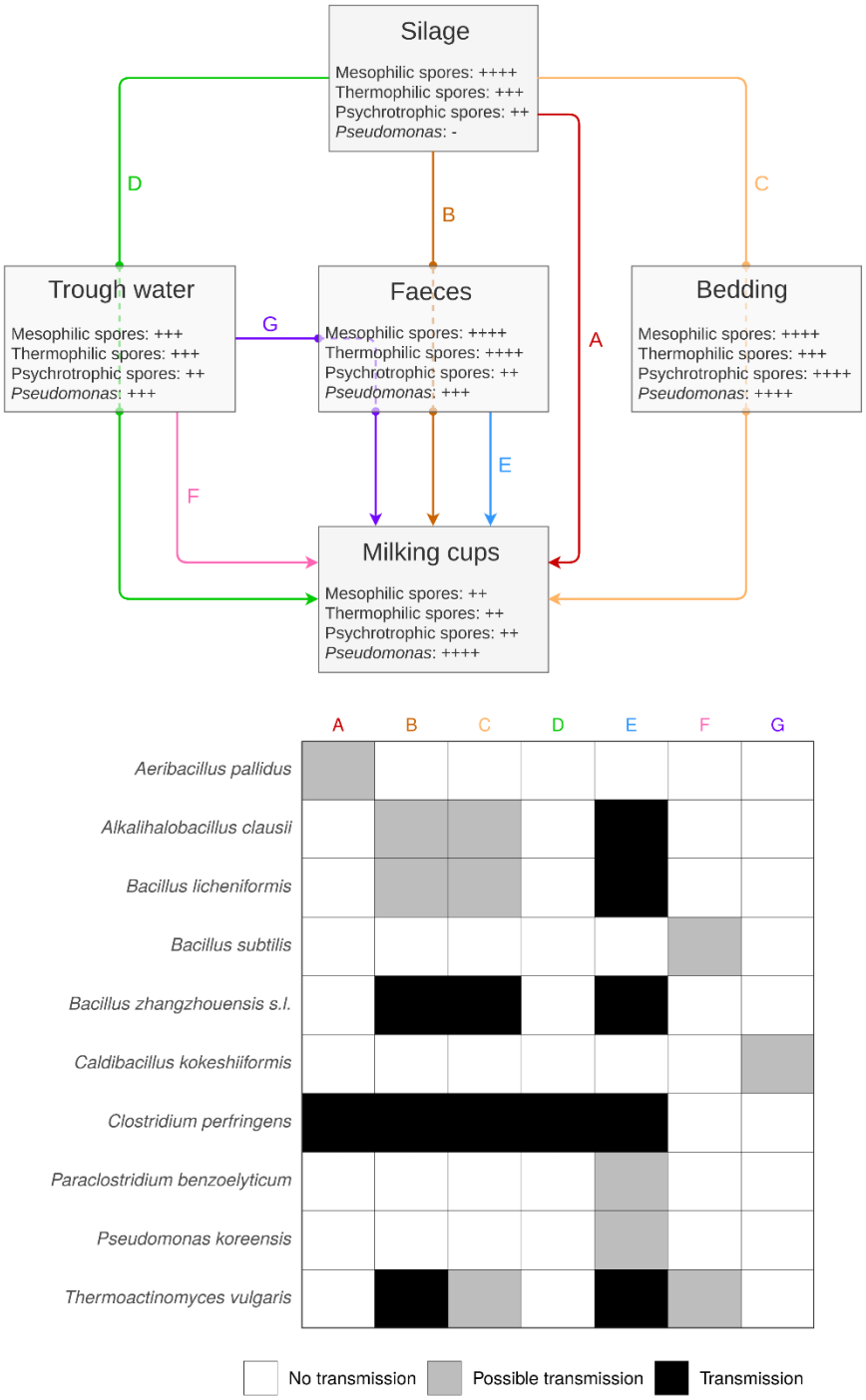


Figure 8.1 Schematic representation of the main reservoirs and transmission of spoilage bacteria in the sheep dairy farm barn environment.

For each source, the abundance of mesophilic, thermophilic, and psychrotrophic spores, as well as *Pseudomonas* is presented as arbitrary units ranging from "–" (not detected) to "++++" (highly abundant). The transmission of bacteria throughout the environment and to milking cups is depicted with coloured arrows on the schematic representation and a letter referring to the table underneath showing the different bacteria and their transmission across the corresponding environments.

Thermoactinomyces vulgaris, *Clostridium perfringens* and *Bacillus zhangzhouensis s.l.* originating from silage were found to enter milking cups from all considered environmental sources, which points to the high transmissibility and to the ubiquitous nature of these bacteria in the barn environment. Surprisingly, no milking cups contaminants were found to originate exclusively from bedding materials, indicating that bedding materials may instead primarily serve as an intermediary favouring contact between environmental bacteria and the udders of dairy ewes.

Together, my results indicate that silage represents a key reservoir and source of dairy spoilage-associated mesophilic and thermophilic spore-forming bacteria in the farm environment and in milking cups. This is in agreement with, and also complements, results previously obtained on bovine dairy farms highlighting silage as a key source of aerobic and anaerobic mesophilic spore-forming bacteria in milk (te Giffel et al., 2002, Vissers et al., 2007b, Borreani et al., 2019), as well as research on ovine dairy fairy farms highlighting silage as a source of butyric acid bacteria in milk (Arias et al., 2013). Previous work has shown that the contribution of silage to the contamination of raw milk could be limited by improving the manufacturing process, and thus the quality and stability of silage (Vissers et al., 2007a, Borreani and Tabacco, 2010). On-farm silage manufacturing is particularly common in New Zealand, but high humidity and precipitations as well as the high nitrogen content and buffering capacity of the crops means the ensiling process is at greater risk of producing silage with high concentrations of spoilage bacteria. My work shows that silage was central to the transmission of spoilage bacteria in the barn environment. This indicates that further research is warranted on the microbial composition of silages fed on sheep dairy farms, and to further probe the extent of the contribution of silage to the contamination of ewe milk. Specifically, silages interventions and practices that can limit the growth of spoilage bacteria during the ensiling process should be investigated as they have the potential to reduce the abundance of spoilage bacteria in silage and thus limit their transfer to raw milk.

8.1.2.2 Reservoirs and sources of spoilage bacteria in the pasture environment

The microbial ecological picture obtained from the farm where dairy ewes grazed on pastures contrasted with that of the farm where the animals were housed and fed silage. Microbial communities present across sources associated with grazing were particularly diverse and heterogeneous, which pointed to a low degree of bacterial cross-contamination in the pasture environment. The main reservoir of spoilage bacteria in the grazing environment was identified to be the natural environment,

i.e., soil and pastures. *Pseudomonas* were highly abundant on pasture and in milking cups, while relatively low concentrations of bacterial spores, and particularly thermophilic spores, were recorded in milking cups and in farm sources other than soil. Surprisingly, the faeces of grazed dairy ewes consistently had the lowest spore counts across all sources, which contrasted with those of housed dairy ewes, as reported above. This suggested that dairy ewes do not shed large quantities of bacterial spores in their faeces when they are grazing on pastures. Similarly, pasture was identified as the main reservoir of *Pseudomonas* in the grazing environment, but faecal counts of these bacteria were markedly low. Still, milking cups were identified as a key reservoir of *Pseudomonas*, indicating that environmental contamination by these bacteria may accumulate in the milking environment and on the milking equipment of pasture-based farms, including perhaps through biofilms, which *Pseudomonas* excel at forming. The main reservoir of spore-forming bacteria was identified to be soil, which consistently had the highest mesophilic, thermophilic and psychrotrophic spore counts across all samples.

Soil is a natural reservoir of spore-forming bacteria but also an integral part of the farm environment. My results emphasise that contact between soil and the animals should be limited during grazing, in order to prevent excessive attachment of soil particles containing high concentrations of bacterial spores to the udders of dairy ewes, and thereby limit the possible transfer of bacteria to raw milk during milking. Potential approaches that may help reduce the contribution of soil to the contamination of raw milk by spoilage bacteria have been identified on cow dairy farms and require further assessment on sheep dairy farms. They include strategic implementation of pre-milking cleaning routines and adequate grazing rotation (both including working around wet weather events) as well as limiting the use of biological soil amendments (such as dairy effluents or manure ploughing) containing high concentrations of bacterial spores on grazed areas.

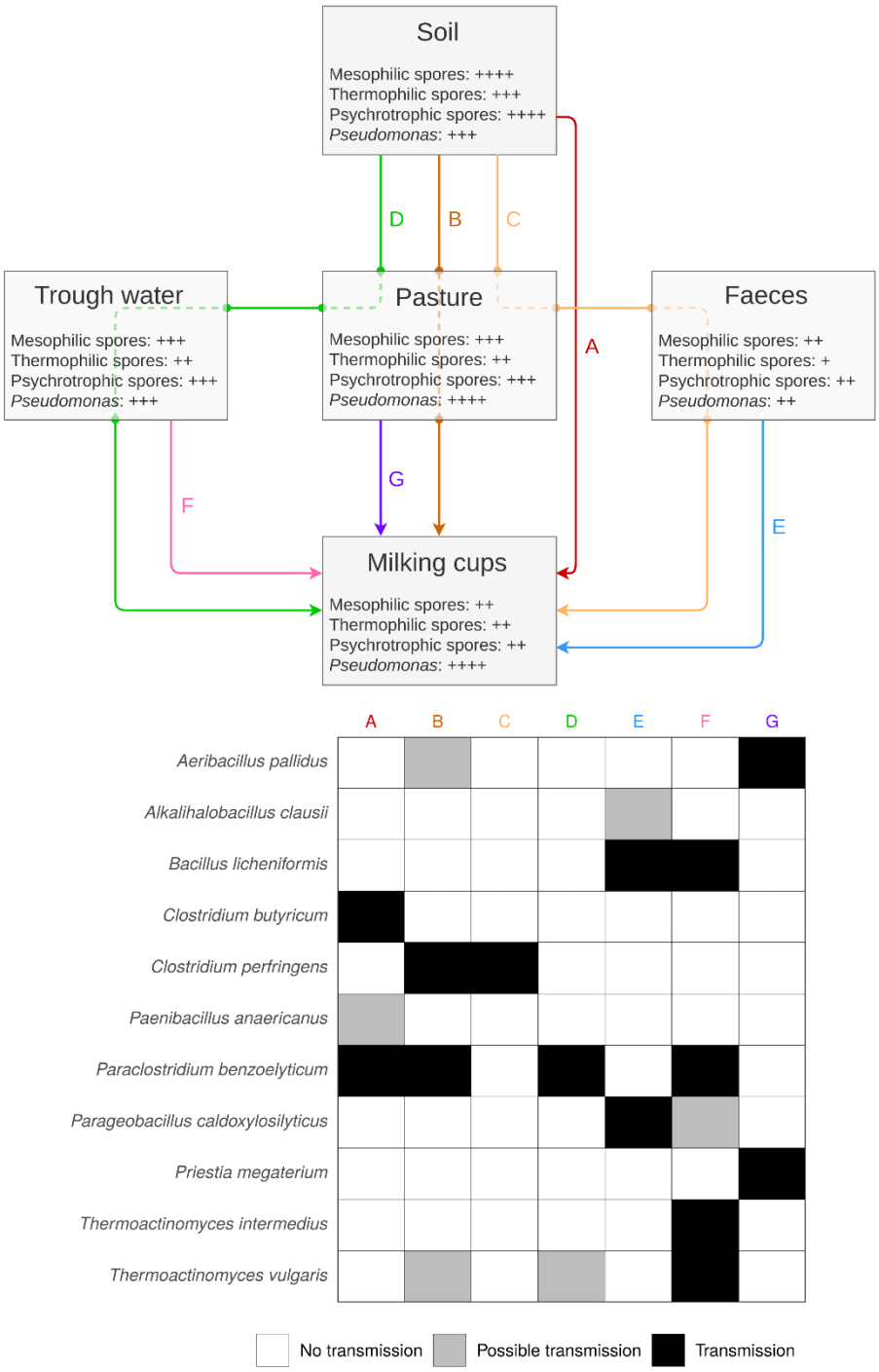


Figure 8.2 Schematic representation of the main reservoirs and transmission of spoilage bacteria in the sheep dairy farm pasture environment.

For each source, the abundance of mesophilic, thermophilic, and psychrotrophic spores, as well as *Pseudomonas* is presented as arbitrary units ranging from "–" (not detected) to "++++" (highly abundant). The transmission of bacteria throughout the environment and to milking cups is depicted with coloured arrows on the schematic representation and a letter referring to the table underneath showing the different bacteria and their transmission across the corresponding environments.

Source-tracking of selected spoilage bacteria revealed that milking cups contaminants often originated from a single environmental source and that there was less cross-contamination across sources associated with the grazing environment. The natural environment, especially soil, represented the main source of *Clostridium s.l.* contamination in miking cups. Soil-borne *C. butyricum* were transferred directly from soil to milking cups (A arrow on Figure 8.2) while the transfer of *C. perfringens* took place via pasture (B arrow), or pasture and shedding in faeces (C arrow). *Paraclostridium benzoelyticum* originating from soil were found to contaminate milking cups either directly (A arrow on Figure 8.2), via pasture (B arrow), or via trough water (D and F arrows). This finding corroborates previous studies in the cow dairy farm environment identifying soil as the main source of *Clostridium s.l.* in milk (Drouin and Lafreniere, 2012), and further emphasises the importance of limiting excessive contact between soil and the animals during grazing. Soil contamination of the udders and subsequent transfer to raw milk during milking may represent an important source of milk contamination, however my results suggest that other environmental sources also play a role in the transfer and dispersal of soil bacteria throughout the farm environment and to milking cups.

Pasture was identified as a source of milking cups contamination by the thermophilic soil bacterium *Aeribacillus pallidus* and the plant endophyte *Priestia megaterium*. Surprisingly, neither soil, pastures, nor faeces were identified as possible sources of milking cups contamination by bacteria members of *B. cereus sensu lato*. This was despite the facts that *B. cereus s.l.* were highly abundant in milking cups, but also animal faeces and the grazing environment, both of which had been previously identified as sources of these bacteria in cow milk (Vissers et al., 2007d, Vissers et al., 2007e). Uncertainties relating to the identification of *B. cereus s.l.*, which refers to at least 11 different species, need to be addressed. Several members of *B. cereus s.l.* are important spoilage bacteria that are implicated in fluid milk quality, and further work is required to identify the origin of these bacteria in the milking cups of pasture-based sheep dairy farms. Discrimination between members of *B. cereus s.l.* can be accomplished with MLST schemes or whole-genome sequencing (Carroll et al., 2020), however future development of media selective for *B. cereus s.l.* is required as the current options fail to prevent the growth of some other interfering spore-forming bacteria.

In spite of their markedly low spore counts, the faeces of grazed dairy ewes were identified as a source of mesophilic and thermophilic spore contamination in milking cups, including by the highly versatile spoilage bacteria *B. licheniformis*. Importantly, my work is the first to demonstrate the transfer of proteolytic members of *Geobacillus s.l.* (*Parageobacillus caldoxylosilyticus*) from the farm

environment to milking cups, and thus possibly to raw milk. A few members of the *Parageobacillus* genus have been identified as contaminants of the powder manufacturing environment, including *Parag. thermoglucosidasius* (Zhao et al., 2013). *Parageobacillus* are also closely related to *Geobacillus* spp. and share similar ecological niches: soil, aerobically deteriorating organic matter, and the faeces of livestock. Shedding of thermophilic spores from these sources and their potential transmission to raw milk (albeit at low levels) may ultimately lead to their transfer to, and subsequent colonisation of, the powder manufacturing environment. This hypothesis will need to be tested, including by surveying the thermophilic microbial communities present in the processing environment of sheep dairy products and by comparing their phenotype and genotype to that of farm isolates.

Trough water was also identified as a source of mesophilic (*B. licheniformis*) and thermophilic (*Thermoactinomyces vulgaris* and *T. intermedius*) spore contamination in milking cups. It is unlikely that thermophilic bacteria, which require temperatures upwards of 50°C to grow, would be actively proliferating in water. However, this may instead indicate that environmental contamination of water troughs by the microbial communities associated with pastures, or with the animals, have the potential to accumulate in trough water. During drinking, these bacteria may gain access to the animals (via skin contact or ingestion and shedding in faeces) and ultimately to the milking environment and raw milk. The possible contribution of animal drinking water to the transmission of bacteria on dairy farms has been largely overlooked as it often contains low concentrations of bacteria. However, identification of trough water as a potential source of milking cups contamination by spoilage bacteria on both sheep dairy farms emphasises on the need to maintain adequate trough hygiene. In addition, these findings warrant further research into the microbiota of water troughs.

8.1.3 Thermophilic actinomycetes and the sheep dairy production

For a long time, thermophilic actinomycetes have been known to dwell in soil and to be particularly abundant in environments associated with agricultural activity (Cross, 1968). It was established shortly thereafter that aerosolised spores of thermophilic actinomycetes such as *Thermoactinomyces* spp. and *Laceyella* spp. were involved in the onset of a range of autoimmune respiratory diseases with high incidence in farm workers, including hypersensitivity pneumonitis, asthma, or chronic bronchitis (Lacey et al., 1972). *Thermoactinomyces* spp. were also isolated from cow raw milk and shown to produce exolipases capable of impacting the lipid profile of milk (Falkowski, 1979). Despite these findings, *Thermoactinomyces* were subsequently largely overlooked as potential milk

contaminants. However, in recent years, there has been an increasing number of studies taking place at various geographical locations and reporting detection of *Thermoactinomyces* in bovine dairy powders (Yuan et al., 2012, Sadiq et al., 2016b, Sadiq et al., 2018) and in cow raw milk (Buehner et al., 2014), though they had never been isolated from sheep milk and dairy products until now. This suggested that the farm environment represents a possible source of milk and dairy product contamination by *Thermoactinomyces* species. However, a lack of contemporary knowledge on the prevalence of thermophilic actinomycetes on dairy farms prevented the identification of possible sources of milk contamination.

In this study, I found that *Thermoactinomyces* spores were present in the grazing environment of dairy ewes and that they were transferred to milking cups from trough water, and possibly from soil. In the barn environment, thermophilic actinomycetes were highly abundant, and they were transferred to milking cups from silage and animal faeces. The range of species that were detected were found to be consistently and strongly proteolytic as well as occasionally lipolytic, and included *T. vulgaris*, *T. intermedius*, *T. khenchelensis*, *Thermobifida fusca*, *Sacchamonospora viridis* and several other potentially novel *Thermoactinomyces* species. Isolation of thermophilic spores in my study was carried out with atypical methods, including the use of Columbia sheep blood agar and incubation at 65°C, both of which may have aided the isolation of *Thermoactinomyces* species. Conversely, incubation on milk agar, which is one the media frequently used to enumerate and isolate thermophilic spore-formers, yielded poor recovery of *Thermoactinomyces* spp., suggesting that the prevalence and abundance of thermophilic actinomyces may have been underestimated in previous studies that used this media. Further investigation is required to update the prevalence and abundance of these bacteria in dairy farm environments as well as in milk and dairy products, where they may be present at higher concentrations than previously reported.

According to ERIC-PCR fingerprinting, *Thermoactinomyces vulgaris* and *T. intermedius* isolated from both farms were highly diverse, as few isolates shared identical ERIC subtypes. However, genome sequencing of the predominant *Thermoactinomyces* isolates and subsequent genome-based phylogenetic studies revealed a remarkably low genomic diversity at the species level ($dDDH_{d4}$ > 94%), including between farm isolates and the references strains, the latter of which had been isolated from Tunisia, the USA, and China. Though additional isolates should be assessed for confirmation, these results highlight the low genomic variability of *Thermoactinomyces* spp. worldwide and suggest that phylogenetic study of *T. vulgaris* using whole genomes or multilocus sequence typing schemes allows for limited tracking of the isolates. I hypothesise that ERIC-PCR fingerprinting may be able

to capture small-scale, and perhaps non-transmissible (i.e., epigenetic markers), genetic variations that are overlooked during genome-based, or gene-based studies, but which could enable tracking of *Thermoactinomyces* at the local scale. Further evaluation of ERIC-PCR is required to test this hypothesis. Specifically, comparison of the phylogenies generated by genome-wide approaches or by ERIC-PCR across a larger set of isolates could provide additional information that may help validate the use of ERIC-PCR for the subtyping of *Thermoactinomyces* species.

In this study, I showed that *Thermoactinomyces vulgaris* were transferred to milking cups from the farm environment. These bacteria were also isolated from sheep raw milk and powder-based sheep dairy products, highlighting their dispersion from the farm environment, and their presence at all stages of the dairy production. Genes coding for putative spoilage enzymes (e.g., *lipA1*, unidentified serine proteases) were present in the genomes of the farm *Thermoactinomyces* isolates, which also readily produced potent proteases, and lipases, pointing to their potential involvement in dairy quality. The range of temperatures at which these bacteria thrive (50 – 70°C) suggests that they are capable of proliferating in the high-heat sections of powders manufacturing plants. In addition, *T. vulgaris* has also been shown to produce biofilm *in-vitro* (Demirci et al., 1993) and may be able to colonise the stainless steel surface of the dairy manufacturing plant pipeline. Preliminary data suggests that the spores of *T. vulgaris* are highly resistant, including to heat (Sadiq et al., 2016b), and to desiccation which stimulates the release of their spores (Reponen et al., 1998). These spoilage-associated phenotypes are similar to those of the obligate thermophilic spore-formers that are frequently found to contaminate milk powders. However, it remains unknown whether *T. vulgaris* and other thermophilic actinomycetes are actively proliferating during powder manufacturing or if they are merely carried as spores from the farm environment and raw milk. Further research is required to evaluate the *in-situ* spoilage potential of *Thermoactinomyces* spp. during the manufacturing of dairy products. This may include further assessment of their ability to form biofilm or to contribute to multi-species biofilms, as well as to produce spoilage enzymes, and the activity of those, in the high-heat sections of the powder manufacturing environment. Because of their unique enzymatic repertoire, possible interactions between *Thermoactinomyces* cells and other members of the contaminating flora (e.g., enzyme-sharing) should also be investigated.

8.1.4 Microbiology of sheep milk and dairy products

My work represents the first survey of the microbiological content of ewe milk and sheep dairy products in New Zealand, and the first of its kind isolating such a diverse array of bacteria from sheep milk and dairy products. These results contribute to address the lack of microbiological data pertaining to the ovine dairy production, which will help the identification of the predominant contaminants implicated in the quality of sheep dairy products and shed light on the transfer of bacteria from raw materials to end products.

Most of the raw milks that were analysed satisfied the newly set-out microbiological requirements (Ministry for Primary Industries, 2022), and had spore counts similar to that of high-grade cow milk produced in New Zealand and overseas. However, the farm that housed dairy ewes more often (F5) was found to have high mesophilic, thermophilic and psychrotrophic spore counts in raw milk. The microbiota present in the environment of this farm was not surveyed, yet this finding echoes the observations made on Farm 1 and reported in Chapter 4. Specifically, this support the hypothesis that housing of dairy ewes may promote increased contamination of raw milk by bacterial spores, which are particularly abundant in the barn environment. There is also some suggestion that higher *Pseudomonas* contamination of raw milk is taking place on the pasture-based farm. However, regardless of grazing or housing of the animals, it was found that bulk tank milk containing a mix of fresh and day-old milk had higher concentrations of *Pseudomonas* than bulk tank milk consisting exclusively of fresh milk. The number of milk samples collected in my study was limited and additional samples should be analysed to confirm these trends. However, this finding suggest that storage of raw milk on-farm may lead to rapid proliferation of psychrotrophic bacteria, including *Pseudomonas*, in the bulk tank before it is collected, which may be deleterious for milk quality. The volume of the New Zealand sheep dairy production did not initially warrant dairy collection and farmers were asked to pool milk over two days before it was taken to the manufacturing plant. Nowadays, as more farms have settled in the same areas, raw milk is collected daily from the farms and processing runs are more frequent due to greater processing power. To obtain a more accurate picture of the potential proliferation of *Pseudomonas* during storage and transport of raw milk, it would be interesting to analyse and compare the psychrotrophic microbiota of fresh raw milk and raw milk sampled after refrigeration, and at the manufacturing plant.

The two powder-based sheep dairy products that were analysed fulfilled the microbiological requirements of dairy powders, indicating that the current manufacturing processes in place

adequately shield sheep dairy products from excessive microbial contamination. However, the presence of bacteria in dairy products that were also isolated from the farm environment suggest that some contaminants are capable of persisting throughout dairy processing. Importantly, my work identified that some of the contaminants of ovine dairy powders are similar to those found in bovine dairy powders, once again highlighting parallels between the microbiology of bovine and ovine dairy production. However, because of the unique composition of sheep milk, which include higher levels of proteins, processing conditions may differ from those used in the manufacturing of cow milk powders, which may have implications on the abundance and diversity of the bacteria contaminating these products. Analysis of a larger dataset of dairy products, including non-powders commodities, will be required in the future to further characterise the spoilage microbiota of ovine dairy products.

8.2 Conclusions

My research set out to describe the microbial communities associated with dairy spoilage and found in sheep dairy farm environments. The goals of this preliminary work were to survey the prevalence of spoilage bacteria associated with the sheep dairy production, identify farm reservoirs of these bacteria and provide a first overview of their potential transmission throughout the dairy farm and to raw milk. These goals can be considered attained. This piece of work significantly furthers our understanding of the sheep dairy farm microbiology and complements current microbiological knowledge of the broader dairy farm environment, highlighting both parallels and differences between the microbial ecology of bovine and ovine dairy farms. As it has been established on bovine dairy farms, the results reported here will help with the identification of risk factors and sources of ewe milk contamination, which can be further investigated and addressed to improve the microbiological quality of raw milk produced on sheep dairy farms.

Several farm-level reservoirs of spoilage bacteria were identified, including silage, bedding materials, animal faeces and soil for spore-forming bacteria, as well as bedding materials and pasture for *Pseudomonas* species. Possible remediation steps aiming at reducing the abundance of these bacteria in these sources were identified in the literature, from work on bovine dairy farms, and will require future assessment on sheep dairy farms.

The transmission of spoilage bacteria from the barn or the pasture environment to milking cups was evidenced using subtyping of the farm isolates. Sources of milking cups contamination, and thus

possibly of raw milk contamination, differed on both farms likely because of their farming system. Predominant sources of milk contamination identified in the barn or pasture environment of dairy ewes were consistent with previous findings on bovine dairy farms operating under similar farming systems. However, for the first time, my work shed light on the transmission of dairy spoilage bacteria that have been rarely isolated from dairy farms (e.g., *Geobacillus s.l.*), and thereby identified the dairy farm as a possible origin of these bacteria in dairy products. In addition, my results suggest the presence, and contribution to milk contamination, of novel potential farm-level routes of milk contamination that will require further assessment.

Potential trends between milk microbiological quality and farming systems or farming practices that were identified in this study will require future assessment. This preliminary study does not offer certainty in the conclusions drawn on this matter; however, it highlights multiple areas that warrant particular attention in the future. With a growing number of farms either being built, or converting from cow to sheep dairying, there will be a higher number of farm locations available for sampling. Close collaboration with the industry will be vital to obtain access to farms for sampling, and to ensure that the research can have practical and tangible applications for farmers.

From my experience presenting at the SheepMilkNZ conference for the past several years, farmers and milk processors are often keen to learn about the farm microbiology and its contribution to milk microbiological quality. However, the implications of farm-level choices involving microbial activity are often opaque for farmers, who have limited time and few opportunities to improve their knowledge on this topic. Farming is not an exception; in the general population, awareness of relevant microbial activities and how they impact our lives – microbiology literacy – is lacking. Addressing this has been identified to be indispensable for making informed personal and business decisions on a variety of topics, for governmental and business policy development, and for knowledgeable input of societal stakeholders in such policymaking (Timmis et al., 2019). As farmers are the keystone of sheep dairy farming, they should be empowered with basic farm microbiology knowledge that can help them make informed decisions on a daily basis, and which may assist in improving milk microbiological quality. The results of my work will be made accessible to the New Zealand sheep dairy industry for guidance. This will provide the industry with some of the microbiological implications of farming practices on sheep dairy farms and assist in the selection of farm practices that are associated with the production of high-quality milk.

Appendices

Appendix 4.1. Mean bacterial counts in samples associated with a sheep dairy farm

Bacterial count (log_{10} cfu/g; /l or /swab)	Fence	Bedding	Silage	Faeces	Trough water	Milking cups	Corn	Rumen Supplement
MSC	5.57	6.69	6.30	6.92	6.08	4.52	1.73	2.26
TSC (65°C, SBA)	4.16	5.41	5.41	6.58	5.39	3.22	N.D.	0.79
TSC (60°C, SBA)	X	X	X	X	X	4.12	X	X
TSC (65°C, MA)	X	4.86	4.12	4.30	X	X	X	X
TSC (60°C, MA)	X	X	X	X	X	4.15	X	X
PSC	4.29	6.00	4.50	4.20	4.62	3.61	1.08	1.68
CFC count	X	7.44	N.D.	7.01	6.72	7.44	X	X
Psychrotrophic count	7.42	X	X	X	X	X	6.10	1.63

MSC: Mesophilic spore count; TSC: Thermophilic spore count; PSC: Psychrotrophic spore count; CFC: Cephaloridine fucidin cetrimide agar
Bacterial counts are expressed in log_{10} cfu/g (bedding, silage, faeces, corn and rumen supplement), log_{10} cfu/l (trough water) or log_{10} cfu/swab (fences and milking cups).

Appendix 4.2. Unidentified bacterial clusters isolated from F1

Name by which the cluster is referred to	Most closely related type strain[1]	Maximum 16S rRNA sequence similarity (%)[2]	Type strain accession number[3]
Obligate anaerobic spore-formers			
Clostridium sp. *(uliginosum)*	*Clostridium uliginosum*^{T} CK55	88.9	AJ276992
Paeniclostridium sp. *(sordellii)*	*Paeniclostridium sordellii*^{T} ATCC 9714	94.7	AB075771
Aerobic mesophilic spore-formers			
Bacillus sp. (*badius*)	*Bacillus badius*^{T} ATCC 14574	94.1	X77790
Bacillus sp. (*pervagus)*	*Bacillus pervagus*^{T} 8-4-E12	96	HF952773
Cytobacillus sp. *(kochii)*	*Cytobacillus kochii*^{T} WCC 4582	96.1	FN995265
Lysinibacillus sp. *(pakistanensis)*	*Lysinibacillus pakistanensis*^{T} NCCP-54	95.2	AB558495
Oceanobacillus sp. *(chironomi*)	*Oceanobacillus chironomi*^{T} T3944D	93.5	DQ298074
Oceanobacillus sp. *(polygoni)*	*Oceanobacillus polygoni*^{T} SA9	93.7	AB750685
Ornithinibacillus sp. (*bavariensis*)	*Ornithinibacillus bavariensis*^{T} WSBC 24001	96.4	Y13066
Paenibacillus sp. (*harenae*)	*Paenibacillus harenae* ^{T} B519	82.2	AY839867
Paenibacillus sp. (*lactis*)	*Paenibacillus lactis*^{T} MB 1871	94.5	AY257868
Paenibacillus sp. (*lentus*)	*Paenibacillus lentus*^{T} CMG1240	92.2	KC800716
Paenibacillus sp. (*quercus*)	*Paenibacillus quercus*^{T} 1-25	82.3	JX409872
Paenibacillus sp. (*vini*)	*Paenibacillus vini*^{T} LAM0504	86.7	KJ005124

Siminovitchia sp. *(fordii)*	*Siminovitchia fordii*^{T} R-7190	95.9	AY443039
Aerobic thermophilic spore-formers			
Ureibacillus sp. (*composti*)	*Ureibacillus composti*^{T} THC145	87.2	DQ348071
Aerobic psychrotrophic spore-formers			
Lederbergia sp. (*lentus*)	*Lederbergia lentus*^{T} NCIMB8773	92	AB021189
Caryophanon sp. (*tenue*)	*Caryophanon tenue*^{T} NCDO 2324	85	X70315
Neobacillus sp. *(ginsengisoli)*	*Neobacillus ginsengisoli*^{T} DCY53	88.2	HQ224517
Oceanobacillus sp. (*chungangensis*)	*Oceanobacillus chungangensis*^{T} CAU1051	95.7	JX233492
Paenibacillus sp. (*agarexedens*)	*Paenibacillus agarexedens*^{T} DSM 1327	82	AJ345020
Paenibacillus sp. (*camelliae*)	*Paenibacillus camelliae*^{T} b11s-2	87.1	EU400621
Paenibacillus sp. (*etheri*)	*Paenibacillus etheri*^{T} SH7	94.3	KC625558
Paenibacillus sp. (*granivorans*)	*Paenibacillus granivorans*^{T} A30	81.1	AF237682
Paenibacillus sp. (*jilunlii*)	*Paenibacillus jilunlii*^{T} Be17	95	GQ985393
Paenibacillus sp. (*macquariensis*)	*Paenibacillus macquariensis*^{T} M4-2	94.6	AB360546
Paenibacillus sp. (*tundrae*)	*Paenibacillus tundrae*^{T} A10b	94.7	EU558284
Paenibacillus sp. (*typhae*)	*Paenibacillus typhae*^{T} xj7	91.5	JN256679
Paenibacillus sp. (*wynnii*)	*Paenibacillus wynnii*^{T} CK55	87	AJ633647
Paenibacillus sp. (*xylanexedens*)	*Paenibacillus xylanexedens*^{T} B22a	95	EU558281
Peribacillus sp. (*gossypii*)	*Peribacillus gossypii*^{T} JM-267	92.1	KT240114
Sporosarcina sp. (*globispora*)	*Sporosarcina globispora*^{T} DSM 4	92.4	X68415

Sporosarcina sp. (*newyorkensis*)	*Sporosarcina newyorkensis*[T] 6062	95.4	GU994085
Sporosarcina sp. (*psychrophila*)	*Sporosarcina psychrophila*[T] IAM 12468	94.6	D16277
Non-spore-forming psychrotrophs			
Acinetobacter sp. *(lwoffii)*	*Acinetobacter lwoffii*[T] DSM 2403	85.6	X81665
Brachybacterium sp. *(tyrofermentans)*	*Brachybacterium tyrofermentans*[T] CNRZ 926	95.4	X91657
Carnobacterium sp. *(maltaromaticum)*	*Carnobacterium maltaromaticum*[T] DSM 20342	90.4	M58825
Jeotgalicoccus sp. *(psychrophilus)*	*Jeotgalicoccus psychrophilus*[T] YKJ-115	94.9	AY028926
Pseudomonas sp. *(balearica)*	*Pseudomonas balearica*[T] SP1402	95.7	U26418
Pseudomonas sp. *(extremaustralis)*	*Pseudomonas extremaustralis*[T] CT14-3	86	AJ583501
Pseudomonas sp. (*fragi*)	*Pseudomonas fragi*[T] ATCC 4973	95.9	AF094733
Pseudomonas sp. *(litoralis)*	*Pseudomonas litoralis*[T] 2SM5	95.1	FN908483
Pseudomonas sp. *(parafulva)*[5]	*Pseudomonas parafulva*[T] AJ2129	86.3	AB060132
Pseudomonas sp. (*vranovensis)*	*Pseudomonas vranovensis*[T] CCM 7279	94.6	AY970951
Pseudomonas sp. *(weihenstephanensis)*	*Pseudomonas weihenstephanensis*[T] DSM 29166	96	KP738720
Pseudomonas sp. *(xanthomarina)*[4]	*Pseudomonas xanthomarina*[T] KMM	92.2	AB176954
Stenotrophomonas sp. *(rhizophila)*	*Stenotrophomonas rhizophila*[T] e-p10	95.8	AJ293463
Stenotrophomonas sp. *(tumulicola)*	*Stenotrophomonas tumulicola*[T] T5916-2-1b	94.7	LC066089

[T]: Type strain

[1] Most closely related type strain 16S rRNA sequence as found in the RDP

[2] Matching percentage given for the isolate with the partial 16S rRNA sequence showing highest similarity to that of a given type strain. The partial 16S rRNA sequence of other isolates in the same cluster showed ≥99% similarities to the 16S rRNA sequence of this isolate.

[3] Accession number for the type strain in the GENBANK database

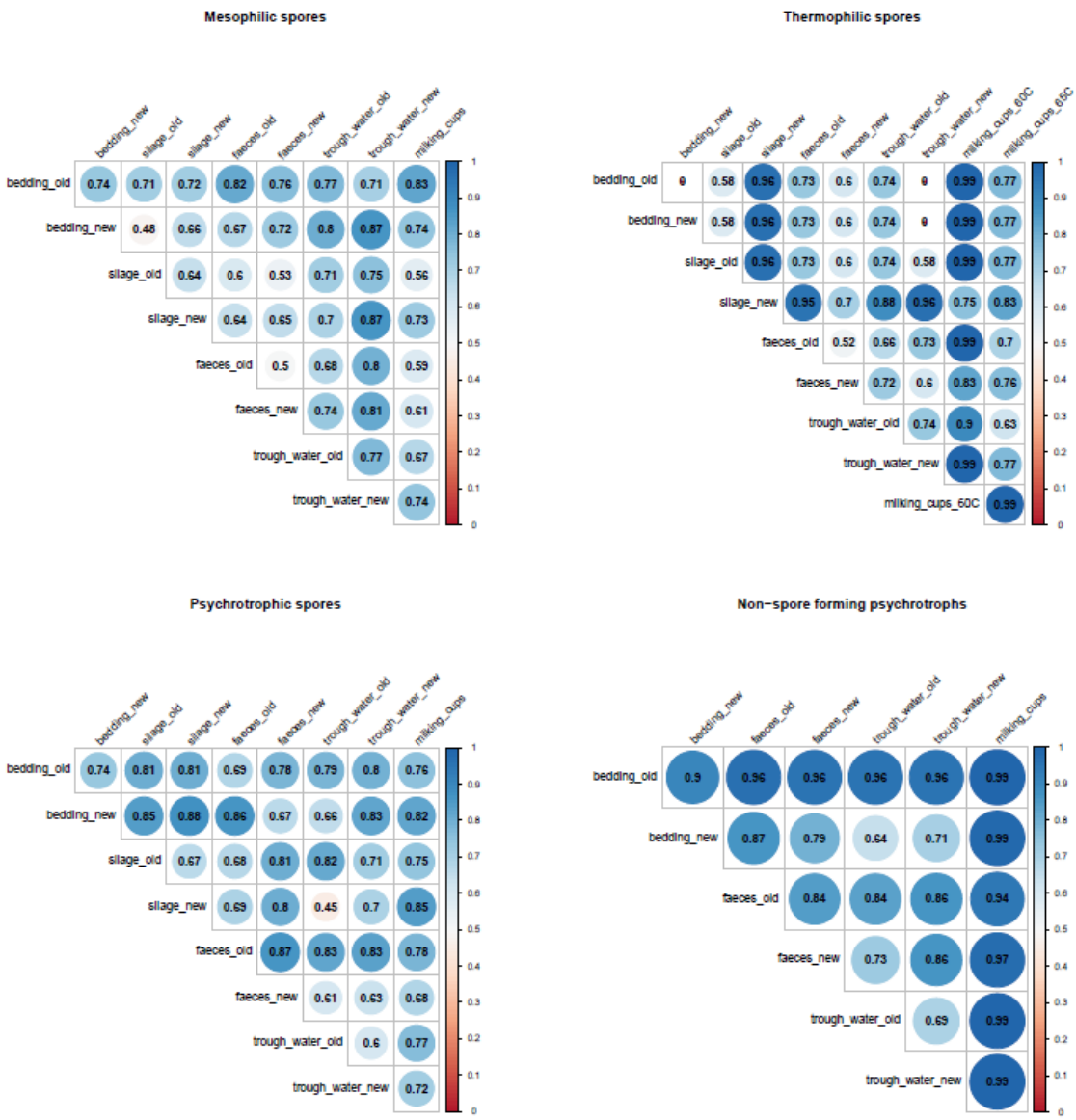
[4] Confirmed as *Pseudomonas nitrititolerans* by whole genome sequencing (Gupta et al., 2020b)

[5] Confirmed as *Pseudomonas saudiphocaensis* by whole genome sequencing (Gupta et al., 2021)

.

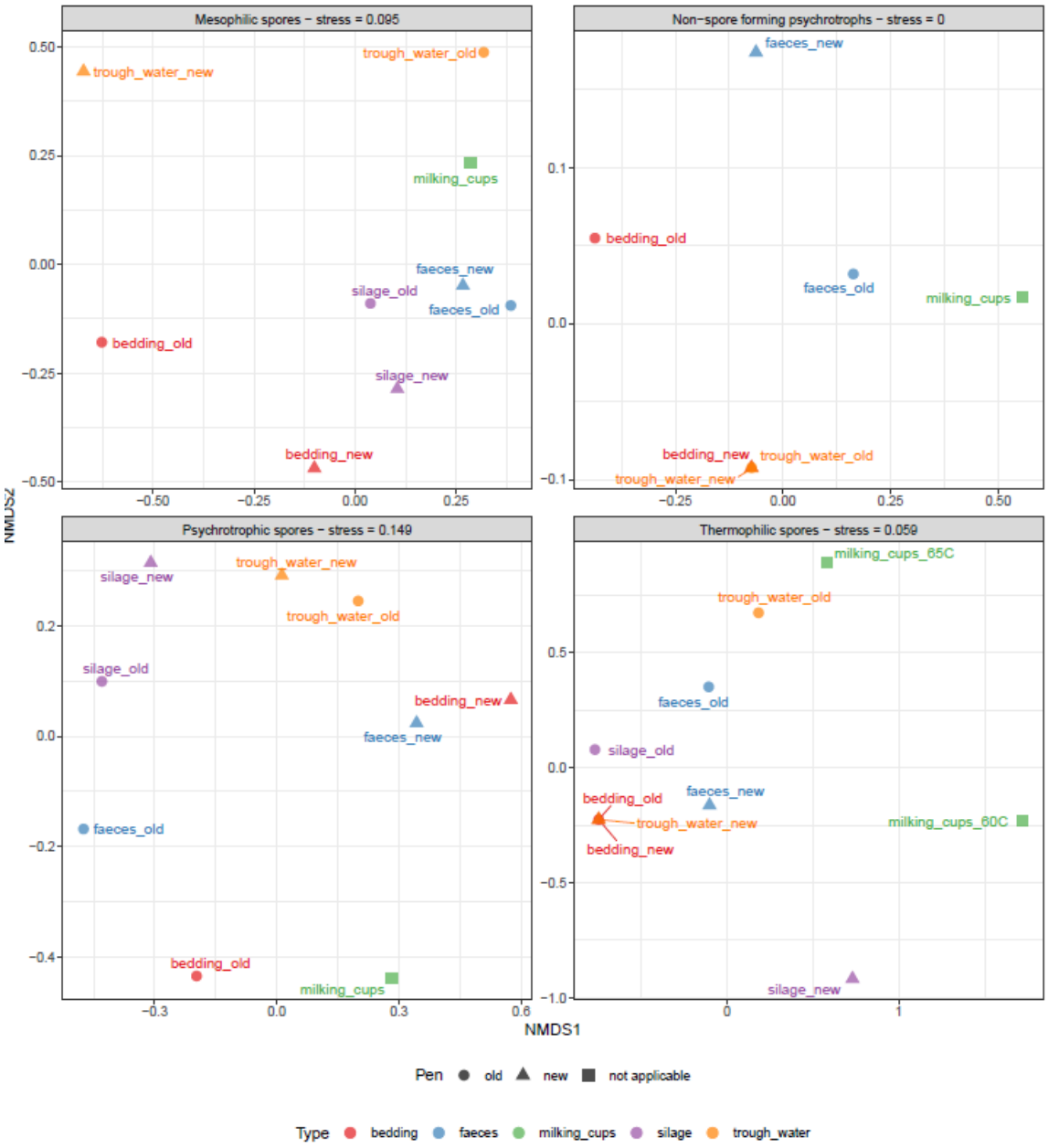
Appendix 4.3. Diversity indexes calculated for each sample type and each bacterial group of interest

Sample	Shannon	Simpson	Inverse.Simpson
Mesophilic spores			
bedding_old	1.778	0.789	4.742
bedding_new	1.579	0.750	4.000
silage_old	1.622	0.720	3.576
silage_new	1.923	0.803	5.085
faeces_old	1.809	0.791	4.775
faeces_new	1.758	0.772	4.392
trough_water_old	2.191	0.845	6.436
trough_water_new	2.278	0.816	5.440
milking_cups	1.730	0.730	3.700
Thermophilic spores			
bedding_old	0.000	0.000	1.000
bedding_new	0.000	0.000	1.000
silage_old	0.150	0.067	1.071
silage_new	1.400	0.659	2.934
faeces_old	0.868	0.500	2.000
faeces_new	0.406	0.174	1.211
trough_water_old	0.705	0.452	1.825
trough_water_new	0.000	0.000	1.000
milking_cups_60C	1.454	0.680	3.125
milking_cups_65C	0.888	0.542	2.182
Psychrotrophic spores			
bedding_old	1.528	0.673	3.060
bedding_new	0.996	0.570	2.326
silage_old	1.956	0.811	5.297
silage_new	1.981	0.815	5.411
faeces_old	2.218	0.856	6.942
faeces_new	1.221	0.615	2.595
trough_water_old	1.366	0.665	2.989
trough_water_new	1.825	0.776	4.459
milking_cups	1.786	0.751	4.017
Non-spore forming psychrotrophs			
bedding_old	1.912	0.788	4.709
bedding_new	2.252	0.850	6.669
faeces_old	0.847	0.518	2.075
faeces_new	1.721	0.774	4.433
trough_water_old	1.895	0.809	5.224
trough_water_new	1.938	0.833	5.976
milking_cups	1.891	0.816	5.430



Appendix 4.4. Pair distances between the species diversity of 4 bacterial group isolated from samples collected on a New Zealand sheep dairy farm.

A colour scale indicates similarity (red) to dissimilarity (blue). Aberrant similarities among the diversity of thermophilic spore-formers were due to the fact that a unique species was present at relative abundance > 1% and was therefore taken into account for the analysis.



Appendix 4.5. Non-metric multidimensional scaling (NMDS) plot of bacterial diversity isolated from farm samples using culture-dependent methods.

Sample types are represented by a colour while sample sets are represented by a shape.

Appendix 4.6 ELISA assessment of *Clostridium perfringens* toxinotypes

Sample	Isolate	*C. perfringens* surface antigens	α toxin production	β toxin production	ε toxin production
NB	SKOBHE9	+	+	-	-
	SKOBHE21.1	+	+	-	-
	SKOBHE21.2	+	+	-	-
	SKOBHE42	+	+	-	-
OS	SKOSHE6.1	+	+	-	-
	SKOSHE7.1	+	-	-	-
	SKOSHE12	+	-	-	-
	SKOSHE20	+	+	-	-
	SKOSHE23	+	+	-	-
	SKOSHE28.1	+	+	-	-
	SKOSHE29	+	+	-	-
OW	SKOWHE1	+	+	-	-
	SKOWHE18	+	+	-	-
	SKOWHE37	+	+	-	-
	SKOWHE65.1	+	+	-	-
NP	SKNPHE3	+	+	-	-
	SKNPHE20	+	+	-	-
	SKNPHE47	+	+	-	-
MC	SKMCHE4	+	+	-	-
	SKMCHE6	+	+	-	-
	SKMCHE9	+	+	-	-
	SKMCHE12	+	+	-	-
	SKMCHE13.2	+	+	-	-
	SKMCHE15	+	+	-	-
	SKMCHE16	+	+	-	-
	SKMCHE18	+	+	-	-
	SKMCHE20	+	+	-	-
	SKMCHE25	+	+	-	-
	SKMCHE29	+	+	-	-

SKMCHE30	+	+	-	-

NB = “new” bedding; OS = “old” silage; OW = “old” water; NP = “new” faeces; MC = milking cups

Appendix 4.7 Taxonomy of the *Pseudomonas* species isolated from F1

***Pseudomonas aeruginosa* lineage**	***Pseudomonas fluorescens* lineage**									
	Pseudomonas fluorescens complex								*P. asplenii* group	*P. rhizosphaerae* group
	P. fluorescens subgroup	*P. fragi* subgroup	*P. protegens* subgroup	*P. mandelii* subgroup	*P. corrugata* subgroup	*P. gessardii* subgroup	*P. jessenii* subgroup	*P. koreensis* subgroup		
P. caeni *P. nitrititolerans* *P. oryzihabitans* *P. saudiphocaensis* *Pseudomonas* sp. (*balearica*) *Pseudomonas* sp. (*litoralis*)	*P. azotoformans* *P. constantini* *P. extremaustralis* *P. extremorientalis* *P. libanensis* *P. lurida* *P. poae* *P. rhodesiae* *P. tolaasii* *Pseudomonas* sp. (*extremaustralis*)	*P. fragi* *P. psychrophila* *P. weihenstephanensis* *P. deceptionensis* *P. lundensis* *Pseudomonas* sp. (*fragi*) *Pseudomonas* sp. (*weihenstephanensis*)	*P. protegens*	*P. migulae*	*P. kilonensis*	*P. gessardii* *P. brenneri* *P. yamanorum*	*P. reinekei* *P. umsongensis* *P. vancouverensis*	*P. koreensis* *P. baetica*	*Pseudomonas* sp. (*vranovensis*)	*P. rhizosphaerae*

Appendix 5.1 Unidentified bacterial clusters isolated from F2

Name of the cluster	Most closely related type strain[1]	Maximum seqmatch score[2]	Type strain accession number[3]
	Obligate anaerobic spore-formers		
Clostridium sp. *(uliginosum)*	*Clostridium uliginosum*^{T} CK55	0.887	AJ276992
Clostridium sp. (*baratii*)	*Clostridium baratii*^{T} ATCC 27638	0.915	X68174
	Aerobic mesophilic spore-formers		
Cytobacillus sp. *(depressus)*	*Cytobacillus depressus*^{T} BZ1	0.879	KP259553
Fredinandcohnia sp. *(onubensis)*	*Fredinandcohnia onubensis*^{T} CECT 8479	0.957	LN650668
Lysinibacillus sp. *(contaminans*)	*Lysinibacillus contaminans*^{T} FSt3A	0.950	KC254732
Lysinibacillus sp. (*macroides*)	*Lysinibacillus macroides*^{T} LMG 18474	0.958	AJ628749
Mesobacillus sp. (*foraminis*)	*Mesobacillus foraminis*^{T} CV53	0.929	AJ717382
Neobacillus sp. (*bataviensis*)	*Neobacillus bataviensis*^{T} NBRC 102449	0.920	AB681791
Neobacillus sp. (*niacini*)	*Neobacillus niacini*^{T} IFO15566	0.962	AB021194
Neobacillus sp. (*notoginsengisoli*)	*Neobacillus notoginsengisoli*^{T} SYP-B691	0.961	KP076294
Paenibacillus sp. (*barcinonensis*)	*Paenibacillus barcinonensis*^{T} BP-23	0.951	AJ716019

Paenibacillus sp. (*etheri*)	*Paenibacillus etheri*^{T} SH7	0.941	KC625558
Paenibacillus sp. *(macerans)*	*Paenibacillus macerans*^{T} IAM 12467	0.934	AB073196
Paenibacillus sp. *(marinisediminis)*	*Paenibacillus marinisediminis*^{T} LHW35	0.954	JF748731
Paenibacillus sp. *(peoriae)*	*Paenibacillus peoriae*^{T} DSM 8320	0.960	AJ320494
Paenibacillus sp. *(phoenicis)*	*Paenibacillus phoenicis*^{T} 3PO2SA	0.946	EU977789
Paenibacillus sp. *(sunsongensis)*	*Paenibacillus susongensis*^{T} M327	0.946	KJ101448
Paenibacillus sp. *(tundrae)*	*Paenibacillus tundrae*^{T} A10b	0.927	EU558284
Peribacillus sp. *(muralis)*	*Peribacillus muralis*^{T} LMG 20238	0.933	AJ316309
	Aerobic thermophilic spore-formers		
Brevibacillus sp. (*thermoruber*)	*Brevibacillus thermoruber*^{T} DSM 7064	0.963	Z26921
Parageobacillus sp. (*caldoxylosilyticus*)	*Parageobacillus caldoxylosilyticus*^{T} S1812	0.936	AF067651
Parageobacillus sp. (*thermoglucosidasius*)	*Parageobacillus thermoglucosidasius*^{T} BGSC 95A1	0.948	AY608981
Siminovitcha sp. (*thermophila*)	*Siminovitcha thermophila*^{T} SgZ-10	0.875	JX274438
	Aerobic psychrotrophic spore-formers		
Acitenobacter sp. (*venetianus*)	*Acinetobacter venetianus*^{T} ATCC 31012	0.925	AJ295007
Neobacillus sp. (*cucumis*)	*Neobacillus cucumis*^{T} AP-6	0.919	KT895286
Paenibacillus sp. (*alkaliterrae*)	*Paenibacillus alkaliterrae*^{T} KSL-134	0.946	AY960748

Paenibacillus sp. (*anaericanus*)	*Paenibacillus anaericanus*^{T} Gsoil 1638	0.949	AB245382
Paenibacillus sp. (*borealis*)	*Paenibacillus borealis*^{T} KK19	0.943	AJ011322
Paenibacillus sp. (*castaneae*)	*Paenibacillus castneae* Ch-32	0.923	EU099594
Paenibacillus sp. (*macquariensis*)	*Paenibacillus macquariensis*^{T} M4-2	0.918	AB360546
Paenibacillus sp. (*odorifer*)	*Paenibacillus odorifer*^{T} TOD45	0.946	AJ223990
Paenibacillus sp. (*salinicaeni*)	*Paenibacillus salinicaeni*^{T} LAM0A28	0.962	KM260652
Paenibacillus sp. (*sonchi*)	*Paenibacillus sonchi*^{T} X19-5	0.950	DQ358736
Paenibacillus sp. (*swuensis*)	*Paenibacillus swuensis*^{T} DY6	0.811	JQ958374
Paenibacillus sp. (*tundrae*)	*Paenibacillus tundrae*^{T} A10b	0.958	EU558284
Peribacillus sp. (*muralis*)	*Peribacillus muralis*^{T} LMG 20238	0.962	AJ316309
Sporosarcina sp. (*globispora*)	*Sporosarcina globispora*^{T} DSM 4	0.950	X68415
Sporosarcina sp. (*newyorkensis*)	*Sporosarcina newyorkensis*^{T} 6062	0.899	GU994085
Sporosarcina sp. (*psychrophila*)	*Sporosarcina psychrophila*^{T} IAM 12468	0.944	D16277
	Non-spore-forming psychrotrophs		
Pseudomonas sp. *(alkyphenolica)*	*Pseudomonas alkyphenolica*^{T} KL28; AY324319	0.954	AB176954
Pseudomonas sp. *(amygdali)*	*Pseudomonas amygdali*^{T} Ogimi 2	0.892	HF558390
Pseudomonas sp. *(cerasi)*	*Pseudomonas cerasi*^{T} 58	0.957	LN713316

Pseudomonas sp. *(congelans)*	*Pseudomonas congelans*[T] DSM 14939	0.940	AJ492828
Pseudomonas sp. *(extremorientalis)*	*Pseudomonas extremorientalis*[T] KMM 3447	0.955	AF405328
Pseudomonas sp. *(graminis)*	*Pseudomonas graminis*[T] DSM 11363	0.936	Y11150
Pseudomonas sp. *(jessenii)*	*Pseudomonas jessenii*[T] CIP 105274	0.912	AF068259
Pseudomonas sp. *(kilonensis)*	*Pseudomonas kilonensis*[T] 520-20	0.907	AJ292426
Pseudomonas sp. *(migulae)*	*Pseudomonas migulae*[T] CIP 105470	0.948	AF074383
Pseudomonas sp. *(oryzihabitans)*	*Pseudomonas oryzihabitans*[T] IAM 1568	0.955	D84004
Pseudomonas sp. *(plecoglossicida)*	*Pseudomonas plecoglossicida*[T] FPC951	0.953	AB009457
Pseudomonas sp. *(rhodesiae)*	*Pseudomonas rhodesiae*[T] CIP 104664	0.946	AF064459
Pseudomonas sp. *(viridiflava)*	*Pseudomonas viridiflava*[T] CECT 458	0.911	AY180972
Stenotrophomonas sp. *(bentonitica)*	*Stenotrophomonas bentonitica*[T] BII-R7	0.873	HG800055

[T]: Type strain

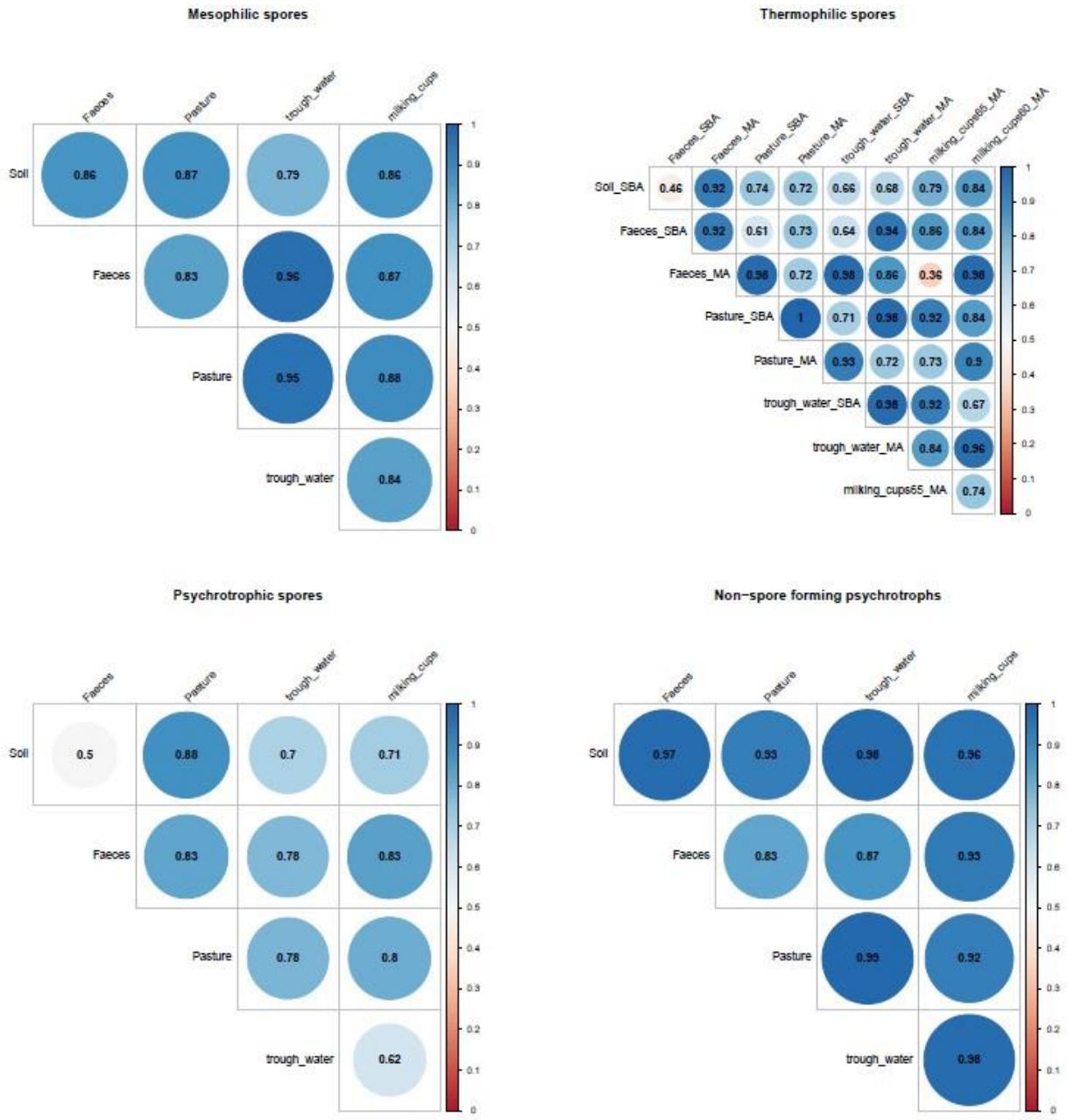
[1] Most closely related type strain 16S rRNA sequence as found in the RDP

[2] Highest seqmatch RDP score (based on shared 7-base oligomers) obtained against a given type strain species.

[3] Accession number for the type strain in the GENBANK database

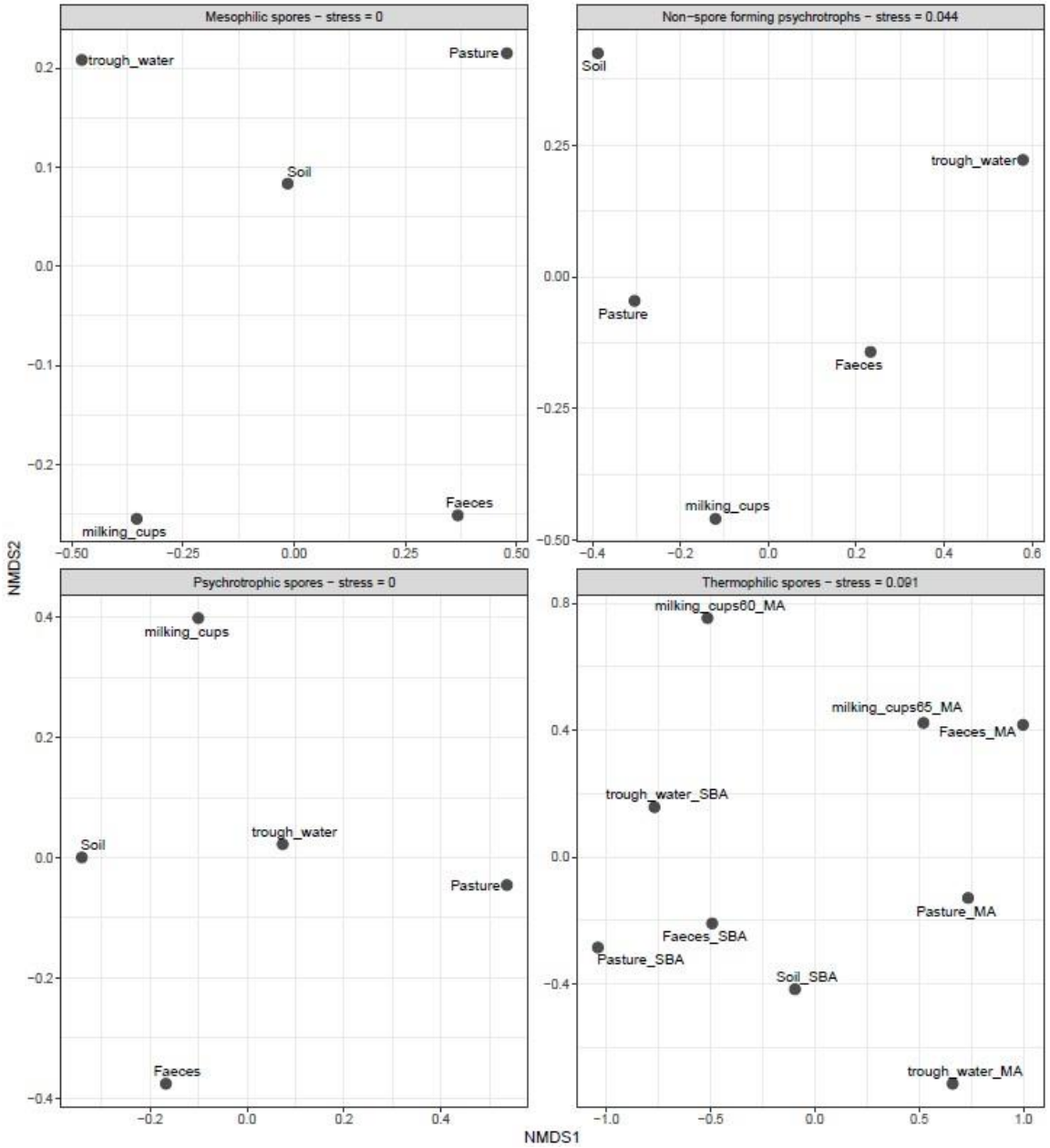
Appendix 5.2. Diversity indexes calculated for each sample type and each bacterial group of interest

Sample	Shannon	Simpson	Inverse.Simpson
Mesophilic spores			
Soil	2.425	0.893	9.375
Faeces	1.794	0.745	3.926
Pasture	0.837	0.398	1.660
trough_water	2.770	0.926	13.442
milking_cups	2.429	0.851	6.693
Thermophilic spores			
Soil_SBA	0.920	0.499	1.996
Faeces_SBA	0.701	0.431	1.757
Faeces_MA	1.523	0.728	3.682
Pasture_SBA	0.000	0.000	1.000
Pasture_MA	1.443	0.696	3.294
trough_water_SBA	0.444	0.215	1.273
trough_water_MA	1.979	0.847	6.545
milking_cups65_MA	1.517	0.704	3.378
milking_cups60_MA	0.957	0.549	2.215
Psychrotrophic spores			
Soil	2.271	0.876	8.048
Faeces	1.557	0.716	3.519
Pasture	1.278	0.647	2.830
trough_water	2.095	0.802	5.062
milking_cups	1.956	0.806	5.142
Non-spore forming psychrotrophs			
Soil	2.386	0.884	8.643
Faeces	1.524	0.687	3.199
Pasture	2.505	0.888	8.924
trough_water	1.052	0.559	2.267
milking_cups	1.780	0.796	4.898



Appendix 5.3. Bray-Curtis distances between the bacterial diversity of four bacterial group isolated from samples collected on a New Zealand sheep dairy farm.

A colour scale indicates similarity (red) to dissimilarity (blue).



Appendix 5.4. Non-metric multidimensional scaling (NMDS) plot of bacterial diversity isolated from farm samples using culture-dependent methods.

The different sample types are visible as dots and annotated. For thermophilic spore-formers, thermophilic diversity is either on Columbia sheep blood agar (SBA) or milk agar (MA).

Appendix 5.5. Taxonomy of the *Pseudomonas* species isolated from F2

***Pseudomonas fluorescens* lineage**						
Pseudomonas fluorescens complex						
P. fluorescens subgroup	*P. fragi* subgroup	*P. koreensis* subgroup	*P. mandelii* subgroup	*P. corrugata* subgroup	*P. gessardii* subgroup	*P. jessenii* subgroup
P. canadensis *P. cedrina* *P. costantinii* *P. extremaustralis* *P. libanensis* *P. orientalis* *P. poae* *P. rhodesiae* *Pseudomonas* sp. (*extremorientalis*) *Pseudomonas* sp. (*rhodesiae*)	*P. fragi* *P. weihenstephanensis* *P. lundensis*	*P. koreensis* *P. helmanticensis* *P. granadensis*	*P. migulae* *Pseudomonas* sp. (*migulae*)	*Pseudomonas* sp. (*kilonensis*)	*P. yamanorum*	*P. jessenii* *P. reinekei* *P. umsongensis* *P. vancouverensis* *Pseudomonas* sp. (*jessenii*)

***Pseudomonas aeruginosa* lineage**	***Pseudomonas fluorescens* lineage**				
	P. syringae group	*P. putida* group	*P. rhizosphaerae* group	*P. lutea* group	*P. asplenii* group
P. oryzihabitans *Pseudomonas* sp. (*oryzihabitans*)	*P. amygdali* *P. asturiensis* *P. cannbina* *P. cerasi* *P. congelans* *P. viriflava* *Pseudomonas* sp. (*amygdali*) *Pseudomonas* sp. (*cerasi*) *Pseudomonas* sp. (*congelans*) *Pseudomonas* sp. (*viridiflava*)	*P. fulva s.l.* *Pseudomonas* sp. (*plecoglossicida*)	*Pseudomonas rhizosphaerae*	*Pseudomonas* sp. (*graminis*)	*P. asplenii* *Pseudomonas* sp. (*alkylphenolica*)

Appendix 6.1. Bacterial and phylogenetic dataset

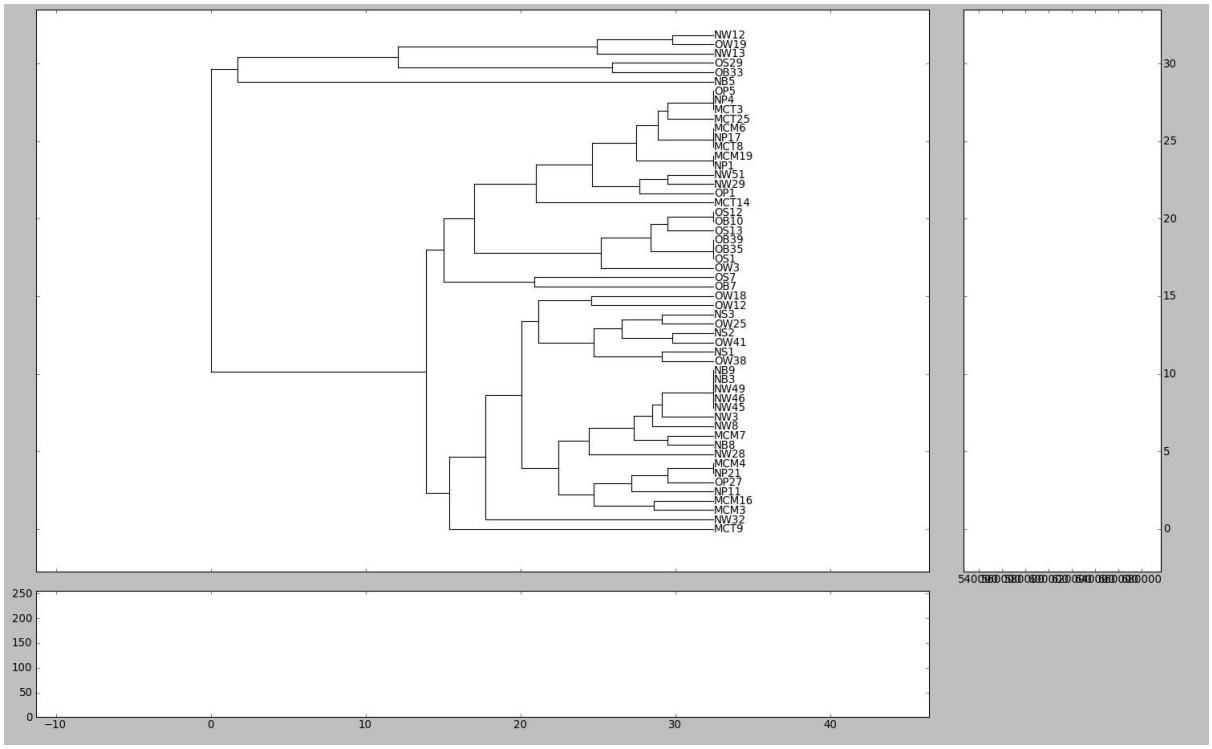
Species	Farm	Isolation samples	Isolation group	No. of isolates	Fingerprinting type	Fingerprinting profiles	Median band number	Average band number	Min similarity to MC	Max similarity to MC	Average similarity to MC
Aeribacillus pallidus	F1	Silage, Faeces	Thermophilic spore-formers	4	ERIC-PCR	3	8	7.5	0.8	0.93	0.9
	F2	Soil, Pasture, Trough water	Thermophilic spore-formers	15	ERIC-PCR	14	9	9.53	0	1	0.49
Alkalihalobacillus clausii	F1	Bedding, Silage, Trough water, Faeces	Aerobic mesophilic spore-formers	28	ERIC-PCR	20	7	7.64	0.3	1	0.65
	F2	Faeces	Aerobic mesophilic spore-formers	3	ERIC-PCR	3	11	12.33	0.73	0.97	0.81
Bacillus cereus	F2	Soil, Faeces, Trough water, Pasture	Psychrotrophic spore-formers, Aerobic mesophilic spore-formers	50	ERIC-PCR	50	9.5	10.16	0	0.71	0.24
Bacillus licheniformis	F1	Bedding, Silage, Trough water, Faeces	Aerobic mesophilic spore-formers	43	ERIC-PCR	35	8	8.47	0.1	1	0.66
	F2	Faeces, Trough water	Aerobic mesophilic spore-formers	2	ERIC-PCR	2	11.5	11.5	0.8	1	0.9

***Bacillus pumilus* type B**	F1	Bedding, Silage, Faeces, Trough water	Psychrotrophic spore-formers, Aerobic mesophilic spore-formers	23	ERIC-PCR	15	4	4.3	0.29	1	0.74
***Bacillus pumilus* types A and C**	F1	Bedding, Faeces, Silage, Trough water	Aerobic mesophilic spore-formers	19	ERIC-PCR	14	4	3.79	0	0.8	0.39
Bacillus smithii	F2	Faeces, Pasture	Thermophilic spore-formers	3	ERIC-PCR	3	13	12.67	0.42	0.6	0.52
Bacillus subtilis	F1	Silage, Faeces, Trough water	Aerobic mesophilic spore-formers	8	ERIC-PCR	8	12	11.88	0.5	0.92	0.75
Caldibacillus kokeshiiformis	F1	Silage, Faeces, Trough water	Thermophilic spore-formers	11	ERIC-PCR	8	7	6.64	0.57	0.92	0.8
Lysinibacillus macroides	F2	Soil	Aerobic mesophilic spore-formers	3	ERIC-PCR	2	12	11.67	0.8	0.84	0.81
***Oceanobacillus* sp. (*chungangensis*)**	F1	Faeces, Silage, Bedding	Psychrotrophic spore-formers, Aerobic mesophilic spore-formers	3	ERIC-PCR	3	10	9.67	0.13	0.44	0.33

Paenibacillus anaericanus	F2	Soil, Faeces, Pasture	Psychrotrophic spore-formers	4	ERIC-PCR	4	10	10.75	0.4	0.9	0.61
Paenibacillus tundrae	F2	Soil, Trough water	Psychrotrophic spore-formers, Aerobic mesophilic spore-formers	5	ERIC-PCR	5	12	14	0.1	0.62	0.43
Paenibacillus xylanedexens	F1	Silage, Faeces, Trough water	Psychrotrophic spore-formers	15	ERIC-PCR	15	10	10.33	0.1	0.43	0.3
Parageobacillus caldoxylosilyticus	F2	Faeces, Trough water	Thermophilic spore-formers	4	ERIC-PCR	4	5.5	5.25	0.33	1	0.64
Peribacillus psychrosaccharolyticus	F1	Bedding, Silage, Faeces, Trough water	Psychrotrophic spore-formers	11	ERIC-PCR	11	11	11.36	0.4	0.83	0.65
	F2	Trough water	Psychrotrophic spore-formers	1	ERIC-PCR	1	6	6	0.25	0.4	0.33
Priestia megaterium	F2	Soil, Faeces, Pasture, Trough water	Aerobic mesophilic spore-formers	48	ERIC-PCR	47	9	9.54	0.21	1	0.59
Pseudomonas azotoformans	F1	Faeces	*Pseudomonas* spp.	1	ERIC-PCR	1	9	9	0.19	0.32	0.27
Pseudomonas koreensis	F1	Bedding, Faeces	*Pseudomonas* spp.	2	ERIC-PCR	2	10.5	10.5	0.38	0.88	0.55
	F2	Pasture, Faeces	*Pseudomonas* spp.	3	ERIC-PCR	3	13	13	0.67	0.79	0.73

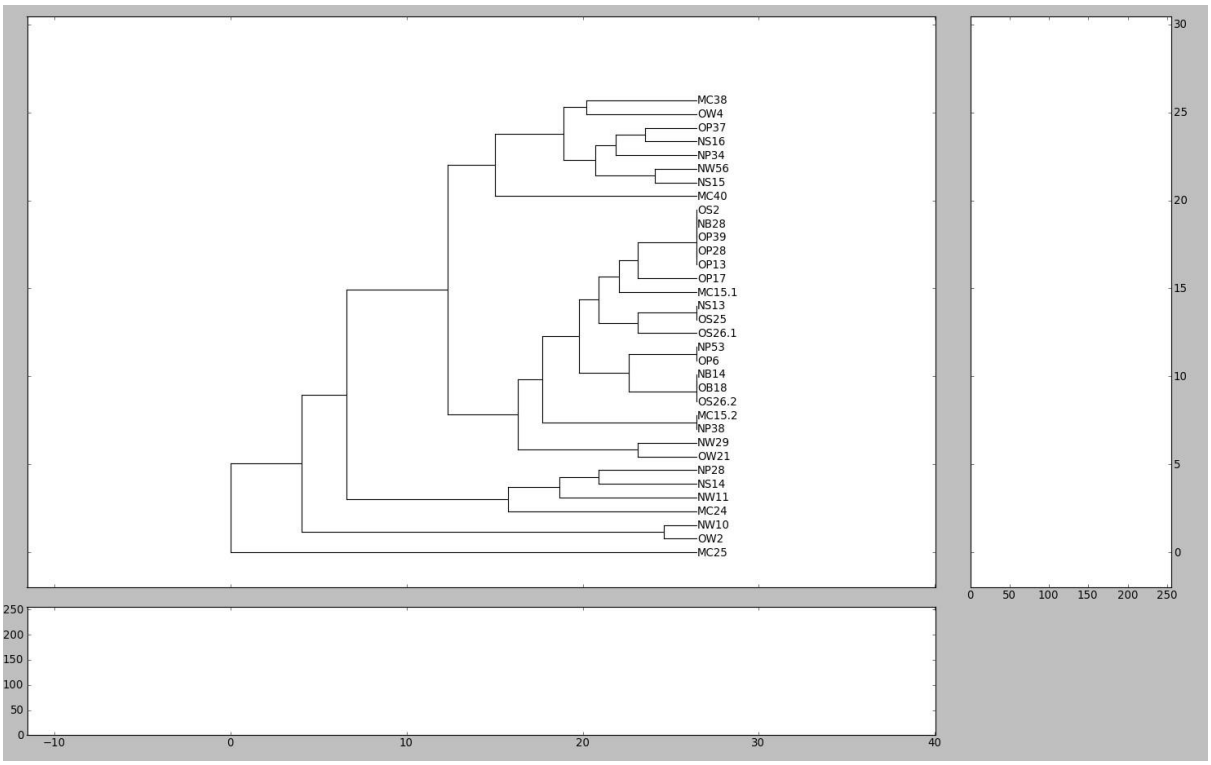
Pseudomonas libanensis	F1	Faeces	*Pseudomonas* spp.	1	ERIC-PCR	1	13	13	0.45	0.69	0.58
Pseudomonas poae	F2	Soil, Pasture, Trough water	*Pseudomonas* spp.	16	ERIC-PCR	15	11.5	11.31	0.24	0.67	0.44
***Thermoactinomyces* spp. (*T. vulgaris, T. intermedius*)**	F1	Trough water, Faeces, Silage, Bedding	Thermophilic spore-formers	72	ERIC-PCR	54	9	8.6	0.25	1	0.76
	F2	Soil, Faeces, Pasture, Trough water	Thermophilic spore-formers	25	ERIC-PCR	24	10	9.32	0.11	1	0.53
Clostridium butyricum	F2	Trough water, Soil	Anaerobic mesophilic spore-formers	8	ARDRA (AluI)	3	12	12.25	0.83	1	0.87
	F2	Trough water, Soil	Anaerobic mesophilic spore-formers	8	ARDRA (HhaI)	2	7	6.75	0.92	1	0.94
Clostridium perfringens	F1	Bedding, Silage, Trough water, Faeces	Anaerobic mesophilic spore-formers	17	ARDRA (HhaI)	6	6	6.18	0.71	1	0.86
	F2	Pasture, Faeces, Soil	Anaerobic mesophilic spore-formers	13	ARDRA (HhaI)	7	7	6.62	0.67	1	0.87

Paraclostridium benzoelyticum	F1	Bedding, Silage, Faeces	Anaerobic mesophilic spore-formers	27	ARDRA (HhaI)	10	9	8.81	0.67	0.95	0.81
	F2	Soil, Pasture, Trough water	Anaerobic mesophilic spore-formers	12	ARDRA (AluI)	1	18	18	1	1	1
	F2	Soil, Pasture, Trough water	Anaerobic mesophilic spore-formers	12	ARDRA (HhaI)	1	6	6	1	1	1



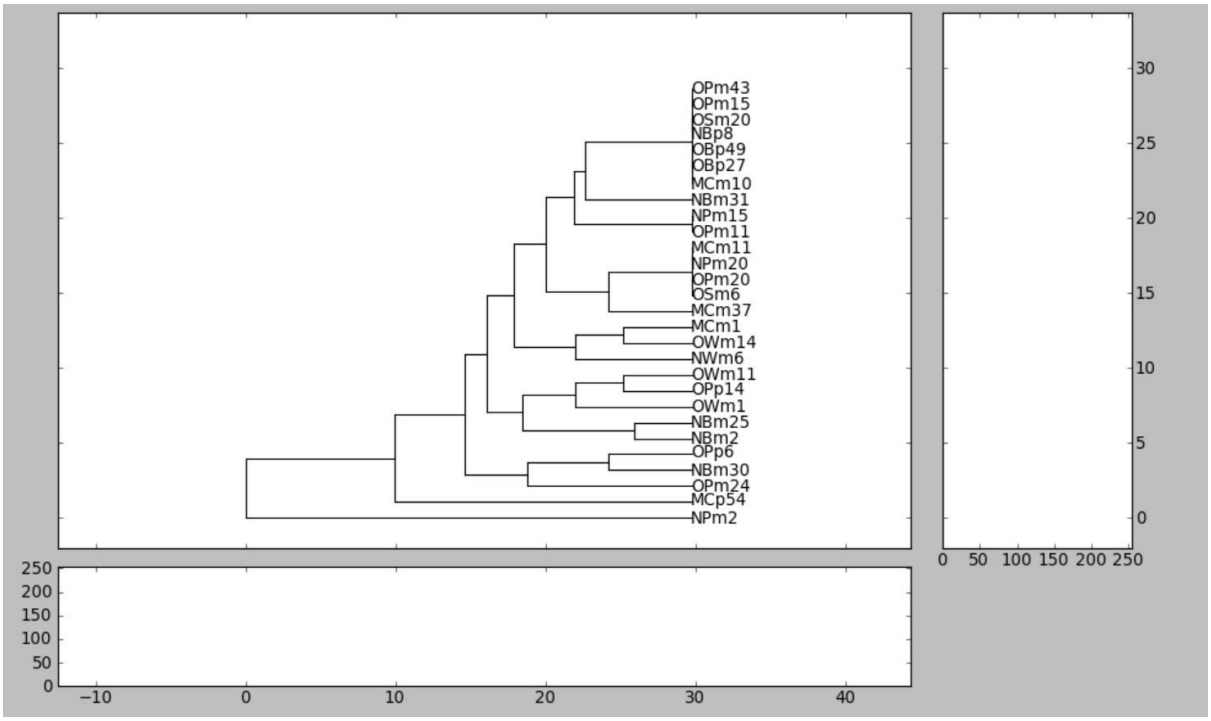
Appendix 6.2. Dendrogram generated with UPGMA clustering showing the genetic similarity of F1 *Bacillus licheniformis* isolates according to their enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) profile.

The bottom x axis displays genetic distances in %. OB = "Old" bedding isolate; NB = "New" bedding isolate; OS = "Old" silage isolate; NS = "New" silage isolate; OP = "Old" faeces isolate; NP = "New" faeces isolate; OW = "Old" trough water isolate; NW = "New" trough water isolate; MCT = Thermophilic milking cups isolate; MCM = Mesophilic milking cups isolate.



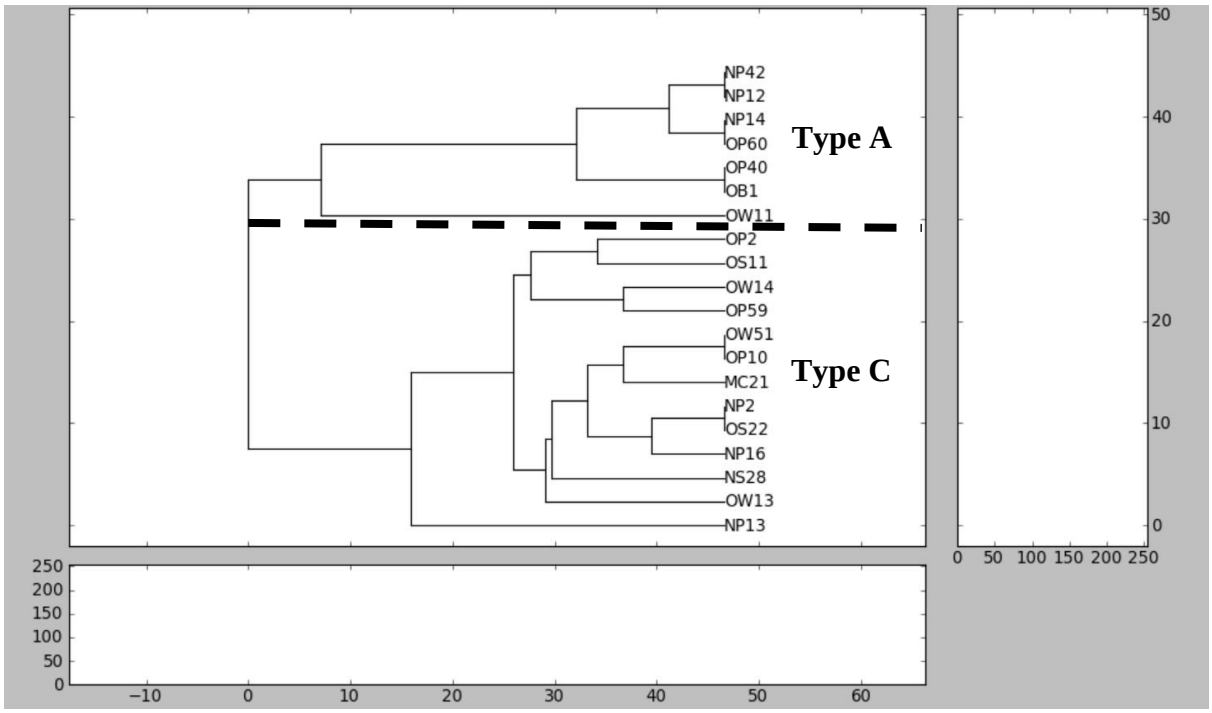
Appendix 6.3. Dendrogram generated with UPGMA clustering showing the genetic similarity of F1 *Alkalihalobacillus clausii* isolates according to their enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) profile.

The bottom x axis displays genetic distances in %. OB = "Old" bedding isolate; NB = "New" bedding isolate; OS = "Old" silage isolate; NS = "New" silage isolate; OP = "Old" faeces isolate; NP = "New" faeces isolate; OW = "Old" trough water isolate; NW = "New" trough water isolate; MC = Milking cups isolate.



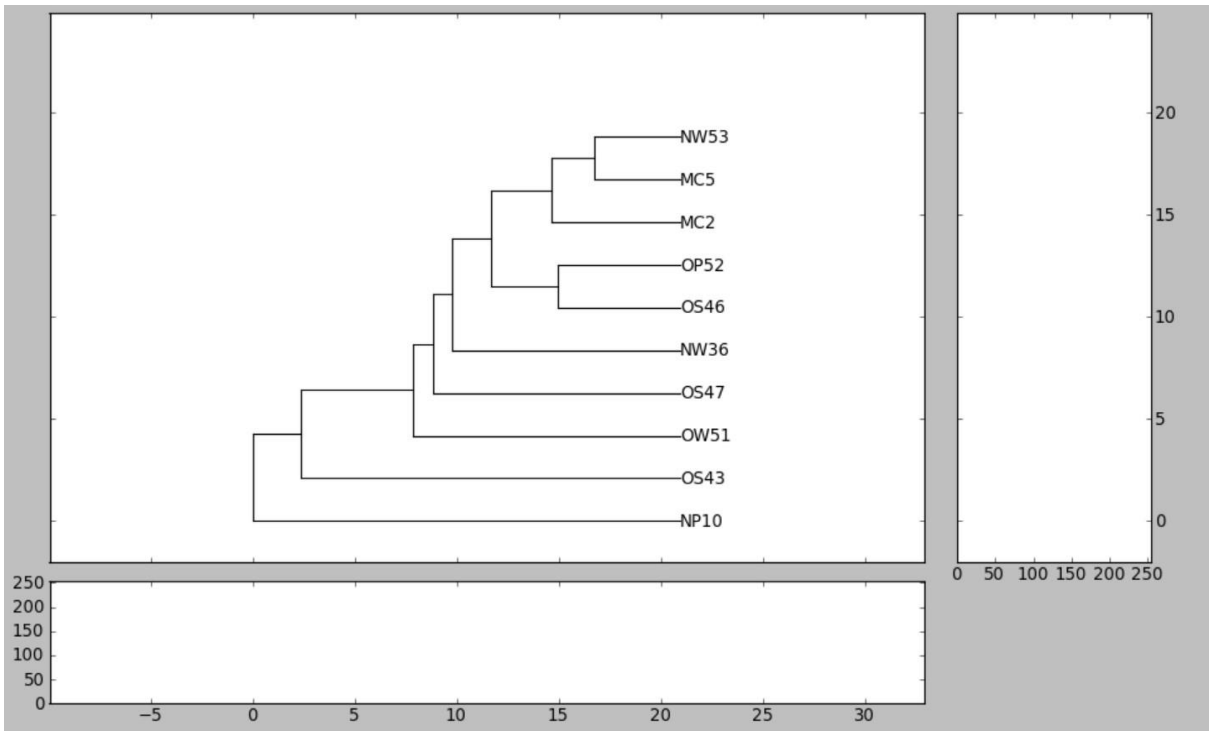
Appendix 6.4. Dendrogram generated with UPGMA clustering showing the genetic similarity of F1 *Bacillus pumilus s.l.* type B isolates according to their enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) profile.

The bottom x axis displays genetic distances in %. OB = "Old" bedding isolate; NB = "New" bedding isolate; OS = "Old" silage isolate; NS = "New" silage isolate; OP = "Old" faeces isolate; NP = "New" faeces isolate; OW = "Old" trough water isolate; NW = "New" trough water isolate; MC = Milking cups; m or p: mesophilic or psychrotrophic isolate, respectively. The isolate NPm2 represents a type *B. pumilus s.l.* type C isolate which is provided for reference.



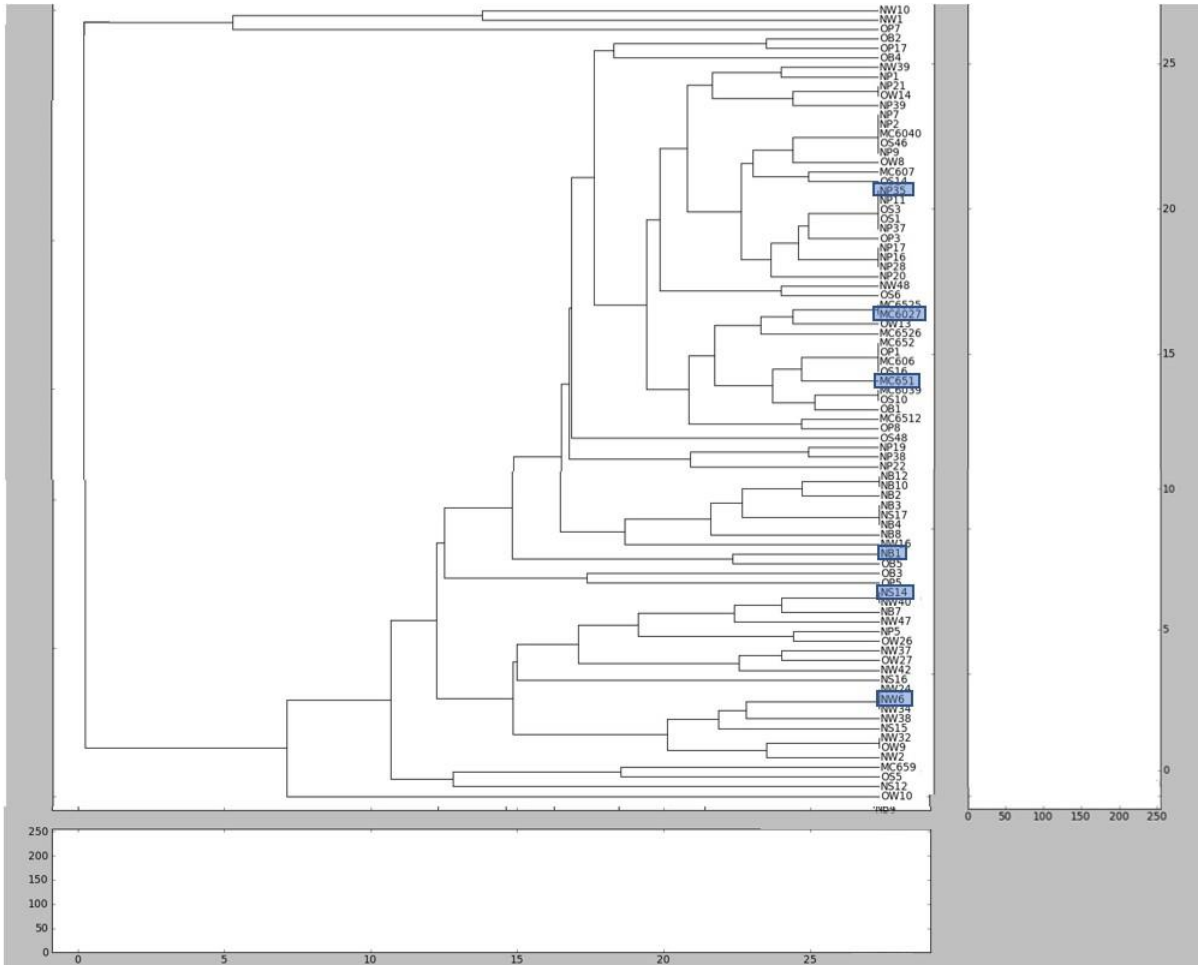
Appendix 6.5. Dendrogram generated with UPGMA clustering showing the genetic similarity of F1 *Bacillus pumilus* types A and C isolates according to their enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) profile.

The bottom x axis displays genetic distances in %. OB = "Old" bedding isolate; NB = "New" bedding isolate; OS = "Old" silage isolate; NS = "New" silage isolate; OP = "Old" faeces isolate; NP = "New" faeces isolate; OW = "Old" trough water isolate; NW = "New" trough water isolate; MC = Milking cups isolate.



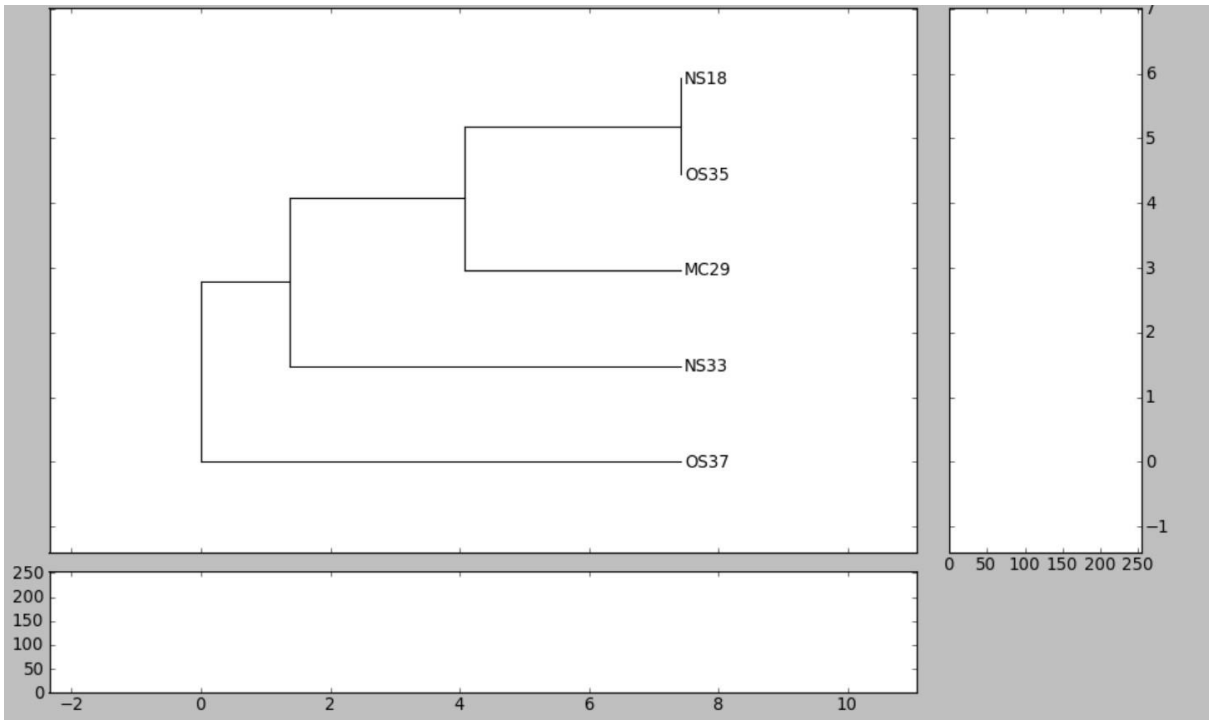
Appendix 6.6. Dendrogram generated with UPGMA clustering showing the genetic similarity of F1 *Bacillus subtilis* isolates according to their enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) profile.

The bottom x axis displays genetic distances in %. OB = "Old" bedding isolate; NB = "New" bedding isolate; OS = "Old" silage isolate; NS = "New" silage isolate; OP = "Old" faeces isolate; NP = "New" faeces isolate; OW = "Old" trough water isolate; NW = "New" trough water isolate; MC = Milking cups isolate.



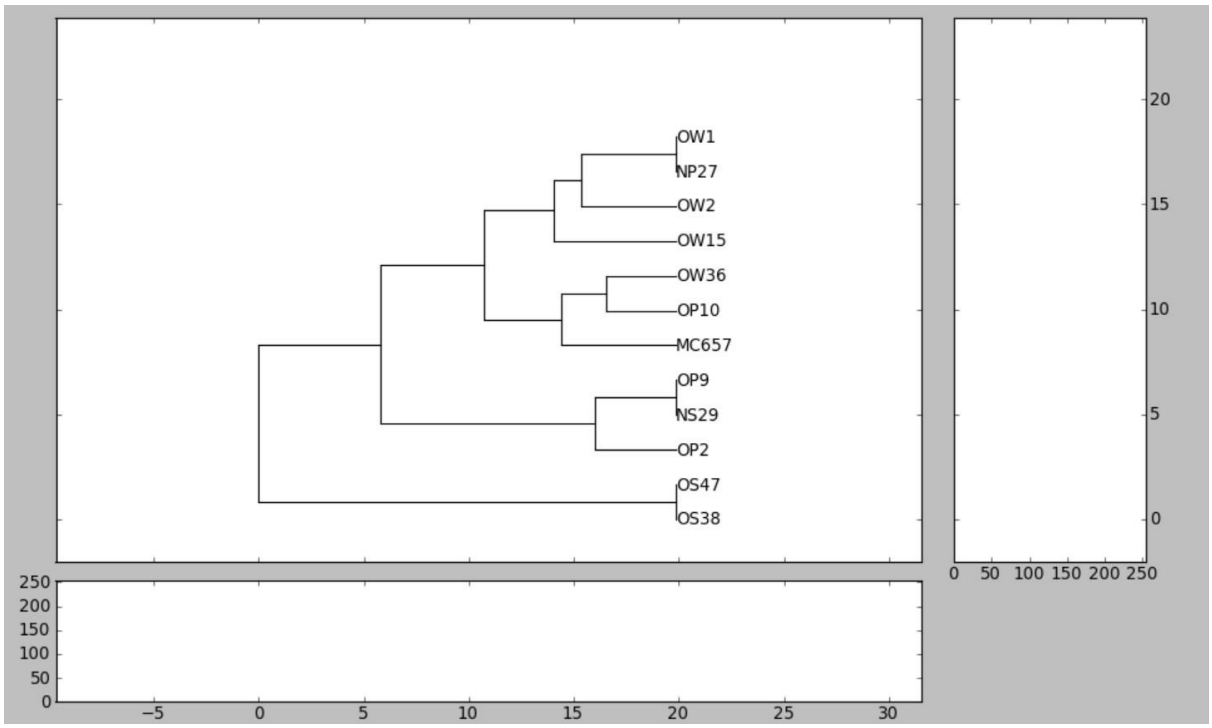
Appendix 6.7. Dendrogram generated with UPGMA clustering showing the genetic similarity of F1 *Thermoactinomyces vulgaris* isolates according to their enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) profile.

The bottom x axis displays genetic distances in %. OB = "Old" bedding isolate; NB = "New" bedding isolate; OS = "Old" silage isolate; NS = "New" silage isolate; OP = "Old" faeces isolate; NP = "New" faeces isolate; OW = "Old" trough water isolate; NW = "New" trough water isolate; MC = Milking cups isolate. The isolates highlighted in blue were selected for whole-genome sequencing.



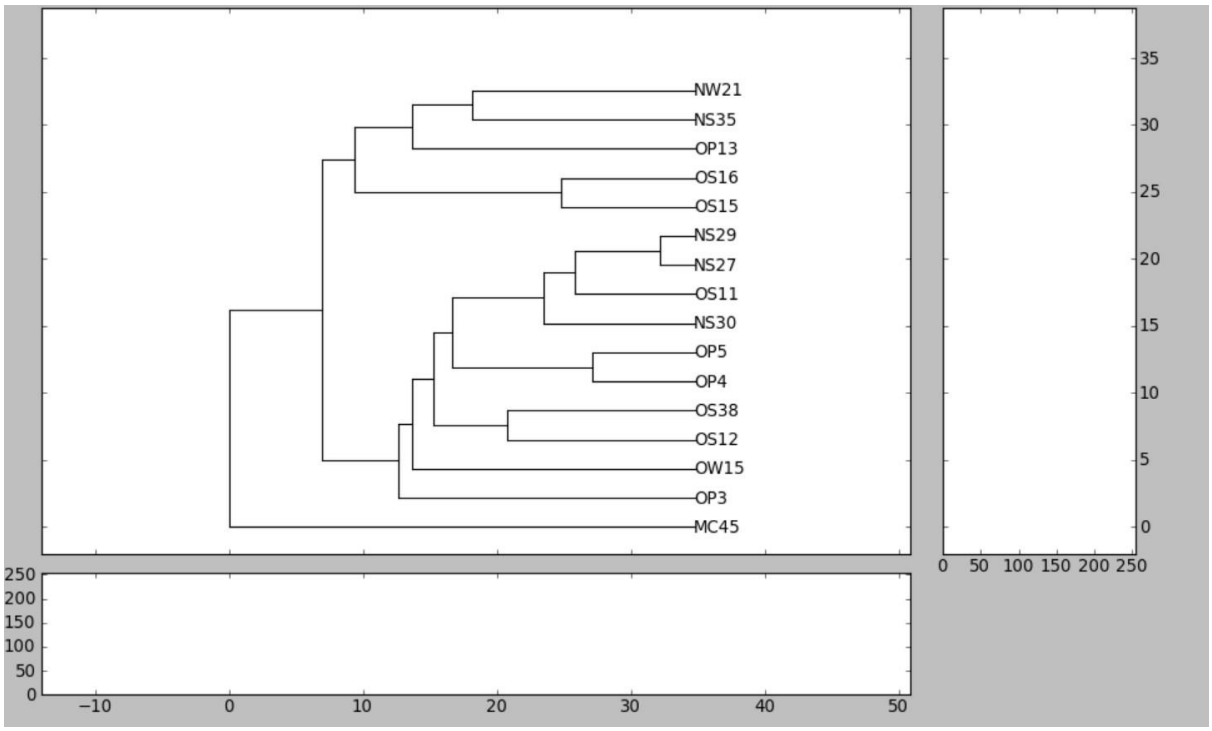
Appendix 6.8. Dendrogram generated with UPGMA clustering showing the genetic similarity of F1 *Aeribacillus pallidus* isolates according to their enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) profile.

The bottom x axis displays genetic distances in %. OB = "Old" bedding isolate; NB = "New" bedding isolate; OS = "Old" silage isolate; NS = "New" silage isolate; OP = "Old" faeces isolate; NP = "New" faeces isolate; OW = "Old" trough water isolate; NW = "New" trough water isolate; MC = Milking cups isolate.



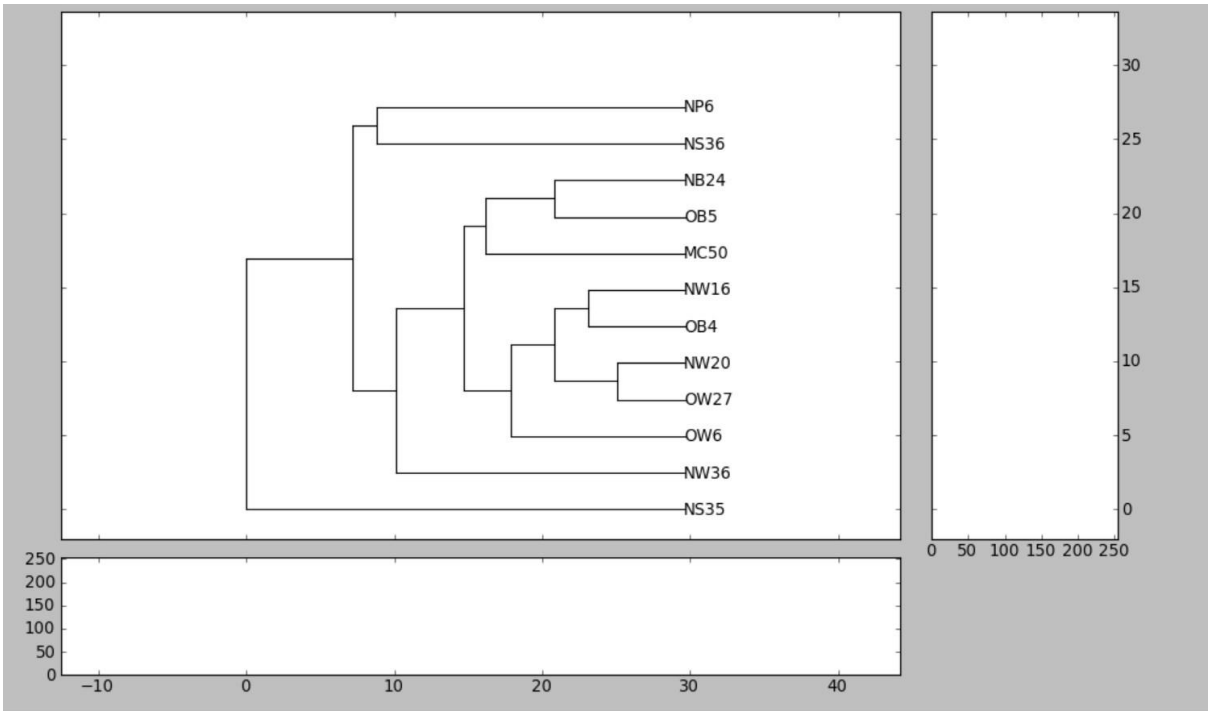
Appendix 6.9. Dendrogram generated with UPGMA clustering showing the genetic similarity of F1 *Caldibacillus kokeshiiformis* isolates according to their enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) profile.

The bottom x axis displays genetic distances in %. OB = "Old" bedding isolate; NB = "New" bedding isolate; OS = "Old" silage isolate; NS = "New" silage isolate; OP = "Old" faeces isolate; NP = "New" faeces isolate; OW = "Old" trough water isolate; NW = "New" trough water isolate; MC = Milking cups isolate.



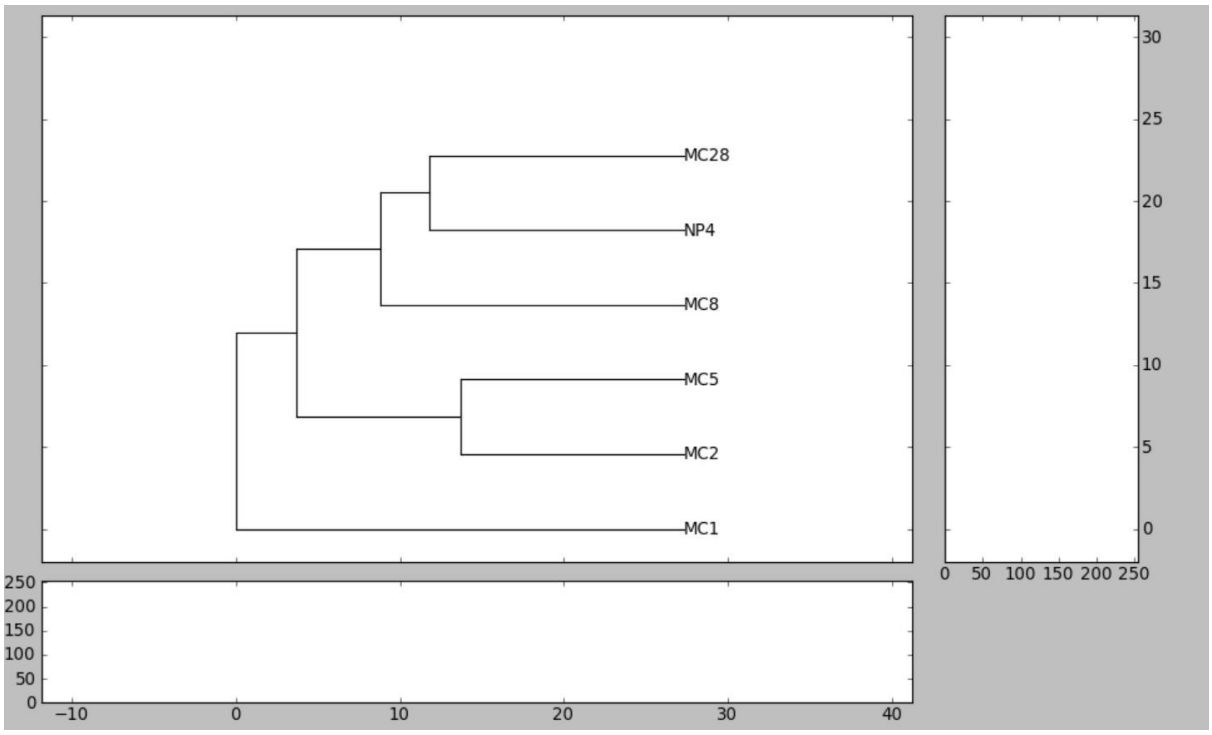
Appendix 6.10. Dendrogram generated with UPGMA clustering showing the genetic similarity of F1 *Paenibacillus xylanexedens* isolates according to their enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) profile.

The bottom x axis displays genetic distances in %. OB = "Old" bedding isolate; NB = "New" bedding isolate; OS = "Old" silage isolate; NS = "New" silage isolate; OP = "Old" faeces isolate; NP = "New" faeces isolate; OW = "Old" trough water isolate; NW = "New" trough water isolate; MC = Milking cups isolate.



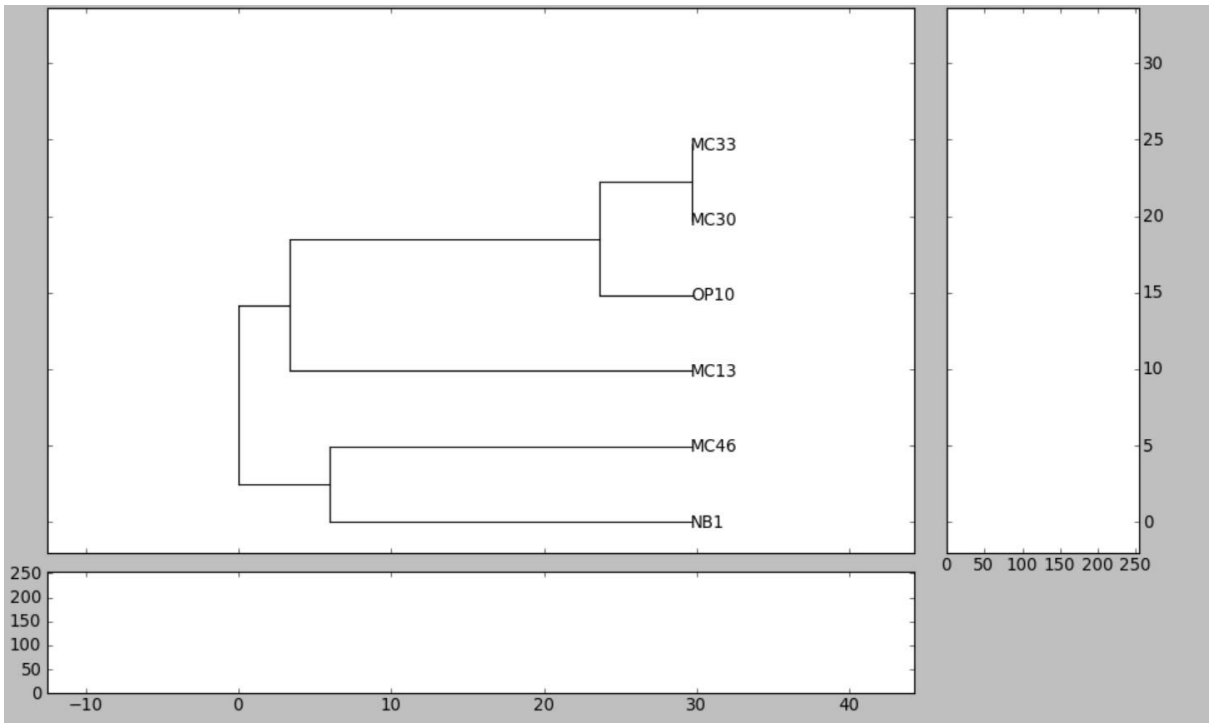
Appendix 6.11. Dendrogram generated with UPGMA clustering showing the genetic similarity of F1 *Peribacillus psychrosaccharolyticus* isolates according to their enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) profile.

The bottom x axis displays genetic distances in %. OB = "Old" bedding isolate; NB = "New" bedding isolate; OS = "Old" silage isolate; NS = "New" silage isolate; OP = "Old" faeces isolate; NP = "New" faeces isolate; OW = "Old" trough water isolate; NW = "New" trough water isolate; MC = Milking cups isolate.



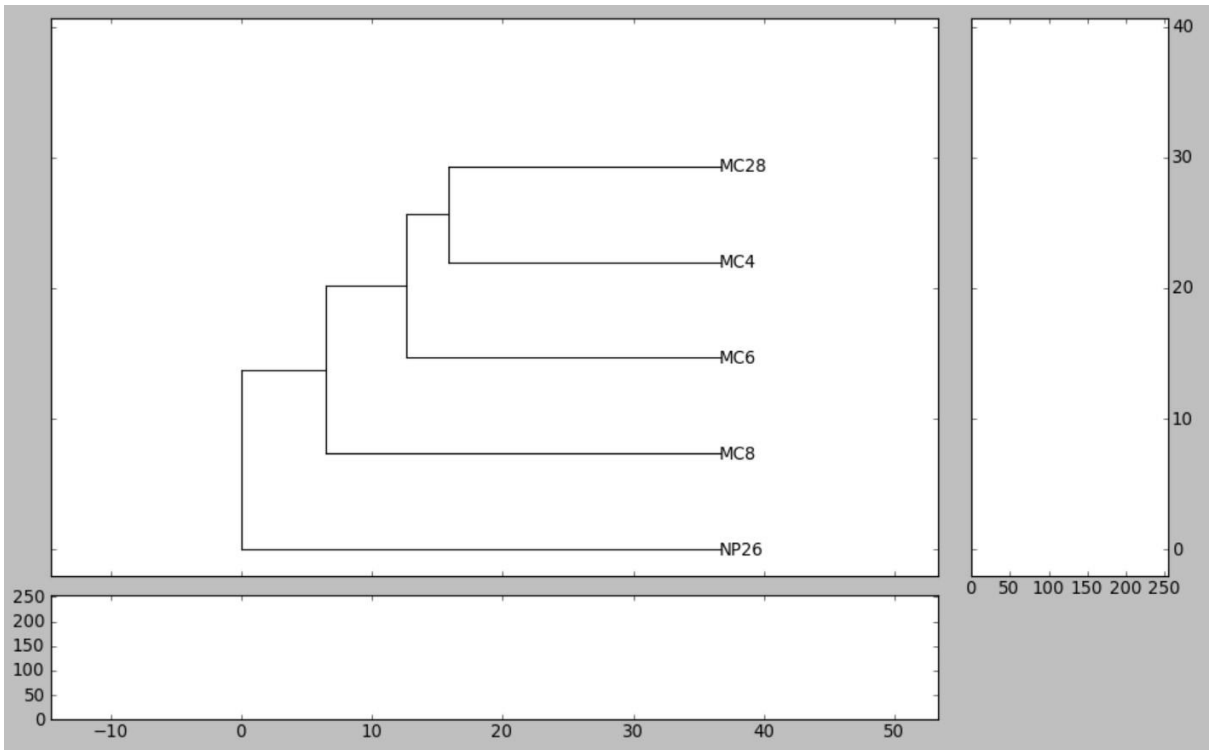
Appendix 6.12. Dendrogram generated with UPGMA clustering showing the genetic similarity of F1 *Oceanobacillus* sp. (*chungangensis*) isolates according to their enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) profile.

The bottom x axis displays genetic distances in %. OB = "Old" bedding isolate; NB = "New" bedding isolate; OS = "Old" silage isolate; NS = "New" silage isolate; OP = "Old" faeces isolate; NP = "New" faeces isolate; OW = "Old" trough water isolate; NW = "New" trough water isolate; MC = Milking cups isolate.



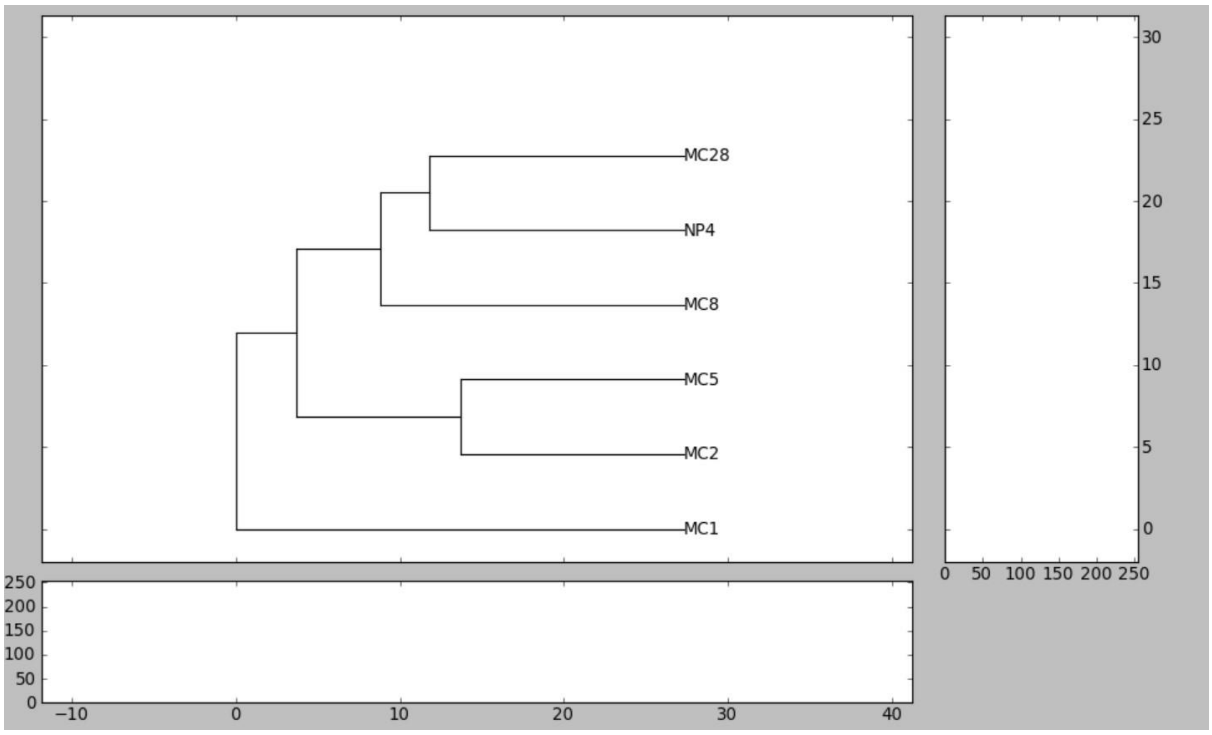
Appendix 6.13. Dendrogram generated with UPGMA clustering showing the genetic similarity of F1 *Pseudomonas koreensis* isolates according to their enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) profile.

The bottom x axis displays genetic distances in %. OB = "Old" bedding isolate; NB = "New" bedding isolate; OS = "Old" silage isolate; NS = "New" silage isolate; OP = "Old" faeces isolate; NP = "New" faeces isolate; OW = "Old" trough water isolate; NW = "New" trough water isolate; MC = Milking cups isolate.



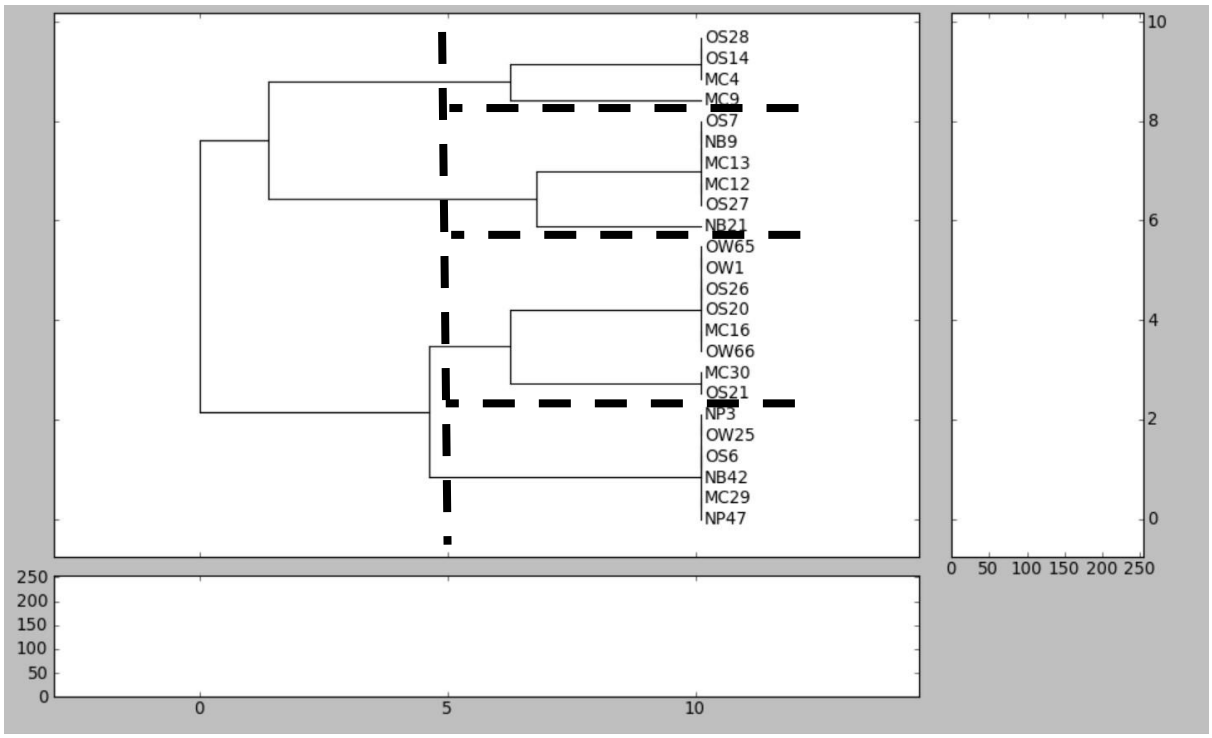
Appendix 6.14. Dendrogram generated with UPGMA clustering showing the genetic similarity of F1 *Pseudomonas azotoformans* isolates according to their enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) profile.

The bottom x axis displays genetic distances in %. OB = "Old" bedding isolate; NB = "New" bedding isolate; OS = "Old" silage isolate; NS = "New" silage isolate; OP = "Old" faeces isolate; NP = "New" faeces isolate; OW = "Old" trough water isolate; NW = "New" trough water isolate; MC = Milking cups isolate.



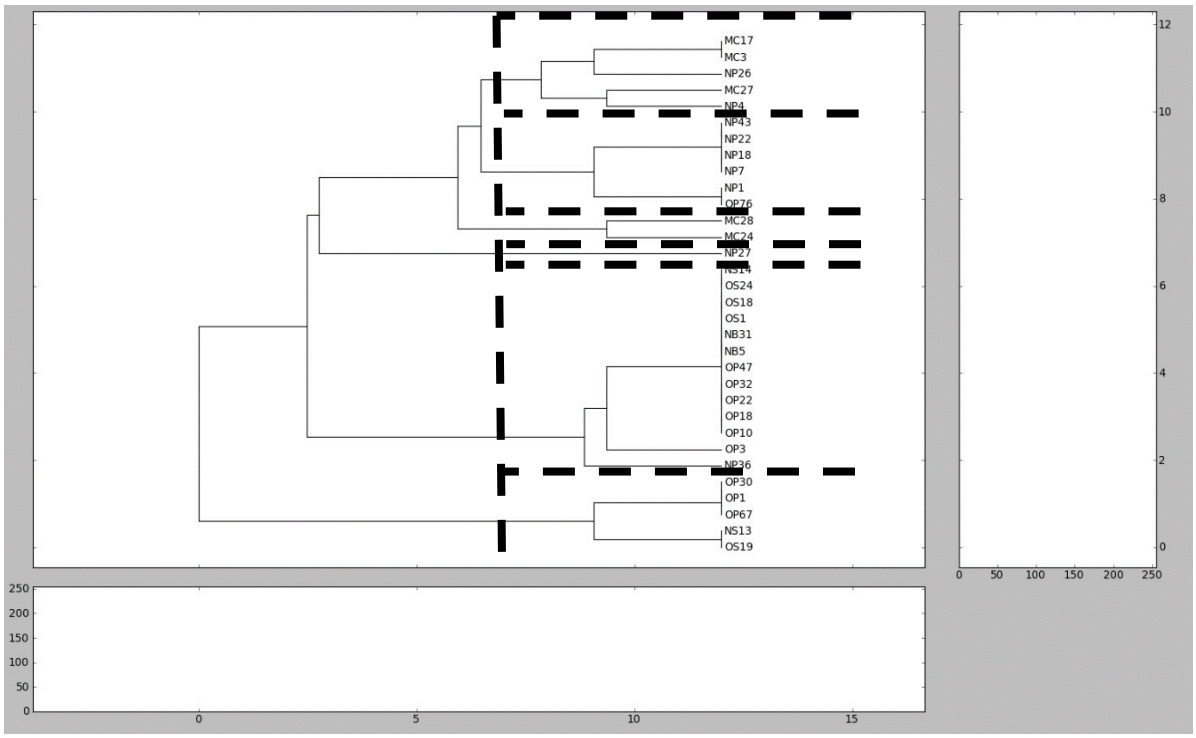
Appendix 6.15. Dendrogram generated with UPGMA clustering showing the genetic similarity of F1 *Pseudomonas libanensis* isolates according to their enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) profile.

The bottom x axis displays genetic distances in %. OB = "Old" bedding isolate; NB = "New" bedding isolate; OS = "Old" silage isolate; NS = "New" silage isolate; OP = "Old" faeces isolate; NP = "New" faeces isolate; OW = "Old" trough water isolate; NW = "New" trough water isolate; MC = Milking cups isolate.



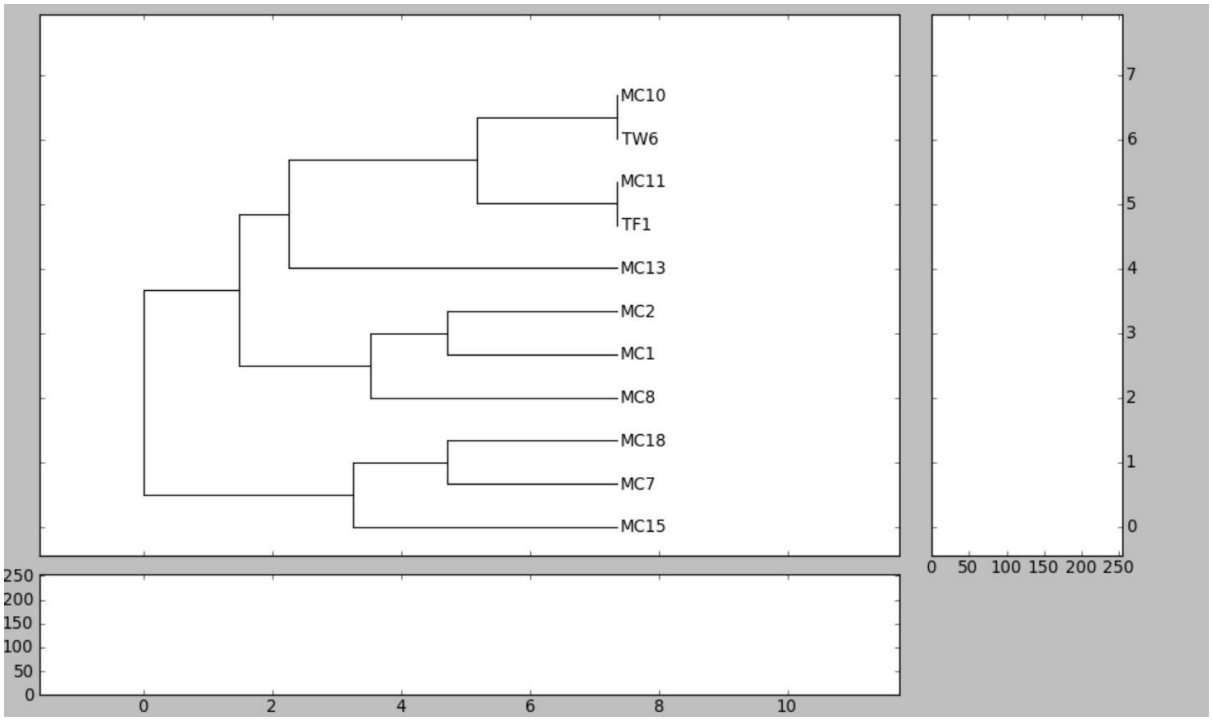
Appendix 6.16. Dendrogram generated with UPGMA clustering showing the genetic similarity of F1 *Clostridium perfringens* isolates according to their Amplified Ribosomal DNA Restriction Analysis (ARDRA) profile with the HhaI restriction enzyme.

The bottom x axis displays genetic distances in %. The four clusters mentioned in the text are delimited by horizontal black dash lines and correspond to 95% ARDRA fingerprint similarity (vertical black dash line). OB = "Old" bedding isolate; NB = "New" bedding isolate; OS = "Old" silage isolate; NS = "New" silage isolate; OP = "Old" faeces isolate; NP = "New" faeces isolate; OW = "Old" trough water isolate; NW = "New" trough water isolate; MC = Milking cups isolate.



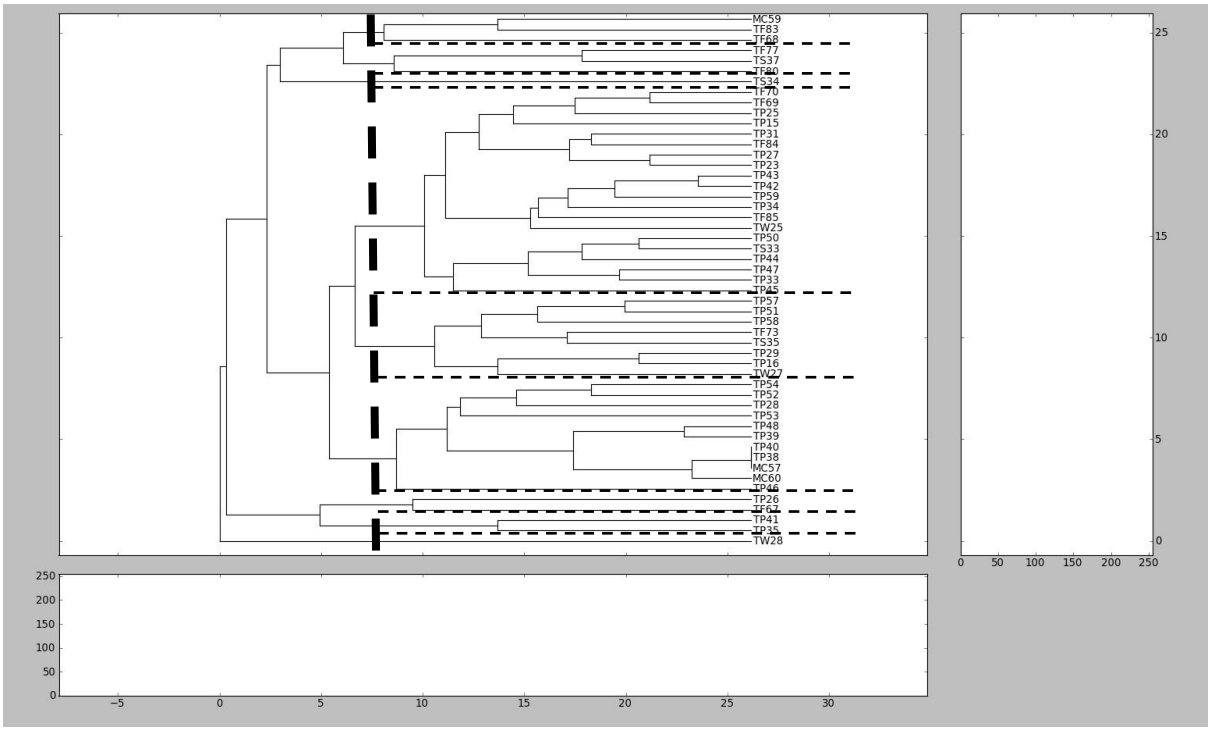
Appendix 6.17. Dendrogram generated with UPGMA clustering showing the genetic similarity of F1 *Paraclostridium benzoelyticum* isolates according to their Amplified Ribosomal DNA Restriction Analysis (ARDRA) profile with the HhaI restriction enzyme.

The bottom x axis displays genetic distances in %. The six clusters mentioned in the text are delimited by horizontal black dash lines and correspond to 95% ARDRA fingerprint similarity (vertical black dash line). OB = "Old" bedding isolate; NB = "New" bedding isolate; OS = "Old" silage isolate; NS = "New" silage isolate; OP = "Old" faeces isolate; NP = "New" faeces isolate; OW = "Old" trough water isolate; NW = "New" trough water isolate; MC = milking cups isolate.



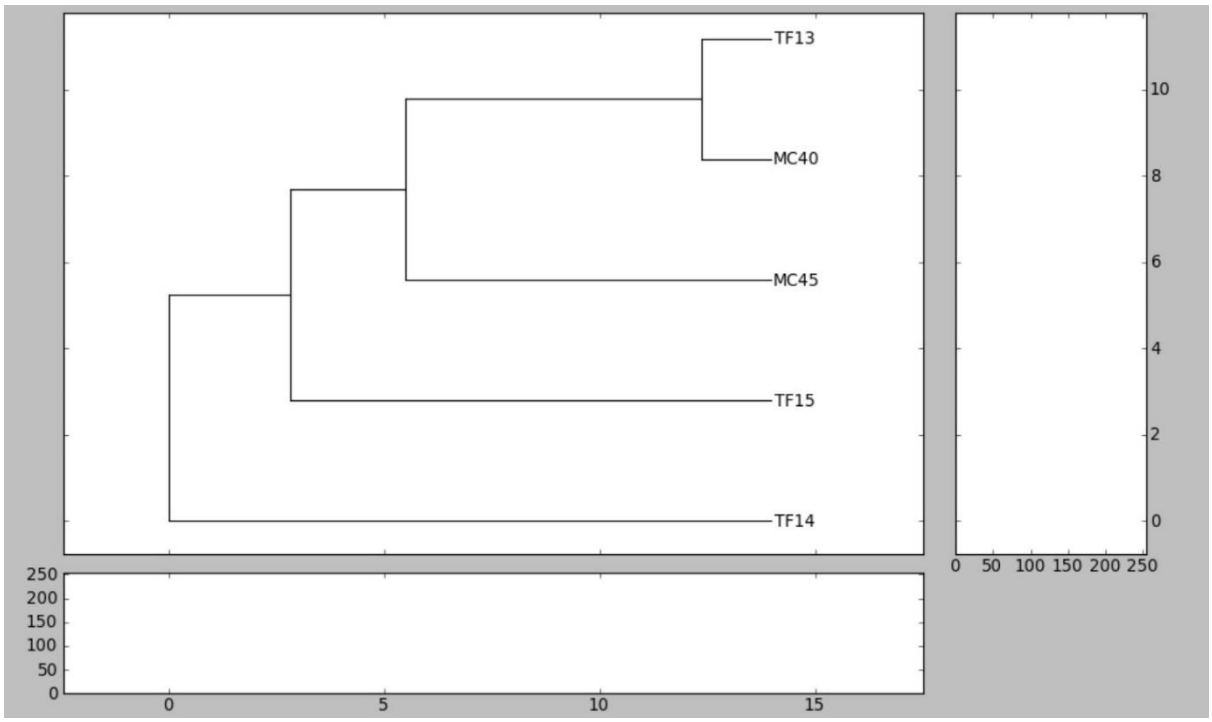
Appendix 6.18. Dendrogram generated with UPGMA clustering showing the genetic similarity of F2 *Bacillus licheniformis* isolates according to their enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) profile

The bottom x axis displays genetic distances in %. TS = soil isolate; TP = pasture isolate; TF = faeces isolate; TW = trough water isolate; MC = milking cups isolate.



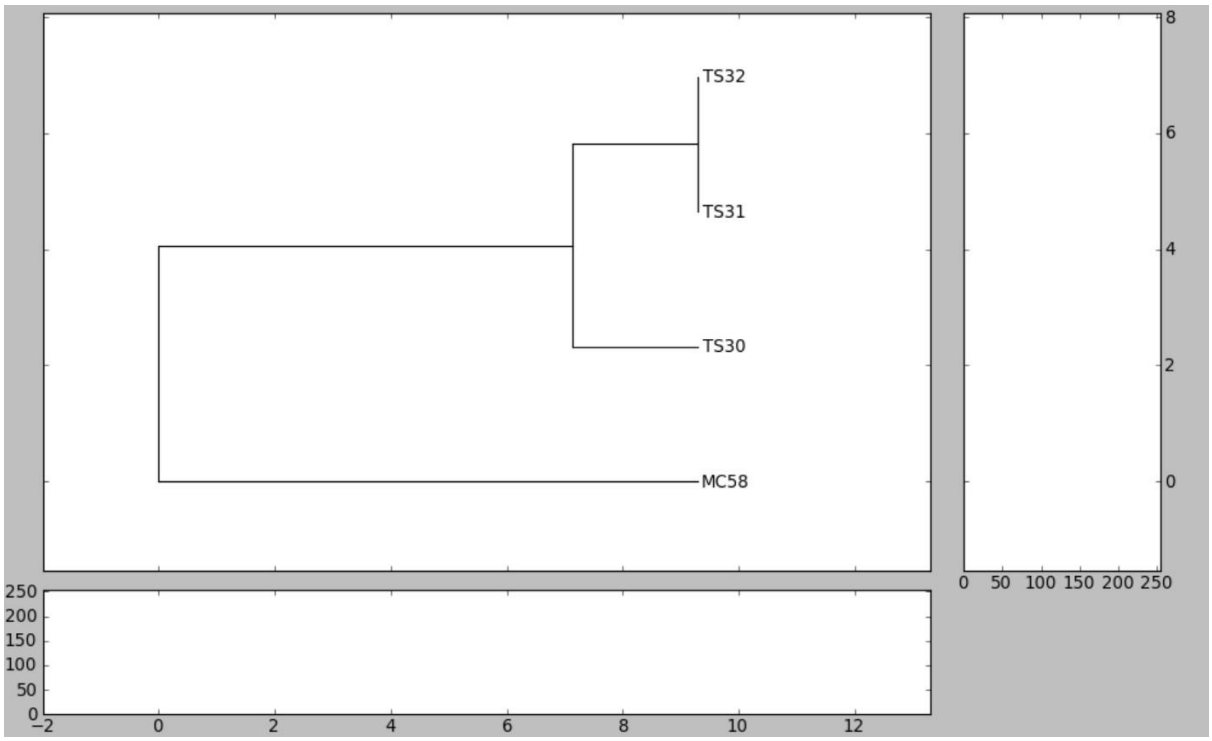
Appendix 6.19. Dendrogram generated with UPGMA clustering showing the genetic similarity of F2 *Priesta megaterium s.l.* isolates according to their enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) profile.

The bottom x axis displays genetic distances in %. The nine clusters mentioned in the text are delimited by horizontal black dash lines and correspond to 80% ERIC PCR fingerprint similarity (vertical black dash line). TS = soil isolate; TP = pasture isolate; TF = faeces isolate; TW = trough water isolate; MC = milking cups isolate.



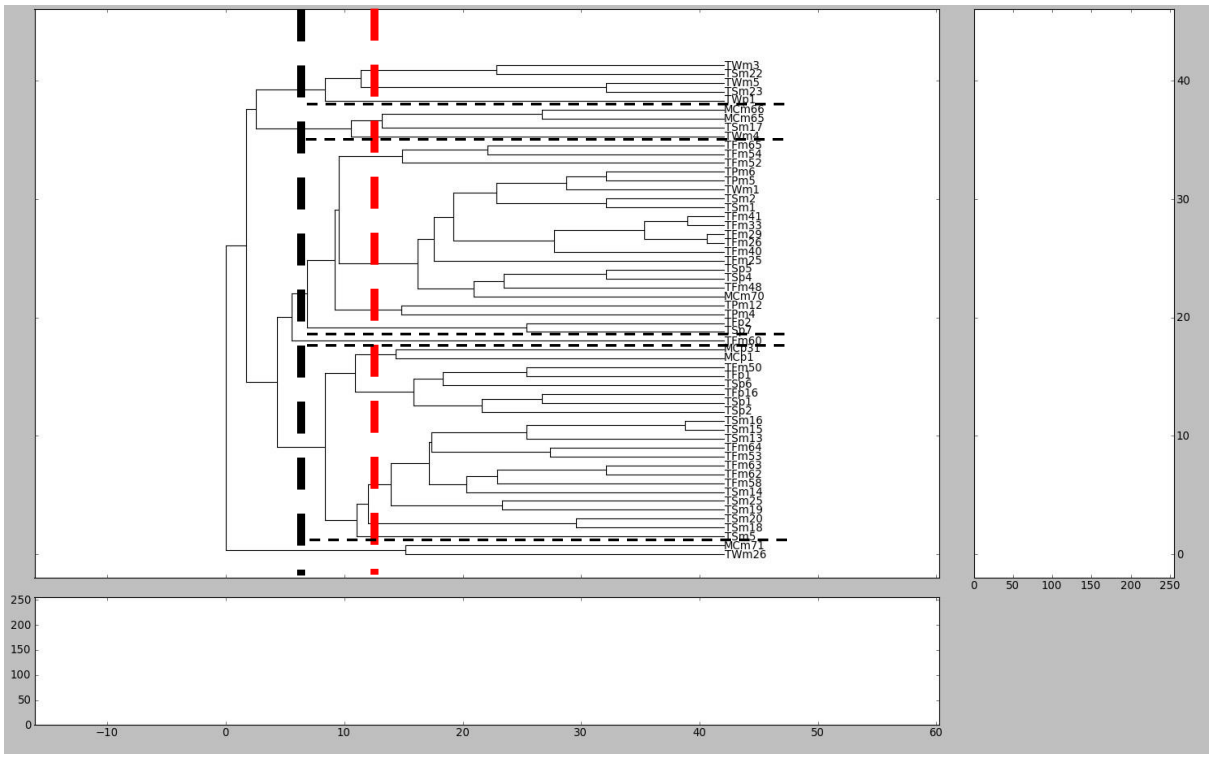
Appendix 6.20. Dendrogram generated with UPGMA clustering showing the genetic similarity of F2 *Alkalihalobacillus clausii* isolates according to their enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) profile.

The bottom x axis displays genetic distances in %. TS = soil isolate; TP = pasture isolate; TF = faeces isolate; TW = trough water isolate; MC = milking cups isolate.



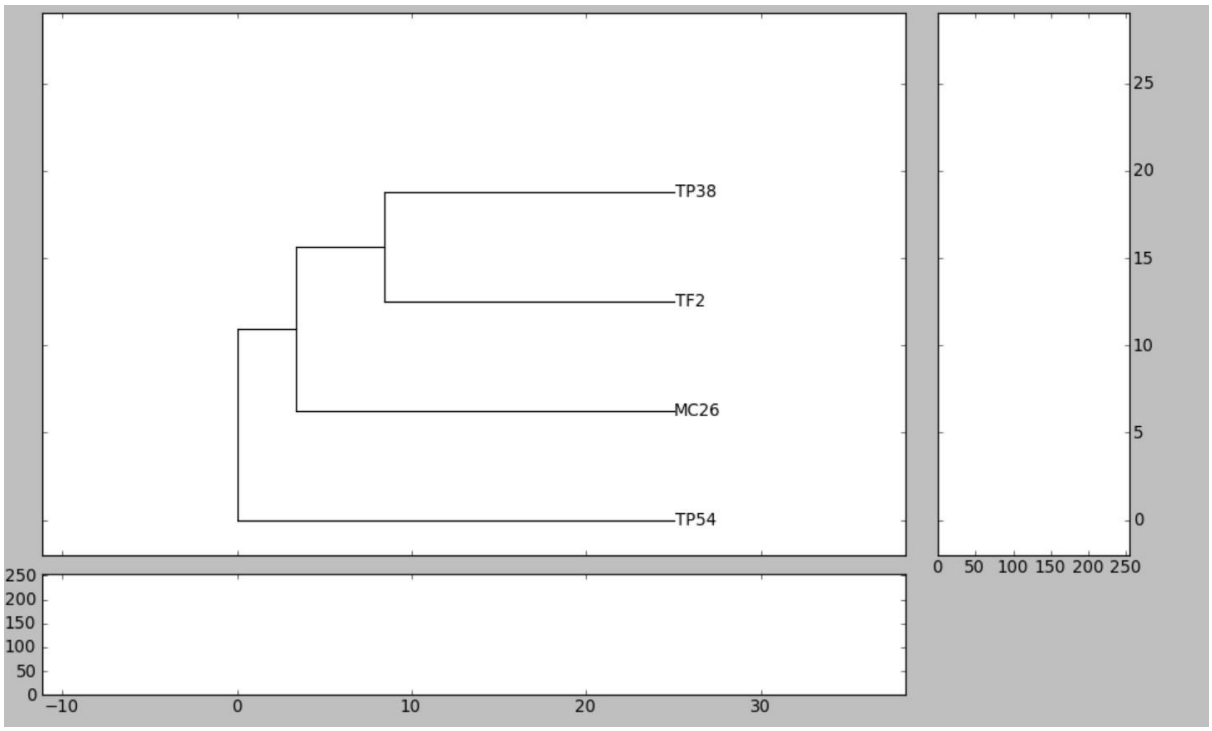
Appendix 6.21. Dendrogram generated with UPGMA clustering showing the genetic similarity of F2 *Lysinibacillus macroides* isolates according to their enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) profile.

The bottom x axis displays genetic distances in %. TS = soil isolate; TP = pasture isolate; TF = faeces isolate; TW = trough water isolate; MC = milking cups isolate.



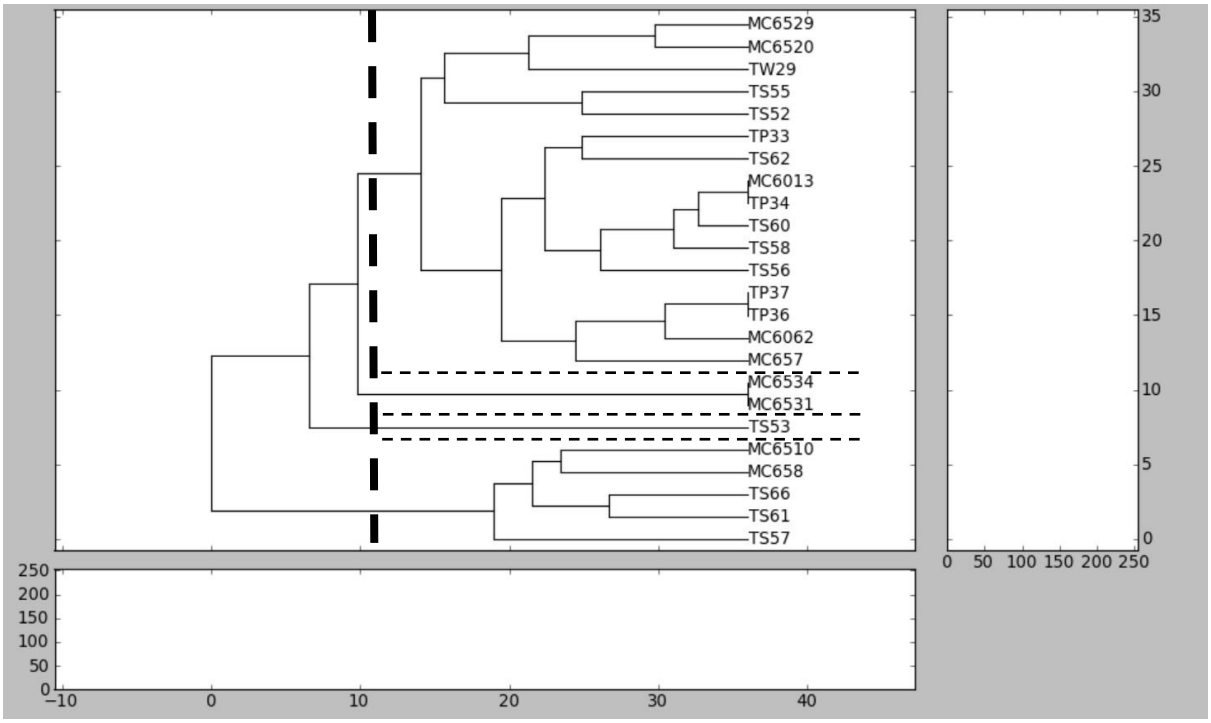
Appendix 6.22. Dendrogram generated with UPGMA clustering showing the genetic similarity of F2 *Bacillus cereus s.l.* isolates according to their enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) profile.

The bottom x axis displays genetic distances in %. The six clusters mentioned in the text are delimited by horizontal black dash lines and correspond to 65% ERIC PCR fingerprint similarity (vertical black dash line). The vertical red dash line corresponds to 70% similarity. TS = soil isolate; TP = pasture isolate; TF = faeces isolate; TW = trough water isolate; MC = milking cups isolate. “m” or “p” suffixes indicate mesophilic or psychrotrophic isolates, respectively.



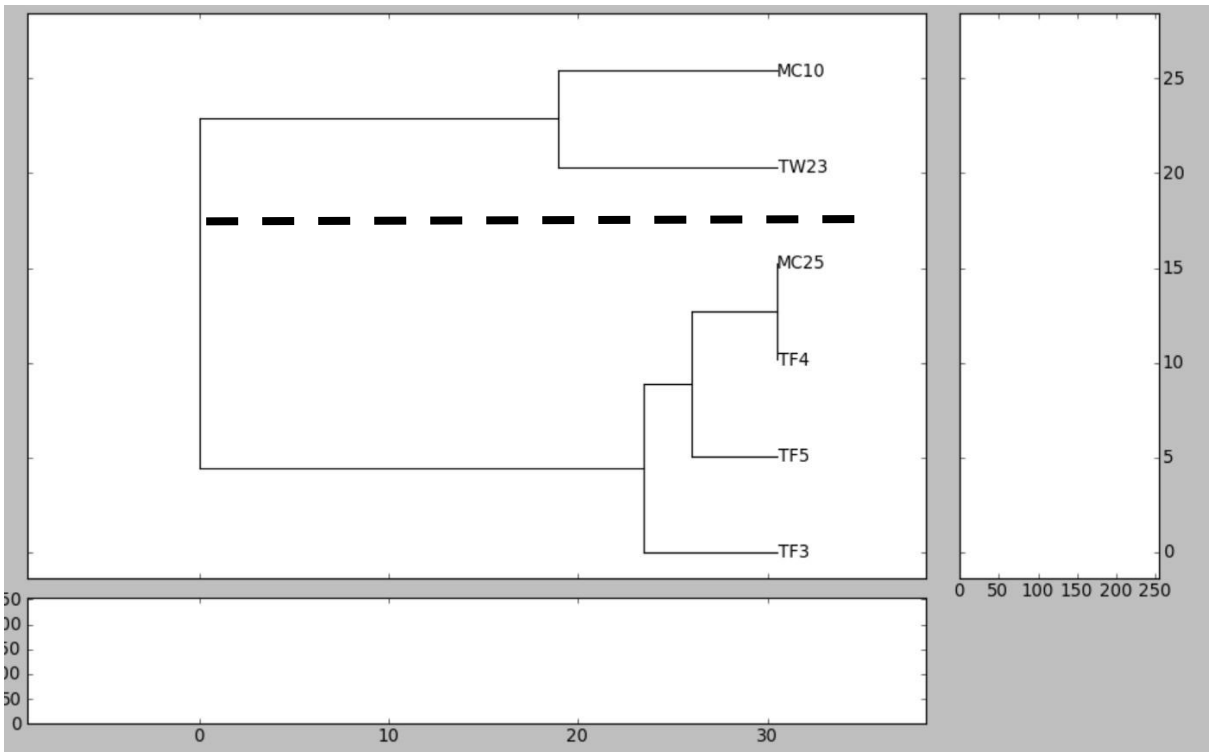
Appendix 6.23. Dendrogram generated with UPGMA clustering showing the genetic similarity of F2 *Bacillus smithii* isolates according to their enterobacterial repetitive intergenic onsensus PCR (ERIC-PCR) profile.

The bottom x axis displays genetic distances in %. TS = soil isolate; TP = pasture isolate; TF = faeces isolate; TW = trough water isolate; MC = milking cups isolate.



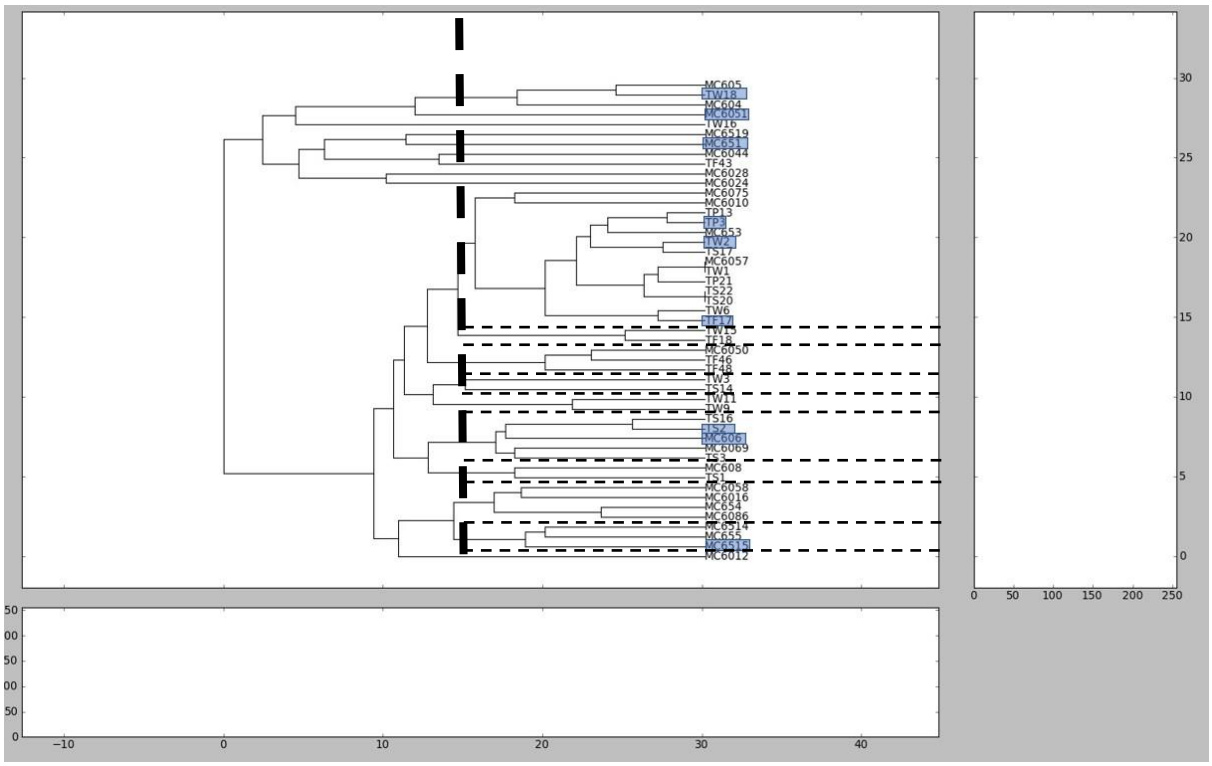
Appendix 6.24. Dendrogram generated with UPGMA clustering showing the genetic similarity of F2 *Aeribacillus pallidus* isolates according to their enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) profile.

The bottom x axis displays genetic distances in %. The four clusters mentioned in the text are delimited by horizontal black dash lines and correspond to 75% ERIC PCR fingerprint similarity (vertical black dash line). TS = soil isolate; TP = pasture isolate; TF = faeces isolate; TW = trough water isolate; MC = milking cups isolate.



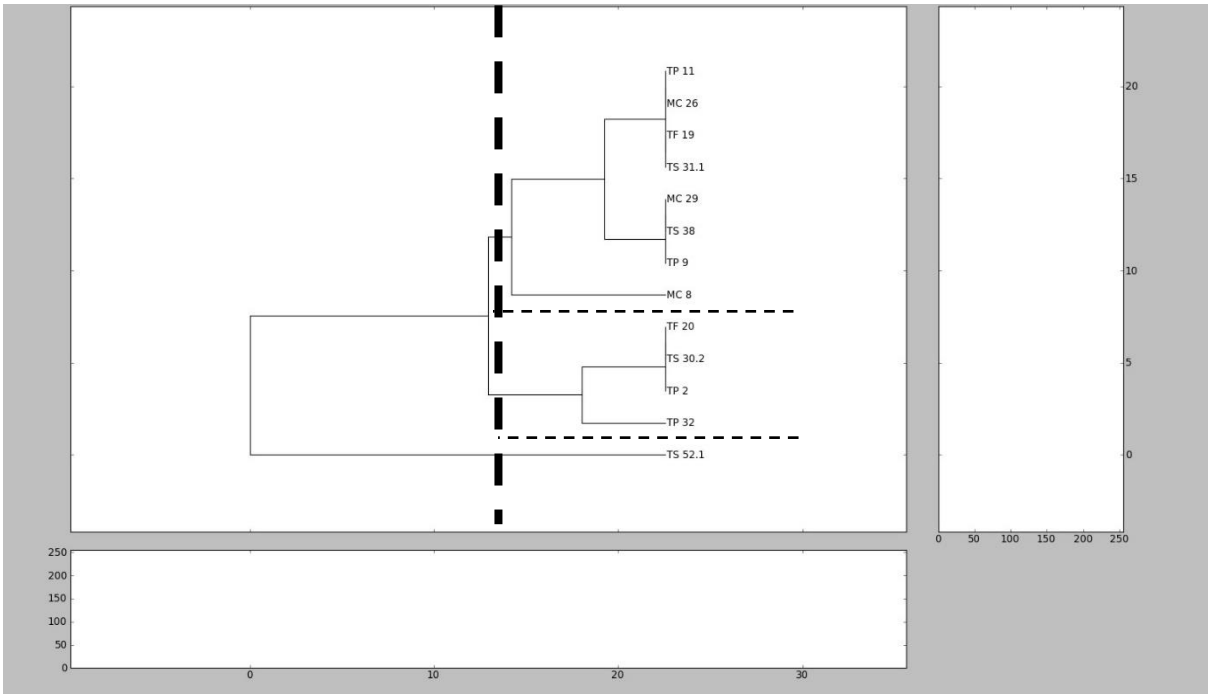
Appendix 6.25. Dendrogram generated with UPGMA clustering showing the genetic similarity of F2 *Parageobacillus caldoxylosilyticus* isolates according to their enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) profile.

The bottom x axis displays genetic distances in %. The two clusters mentioned in the text are delimited by a horizontal black dash line. TS = soil isolate; TP = pasture isolate; TF = faeces isolate; TW = trough water isolate; MC = milking cups isolate.



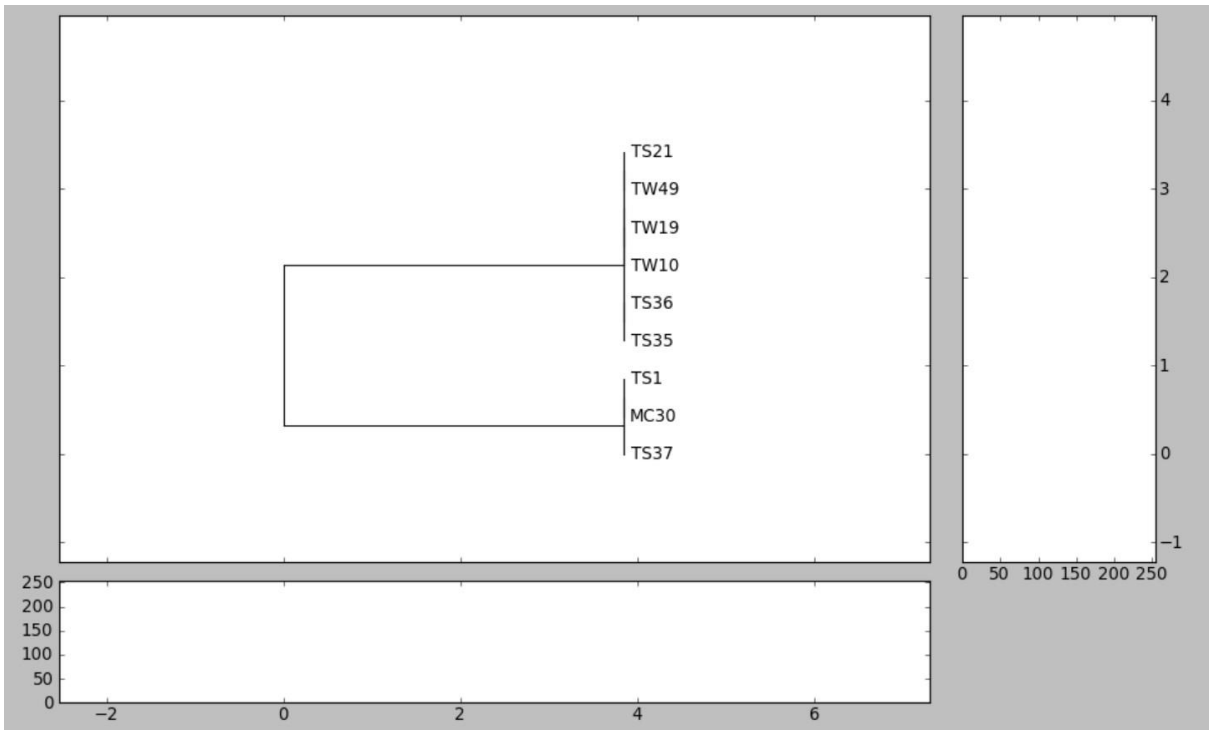
Appendix 6.26. Dendrogram generated with UPGMA clustering showing the genetic similarity of F2 *Thermoactinomyces* spp. isolates according to their enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) profile.

The bottom x axis displays genetic distances in %. The two main clades are clearly visible and diverge at the root of the tree. The 10 clusters in the lower clade mentioned in the text are delimited by horizontal black dash lines and correspond to 85% ERIC PCR fingerprint similarity (vertical black dash line). TS = soil isolate; TP = pasture isolate; TF = faeces isolate; TW = trough water isolate; MC = milking cups isolate. The isolates highlighted in blue represent those selected for whole-genome sequencing.



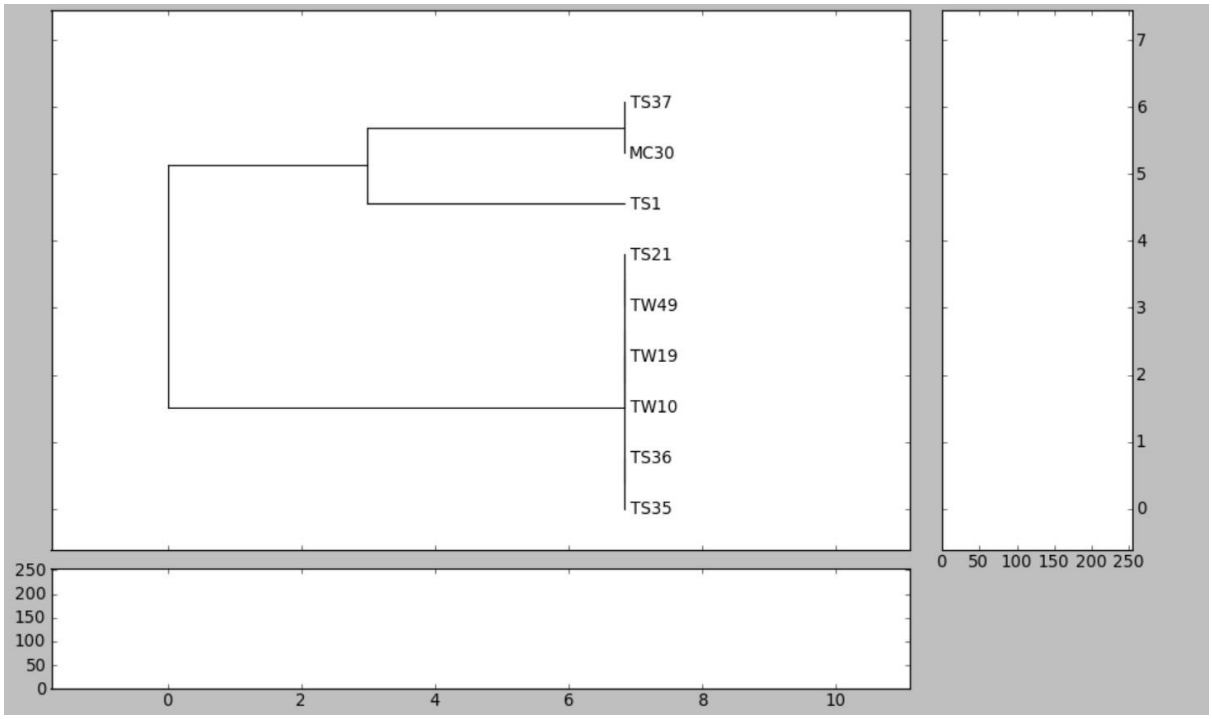
Appendix 6.27. Dendrogram generated with UPGMA clustering showing the genetic similarity of F2 *Clostridium perfringens* isolates according to their Amplified Ribosomal DNA Restriction Analysis (ARDRA) profile with the HhaI restriction enzyme.

The bottom x axis displays genetic distances in %. The three clusters mentioned in the text are delimited by horizontal black dash lines and correspond to 90% ARDRA fingerprint similarity (vertical black dash line). TS = soil isolate; TP = pasture isolate; TF = faeces isolate; TW = trough water isolate; MC = milking cups isolate.



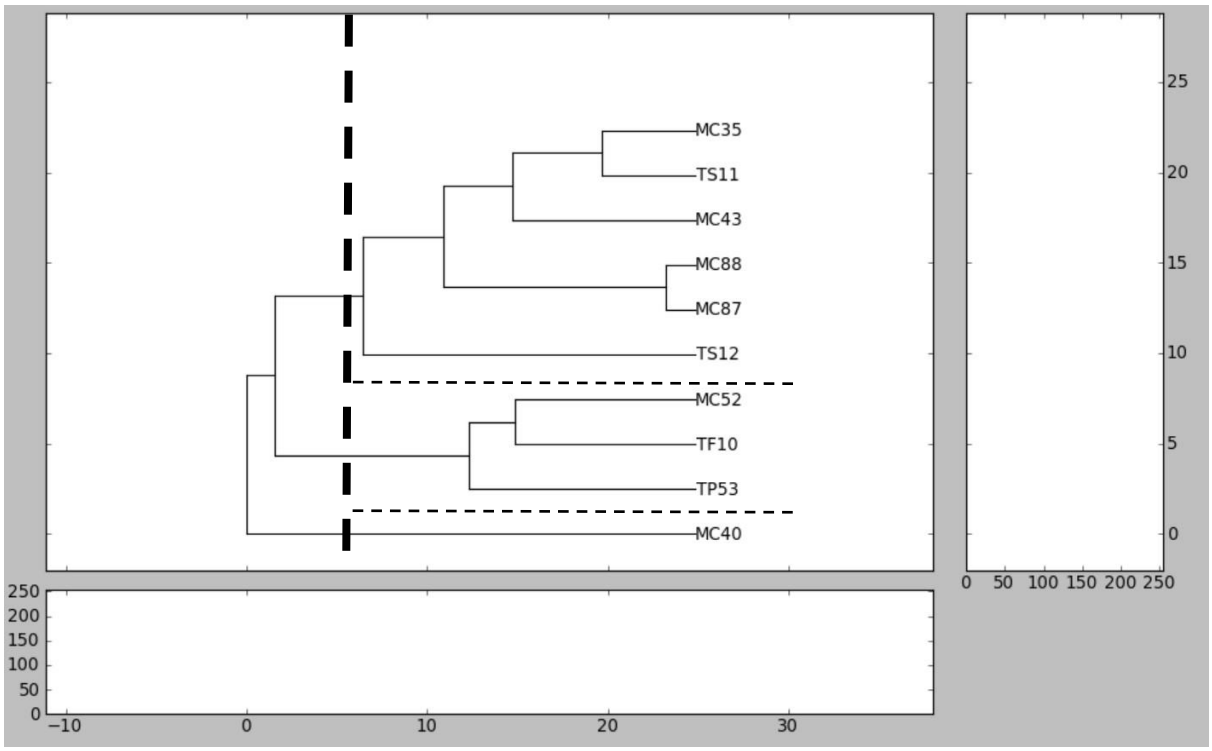
Appendix 6.28. Dendrogram generated with UPGMA clustering showing the genetic similarity of F2 *Clostridium butyricum* isolates according to their Amplified Ribosomal DNA Restriction Analysis (ARDRA) profile with the HhaI restriction enzyme.

The bottom x axis displays genetic distances in %. TS = soil isolate; TP = pasture isolate; TF = faeces isolate; TW = trough water isolate; MC = milking cups isolate.



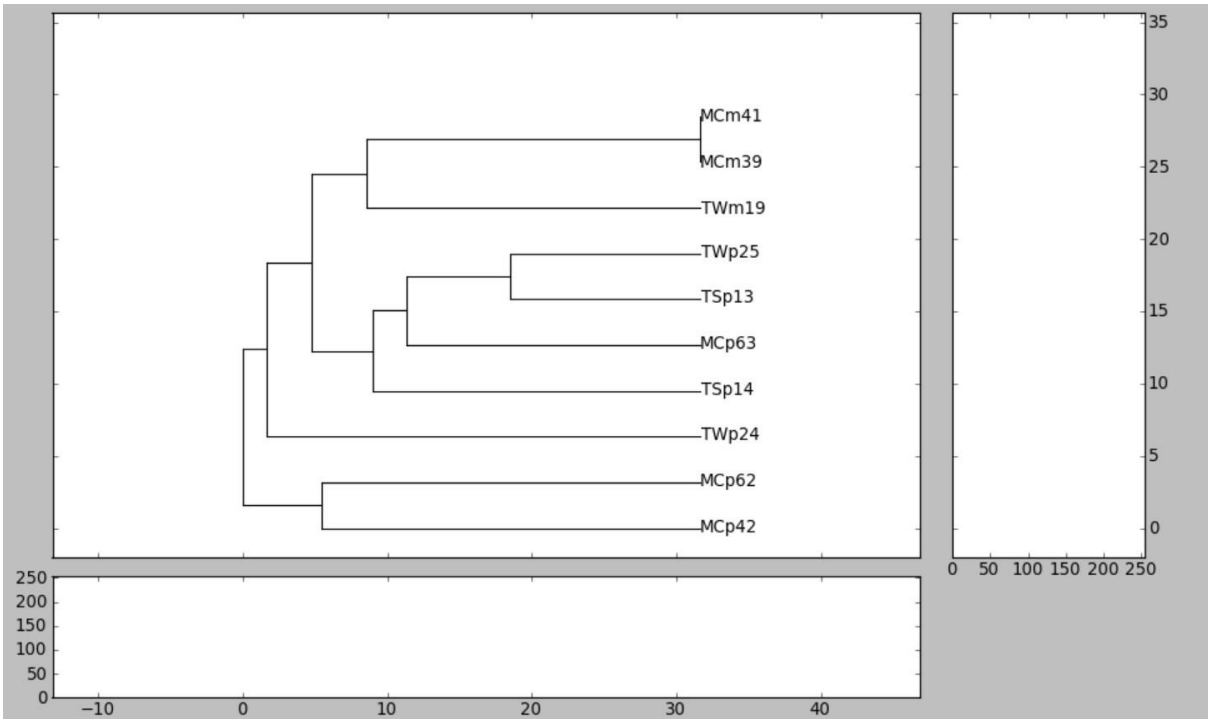
Appendix 6.29. Dendrogram generated with UPGMA clustering showing the genetic similarity of F2 *Clostridium butyricum* isolates according to their Amplified Ribosomal DNA Restriction Analysis (ARDRA) profile with the AluI restriction enzyme.

The bottom x axis displays genetic distances in %. TS = soil isolate; TP = pasture isolate; TF = faeces isolate; TW = trough water isolate; MC = milking cups isolate.



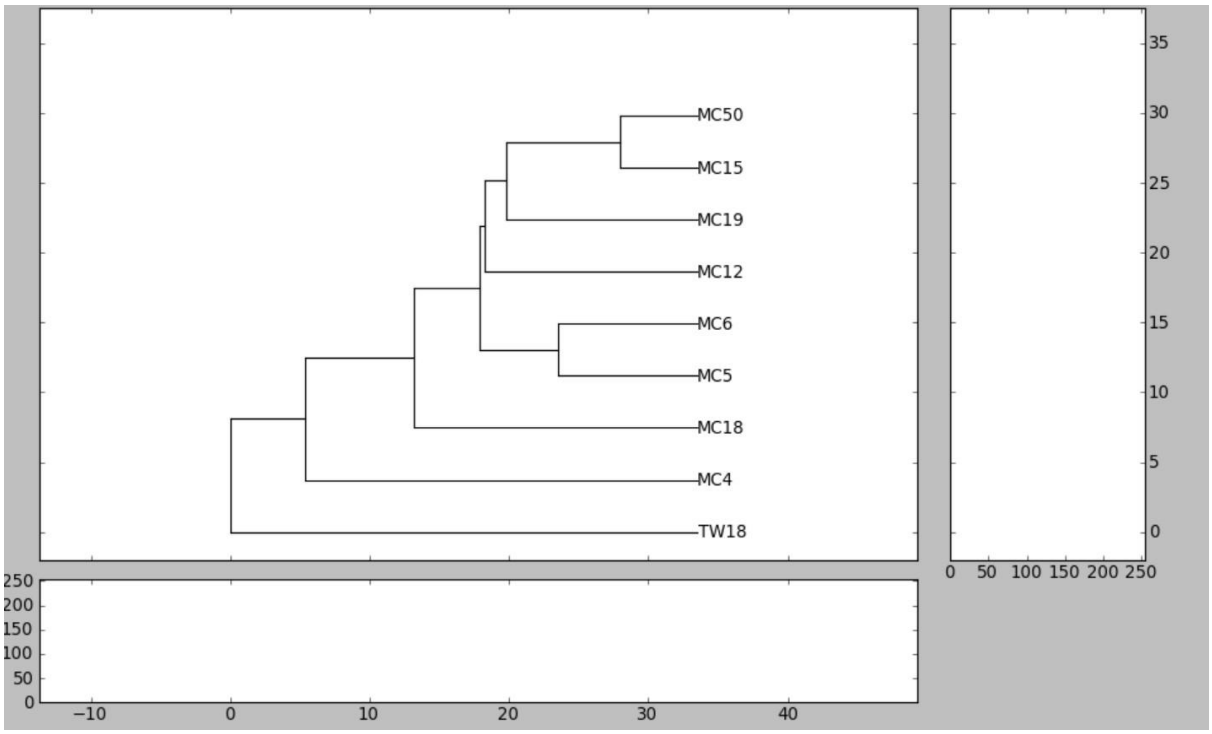
Appendix 6.30. Dendrogram generated with UPGMA clustering showing the genetic similarity of F2 *Paenibacillus anaericanus* isolates according to their enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) profile.

The bottom x axis displays genetic distances in %. The three clusters mentioned in the text are delimited by horizontal black dash lines and correspond to 80% ERIC PCR fingerprint similarity (vertical black dash line). TS = soil isolate; TP = pasture isolate; TF = faeces isolate; TW = trough water isolate; MC = milking cups isolate.



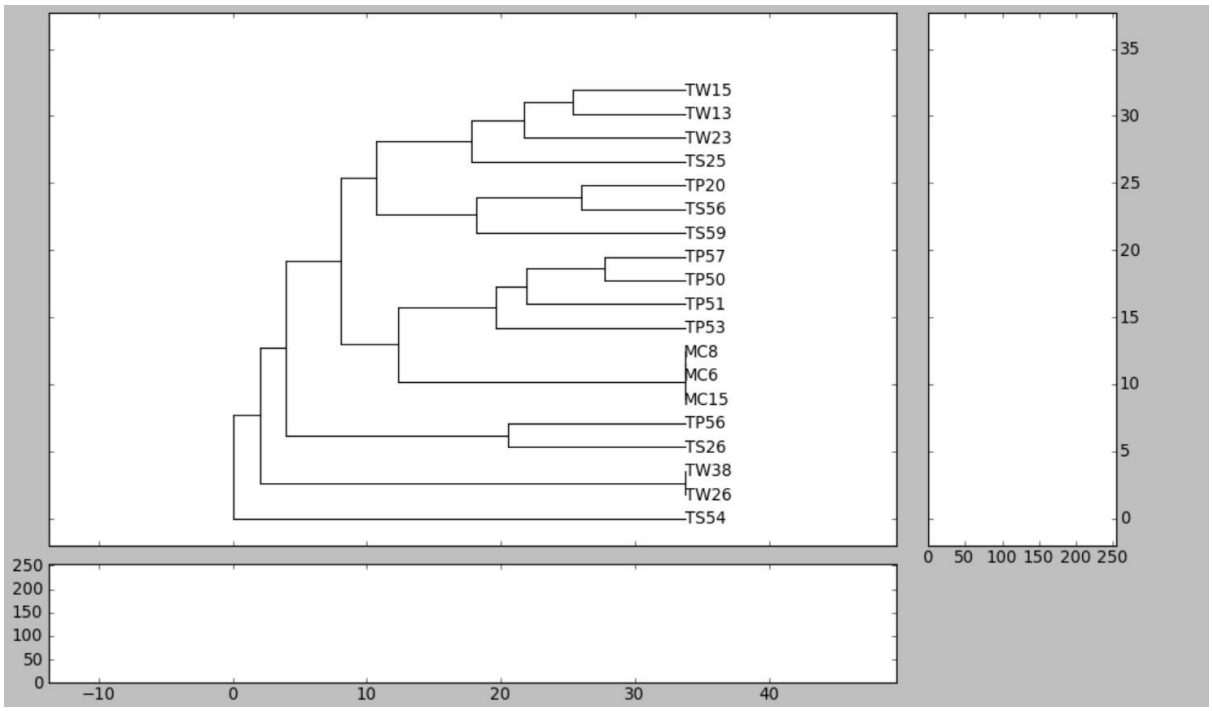
Appendix 6.31. Dendrogram generated with UPGMA clustering showing the genetic similarity of F2 *Paenibacillus tundrae* isolates according to their enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) profile.

The bottom x axis displays genetic distances in %. TS = soil isolate; TP = pasture isolate; TF = faeces isolate; TW = trough water isolate; MC = milking cups isolate. m or p = mesophilic or psychrotrophic isolates, respectively.



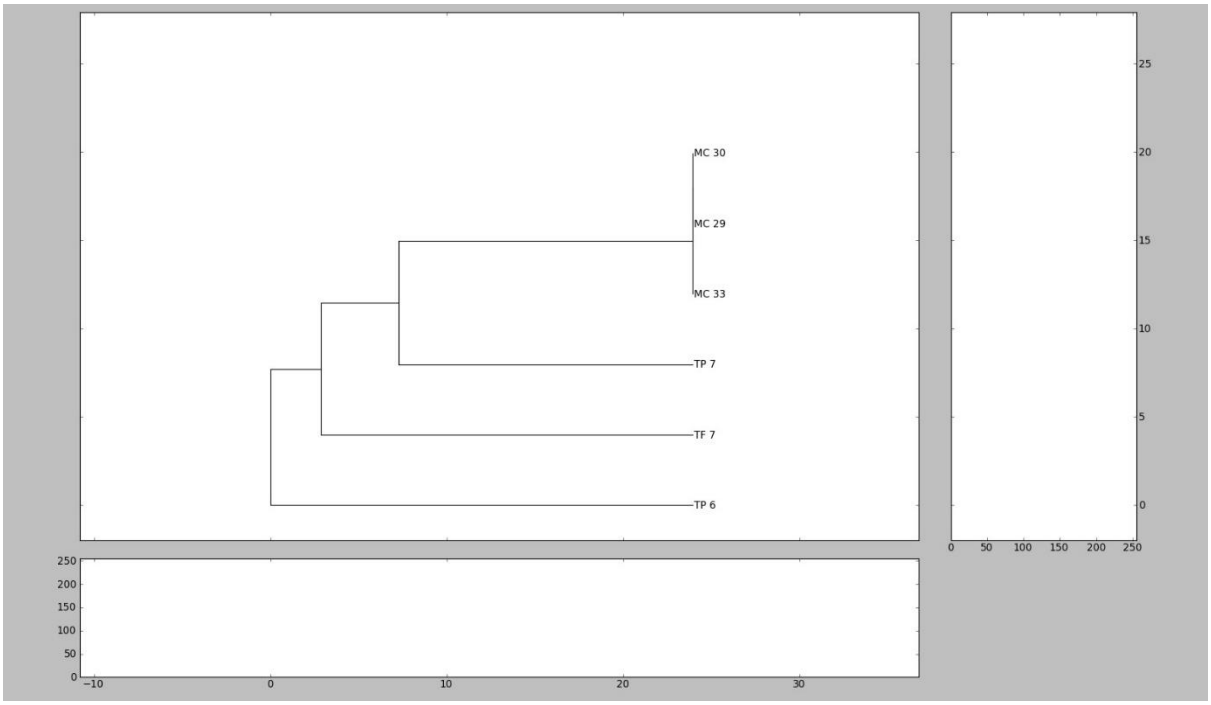
Appendix 6.32. Dendrogram generated with UPGMA clustering showing the genetic similarity of F2 *Peribacillus psychrosaccharolyticus* isolates according to their enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) profile.

The bottom x axis displays genetic distances in %. TS = soil isolate; TP = pasture isolate; TF = faeces isolate; TW = trough water isolate; MC = milking cups isolate.



Appendix 6.33. Dendrogram generated with UPGMA clustering showing the genetic similarity of F2 *Pseudomonas poae s.l.* isolates according to their enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) profile.

The bottom x axis displays genetic distances in %. TS = soil isolate; TP = pasture isolate; TF = faeces isolate; TW = trough water isolate; MC = milking cups isolate.



Appendix 6.34. Dendrogram generated with UPGMA clustering showing the genetic similarity of F2 *Pseudomonas koreensis.* isolates according to their enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) profile.

The bottom x axis displays genetic distances in %. TS = soil isolate; TP = pasture isolate; TF = faeces isolate; TW = trough water isolate; MC = milking cups isolate.

Appendix 6.35. dDDH values between the genome sequence of farm isolates and reference strains of *Thermoactinomyces vulgaris* and *Thermoactinomyces intermedius*, as well as *Bacillus subtilis*, as computed by the TYGS tool (tygs.dsmz.de)

Query strain	Subject strain	dDDH (d0, in %)	C.I. (d0, in %)	dDDH (d4, in %)	C.I. (d4, in %)	dDDH (d6, in %)	C.I. (d6, in %)	G+C content difference (in %)
'F1NPHDT35'	'F1NSHDT14'	98.2	[96.9 - 98.9]	100	[99.9 - 100.0]	99.2	[98.6 - 99.5]	0.25
'*Thermoactinomyces intermedius*' (GCA 013867715.1)	'*Thermoactinomyces intermedius*' (GCA 016055025.1)	100	[100.0 - 100.0]	100	[99.9 - 100.0]	100	[100.0 - 100.0]	0.02
'F2MC60HDT51'	'F2MC65HDT1'	100	[100.0 - 100.0]	100	[99.9 - 100.0]	100	[100.0 - 100.0]	0.01
'F1NSHDT14'	'F2TPHDT3'	99.5	[98.9 - 99.7]	100	[100.0 - 100.0]	99.8	[99.6 - 99.9]	0.14
'F2TPHDT3'	'F2TWHDT2'	100	[100.0 - 100.0]	100	[99.9 - 100.0]	100	[100.0 - 100.0]	0
'F2MC60HDT51'	'F2TWHDT18'	100	[100.0 - 100.0]	100	[99.9 - 100.0]	100	[100.0 - 100.0]	0.01

'F1NPHDT35'	'F2TPHDT3'	98.2	[97.0 - 98.9]	100	[100.0 - 100.0]	99.2	[98.6 - 99.5]	0.4
'F1NPHDT35'	'F2TWHDT2'	98.3	[97.2 - 99.0]	99.9	[99.8 - 99.9]	99.2	[98.7 - 99.6]	0.4
'F1NSHDT14'	'F2TWHDT2'	99.5	[99.1 - 99.8]	99.9	[99.8 - 99.9]	99.8	[99.6 - 99.9]	0.14
'F2MC65HDT1'	'F2TWHDT18'	100	[100.0 - 100.0]	99.9	[99.8 - 99.9]	100	[100.0 - 100.0]	0
'F1NSHDT14'	'F2TFHDT17'	96.9	[95.2 - 98.0]	98.4	[97.6 - 98.9]	98.3	[97.3 - 98.9]	0.02
'F1NPHDT35'	'F2TFHDT17'	95	[92.7 - 96.6]	98.4	[97.6 - 98.9]	97.1	[95.7 - 98.0]	0.27
'F2TFHDT17'	'F2TPHDT3'	98.1	[96.8 - 98.9]	98.4	[97.6 - 98.9]	99	[98.3 - 99.4]	0.13
'F1NBHDT1'	'F2MC65HDT15'	99.1	[98.4 - 99.5]	98.3	[97.5 - 98.9]	99.5	[99.1 - 99.7]	0.09
'F2TFHDT17'	'F2TWHDT2'	98.2	[97.0 - 99.0]	98.3	[97.5 - 98.8]	99	[98.4 - 99.4]	0.12
'F2MC60HDT7'	'F2TSHDT2'	97.9	[96.6 - 98.7]	98.3	[97.6 - 98.9]	98.9	[98.1 - 99.3]	0.03

'F2MC60HDT7'	'*Thermoactinomyces vulgaris*' (GCA 001187615.2)	99	[98.2 - 99.5]	98.1	[97.3 - 98.7]	99.5	[99.1 - 99.7]	0.2
'F1MC65HDT1'	'F2MC60HDT7'	98.4	[97.2 - 99.0]	97.3	[96.2 - 98.0]	99	[98.4 - 99.4]	0.12
'F1MC65HDT1'	'*Thermoactinomyces vulgaris*' (GCA 001187615.2)	99.6	[99.1 - 99.8]	97.2	[96.1 - 98.0]	99.7	[99.5 - 99.9]	0.08
'F2TSHDT2'	'*Thermoactinomyces vulgaris*' (GCA 001187615.2)	98.5	[97.4 - 99.1]	97.2	[96.1 - 98.0]	99.1	[98.5 - 99.5]	0.23
'F1MC60HDT27'	'F1MC65HDT1'	99.4	[98.9 - 99.7]	97.2	[96.1 - 98.0]	99.6	[99.3 - 99.8]	0.05
'F1NWHDT6'	'*Thermoactinomyces vulgaris*' (GCA 003688725.1)	96.3	[94.4 - 97.6]	96.7	[95.5 - 97.6]	97.8	[96.6 - 98.5]	0.32
'F2TFHDT17'	'*Thermoactinomyces vulgaris*' (GCA 003688725.1)	98.6	[97.5 - 99.2]	96.7	[95.4 - 97.6]	99.1	[98.5 - 99.5]	0.03

'F1NWHDT6'	'F2TFHDT17'	95.8	[93.7 - 97.2]	96.6	[95.4 - 97.5]	97.4	[96.1 - 98.3]	0.29
'F1MC60HDT27'	'F1NSHDT14'	98.2	[96.9 - 98.9]	96.5	[95.2 - 97.4]	98.9	[98.2 - 99.3]	0.12
'F1MC65HDT1'	'F2TSHDT2'	98.2	[96.9 - 98.9]	96.5	[95.2 - 97.5]	98.9	[98.2 - 99.3]	0.15
'F2MC60HDT7'	'F2TFHDT17'	97.5	[95.9 - 98.4]	96.5	[95.3 - 97.5]	98.4	[97.5 - 99.0]	0.04
'F1MC60HDT27'	'F1NPHDT35'	96.5	[94.6 - 97.7]	96.5	[95.2 - 97.4]	97.8	[96.7 - 98.6]	0.38
'F1NBHDT1'	'*Thermoactinomyces vulgaris*' (GCA 001187615.2)	96.7	[94.9 - 97.9]	96.5	[95.2 - 97.4]	98	[96.9 - 98.7]	0.15
'F1MC60HDT27'	'F2TPHDT3'	98.9	[98.0 - 99.4]	96.5	[95.2 - 97.4]	99.3	[98.8 - 99.6]	0.02
'F2TFHDT17'	'*Thermoactinomyces vulgaris*' (GCA 001187615.2)	98.9	[98.0 - 99.4]	96.5	[95.2 - 97.4]	99.3	[98.8 - 99.6]	0.23
'F1MC60HDT27'	'F2TWHDT2'	99	[98.2 - 99.5]	96.3	[94.9 - 97.3]	99.4	[98.9 - 99.6]	0.02

'F1MC60HDT27'	'F2MC60HDT7'	97.9	[96.6 - 98.8]	96.3	[95.0 - 97.3]	98.7	[97.9 - 99.2]	0.07
'F1MC65HDT1'	'F2TFHDT17'	98.9	[98.0 - 99.4]	96.2	[94.8 - 97.2]	99.3	[98.7 - 99.6]	0.16
'F1MC60HDT27'	'F2TFHDT17'	98.3	[97.1 - 99.0]	96.2	[94.9 - 97.2]	98.9	[98.2 - 99.3]	0.1
'F1MC65HDT1'	'F1NWHDT6'	96	[93.9 - 97.4]	96.2	[94.9 - 97.2]	97.5	[96.2 - 98.3]	0.45
'F2TFHDT17'	'F2TSHDT2'	97.3	[95.7 - 98.3]	96.1	[94.7 - 97.1]	98.3	[97.4 - 98.9]	0.01
'F1NBHDT1'	'F2MC60HDT7'	95.4	[93.2 - 96.9]	96.1	[94.8 - 97.2]	97.1	[95.7 - 98.1]	0.05
'F1MC60HDT27'	'*Thermoactinomyces vulgaris*' (GCA 001187615.2)	99.2	[98.6 - 99.6]	96.1	[94.7 - 97.1]	99.5	[99.1 - 99.7]	0.13
'F2MC65HDT15'	'*Thermoactinomyces vulgaris*' (GCA 001187615.2)	95.2	[93.0 - 96.8]	96	[94.5 - 97.0]	97	[95.6 - 98.0]	0.24
'F1NWHDT6'	'F2MC60HDT7'	93.5	[90.9 - 95.5]	96	[94.7 - 97.1]	95.9	[94.1 - 97.1]	0.33

'F1NPHDT35'	'*Thermoactinomyces vulgaris*' (GCA 001187615.2)	96	[94.0 - 97.4]	95.9	[94.5 - 96.9]	97.5	[96.2 - 98.3]	0.5
'F1NSHDT14'	'*Thermoactinomyces vulgaris*' (GCA 001187615.2)	97.8	[96.4 - 98.7]	95.9	[94.5 - 96.9]	98.6	[97.8 - 99.1]	0.25
'F2TPHDT3'	'*Thermoactinomyces vulgaris*' (GCA 001187615.2)	98.8	[97.8 - 99.3]	95.9	[94.4 - 96.9]	99.2	[98.6 - 99.5]	0.11
'F1MC65HDT1'	'*Thermoactinomyces vulgaris*' (GCA 003688725.1)	98.8	[97.8 - 99.3]	95.9	[94.5 - 97.0]	99.2	[98.6 - 99.5]	0.12
'F1NPHDT35'	'F2MC60HDT7'	94.6	[92.2 - 96.3]	95.8	[94.4 - 96.9]	96.6	[95.0 - 97.6]	0.31
'F1NWHDT6'	'F2TPHDT3'	94.1	[91.5 - 95.9]	95.8	[94.4 - 96.9]	96.2	[94.5 - 97.3]	0.42
'F2MC60HDT7'	'F2TPHDT3'	97.8	[96.4 - 98.6]	95.8	[94.4 - 96.9]	98.6	[97.7 - 99.1]	0.09
'F1NSHDT14'	'F2MC60HDT7'	96.6	[94.7 - 97.8]	95.8	[94.4 - 96.9]	97.8	[96.7 - 98.6]	0.05

'F1NWHDT6'	'*Thermoactinomyces vulgaris*' (GCA 001187615.2)	95.7	[93.5 - 97.1]	95.8	[94.4 - 96.9]	97.2	[95.9 - 98.2]	0.52
'F2MC60HDT7'	'F2TWHDT2'	97.9	[96.6 - 98.8]	95.7	[94.2 - 96.8]	98.7	[97.9 - 99.2]	0.09
'F2TWHDT2'	'*Thermoactinomyces vulgaris*' (GCA 001187615.2)	98.9	[98.0 - 99.4]	95.7	[94.2 - 96.8]	99.2	[98.7 - 99.6]	0.11
'F2MC60HDT7'	'F2MC65HDT15'	93.8	[91.3 - 95.7]	95.7	[94.3 - 96.8]	96	[94.4 - 97.2]	0.04
'F1NSHDT14'	'F1NWHDT6'	95	[92.7 - 96.6]	95.7	[94.2 - 96.8]	96.8	[95.3 - 97.8]	0.27
'F2TPHDT3'	'*Thermoactinomyces vulgaris*' (GCA 003688725.1)	97.5	[96.0 - 98.5]	95.6	[94.1 - 96.7]	98.4	[97.5 - 99.0]	0.1
'F1MC65HDT1'	'F2TPHDT3'	98.6	[97.5 - 99.2]	95.6	[94.1 - 96.7]	99	[98.4 - 99.4]	0.03
'F1MC65HDT1'	'F1NPHDT35'	96	[94.0 - 97.4]	95.6	[94.1 - 96.7]	97.4	[96.2 - 98.3]	0.43

'F1MC60HDT27'	'F1NWHDT6'	95.4	[93.2 - 96.9]	95.6	[94.2 - 96.7]	97.1	[95.7 - 98.0]	0.4
'F2MC60HDT7'	'*Thermoactinomyces vulgaris*' (GCA 003688725.1)	97.7	[96.2 - 98.6]	95.6	[94.1 - 96.7]	98.5	[97.6 - 99.1]	0.01
'F1NWHDT6'	'F2TWHDT2'	94.3	[91.8 - 96.0]	95.6	[94.1 - 96.7]	96.3	[94.7 - 97.4]	0.42
'F1MC65HDT1'	'F1NSHDT14'	97.8	[96.4 - 98.6]	95.6	[94.1 - 96.7]	98.6	[97.7 - 99.1]	0.17
'F1NWHDT6'	'F2TSHDT2'	94.5	[92.0 - 96.2]	95.5	[94.1 - 96.7]	96.4	[94.9 - 97.5]	0.3
'F2TWHDT2'	'*Thermoactinomyces vulgaris*' (GCA 003688725.1)	97.7	[96.2 - 98.6]	95.5	[94.0 - 96.6]	98.5	[97.6 - 99.0]	0.09
'F1MC60HDT27'	'F1NBHDT1'	97.2	[95.5 - 98.2]	95.5	[94.1 - 96.7]	98.2	[97.2 - 98.8]	0.02
'F2MC65HDT15'	'F2TWHDT2'	94.8	[92.4 - 96.4]	95.5	[93.9 - 96.6]	96.6	[95.1 - 97.7]	0.13
'F1MC60HDT27'	'F2MC65HDT15'	95.7	[93.5 - 97.1]	95.5	[94.0 - 96.7]	97.2	[95.9 - 98.1]	0.11

'F1MC65HDT1'	'F1NBHDT1'	96.4	[94.4 - 97.6]	95.4	[93.9 - 96.6]	97.7	[96.5 - 98.5]	0.07
'F1NPHDT35'	'F1NWHDT6'	94.2	[91.7 - 96.0]	95.4	[93.8 - 96.5]	96.2	[94.6 - 97.4]	0.02
'F1MC65HDT1'	'F2TWHDT2'	98.7	[97.7 - 99.3]	95.4	[93.9 - 96.5]	99.1	[98.5 - 99.5]	0.03
'F1NPHDT35'	'*Thermoactinomyces vulgaris*' (GCA 003688725.1)	94.3	[91.8 - 96.0]	95.4	[93.9 - 96.6]	96.3	[94.7 - 97.4]	0.3
'*Thermoactinomyces vulgaris*' (GCA 001187615.2)	'*Thermoactinomyces vulgaris*' (GCA 003688725.1)	99.1	[98.3 - 99.5]	95.4	[93.9 - 96.6]	99.3	[98.8 - 99.6]	0.2
'F1MC65HDT1'	'F2MC65HDT15'	94.7	[92.4 - 96.4]	95.4	[93.9 - 96.6]	96.6	[95.1 - 97.7]	0.16
'F1MC60HDT27'	'F2TSHDT2'	97.7	[96.2 - 98.6]	95.4	[93.9 - 96.5]	98.5	[97.6 - 99.0]	0.1
'F2TSHDT2'	'*Thermoactinomyces vulgaris*' (GCA 003688725.1)	97.1	[95.4 - 98.2]	95.4	[93.9 - 96.5]	98.1	[97.1 - 98.8]	0.02

'F1MC60HDT27'	'*Thermoactinomyces vulgaris*' (GCA 003688725.1)	98.3	[97.1 - 99.0]	95.3	[93.8 - 96.5]	98.8	[98.1 - 99.3]	0.07
'F2MC65HDT15'	'F2TPHDT3'	94.5	[92.1 - 96.3]	95.3	[93.8 - 96.5]	96.5	[94.9 - 97.6]	0.13
'F1NSHDT14'	'*Thermoactinomyces vulgaris*' (GCA 003688725.1)	96.6	[94.7 - 97.8]	95.2	[93.7 - 96.4]	97.8	[96.6 - 98.5]	0.05
'F1NBHDT1'	'F1NPHDT35'	92.6	[89.7 - 94.7]	95.2	[93.6 - 96.4]	95.1	[93.2 - 96.5]	0.36
'F1NBHDT1'	'F2TPHDT3'	95.8	[93.7 - 97.2]	95.2	[93.6 - 96.4]	97.2	[95.9 - 98.2]	0.04
'F2MC60HDT51'	'*Thermoactinomyces intermedius*' (GCA 013867715.1)	95.6	[93.4 - 97.1]	95.2	[93.6 - 96.4]	97.1	[95.8 - 98.1]	0.09
'F1NBHDT1'	'F2TSHDT2'	94.4	[92.0 - 96.2]	95.1	[93.5 - 96.3]	96.4	[94.8 - 97.5]	0.08
'F2TWHDT18'	'*Thermoactinomyces intermedius*' (GCA 013867715.1)	95.6	[93.4 - 97.1]	95.1	[93.5 - 96.3]	97.1	[95.8 - 98.1]	0.11

'F1NBHDT1'	'F2TFHDT17'	94.9	[92.6 - 96.5]	95.1	[93.5 - 96.3]	96.7	[95.2 - 97.7]	0.09
'F2MC65HDT1'	'*Thermoactinomyces intermedius*' (GCA 013867715.1)	95.6	[93.4 - 97.0]	95.1	[93.6 - 96.3]	97.1	[95.7 - 98.1]	0.1
'F2MC65HDT1'	'*Thermoactinomyces intermedius*' (GCA 016055025.1)	95.5	[93.4 - 97.0]	95.1	[93.5 - 96.3]	97.1	[95.7 - 98.0]	0.09
'F2MC60HDT51'	'*Thermoactinomyces intermedius*' (GCA 016055025.1)	95.5	[93.4 - 97.0]	95.1	[93.5 - 96.3]	97.1	[95.7 - 98.0]	0.08
'F2TWHDT18'	'*Thermoactinomyces intermedius*' (GCA 016055025.1)	95.6	[93.4 - 97.0]	95	[93.4 - 96.3]	97.1	[95.7 - 98.0]	0.09
'F1NSHDT14'	'F2MC65HDT15'	95	[92.7 - 96.6]	94.9	[93.3 - 96.1]	96.7	[95.2 - 97.7]	0.01
'F1NBHDT1'	'F2TWHDT2'	96	[93.9 - 97.3]	94.9	[93.3 - 96.2]	97.3	[96.0 - 98.2]	0.04
'F2TPHDT3'	'F2TSHDT2'	97.2	[95.6 - 98.2]	94.9	[93.3 - 96.1]	98.1	[97.1 - 98.8]	0.12

'F1NPHDT35'	'F2MC65HDT15'	94.2	[91.6 - 95.9]	94.9	[93.3 - 96.1]	96.1	[94.5 - 97.3]	0.27
'F1NBHDT1'	'F1NSHDT14'	96	[94.0 - 97.4]	94.8	[93.1 - 96.0]	97.4	[96.1 - 98.3]	0.1
'F1NPHDT35'	'F2TSHDT2'	94.4	[91.9 - 96.1]	94.8	[93.1 - 96.0]	96.3	[94.7 - 97.4]	0.28
'F2MC65HDT15'	'F2TFHDT17'	93.5	[90.8 - 95.4]	94.8	[93.2 - 96.1]	95.7	[93.9 - 96.9]	0.01
'F1NSHDT14'	'F2TSHDT2'	96.3	[94.4 - 97.6]	94.8	[93.2 - 96.1]	97.6	[96.4 - 98.4]	0.02
'F2TSHDT2'	'F2TWHDT2'	97.3	[95.8 - 98.3]	94.8	[93.2 - 96.0]	98.2	[97.2 - 98.9]	0.12
'F2MC65HDT15'	'F2TSHDT2'	93.1	[90.4 - 95.1]	94.6	[92.9 - 95.9]	95.4	[93.5 - 96.7]	0.01
'F1NBHDT1'	'F1NWHDT6'	93.3	[90.6 - 95.3]	94.1	[92.4 - 95.5]	95.5	[93.7 - 96.8]	0.38
'F1NWHDT6'	'F2MC65HDT15'	92.2	[89.3 - 94.4]	94	[92.2 - 95.4]	94.7	[92.7 - 96.2]	0.29

'F2MC65HDT15'	'*Thermoactinomyces vulgaris*' (GCA 003688725.1)	94	[91.5 - 95.9]	93.9	[92.1 - 95.3]	96	[94.3 - 97.2]	0.04
'F1NBHDT1'	'*Thermoactinomyces vulgaris*' (GCA 003688725.1)	95.5	[93.3 - 97.0]	93.7	[91.8 - 95.1]	96.9	[95.5 - 97.9]	0.06
'F2MC60HDT51'	'F2TSHDT2'	95.4	[93.2 - 96.9]	62.9	[60.0 - 65.7]	92.4	[90.0 - 94.3]	0.02
'F2TSHDT2'	'F2TWHDT18'	95.4	[93.2 - 96.9]	62.8	[59.9 - 65.7]	92.4	[89.9 - 94.2]	0.01
'F2MC65HDT1'	'F2TSHDT2'	95.4	[93.2 - 96.9]	62.8	[60.0 - 65.7]	92.4	[89.9 - 94.2]	0.01
'F2TSHDT2'	'*Thermoactinomyces intermedius*' (GCA 016055025.1)	92.9	[90.1 - 94.9]	62.3	[59.4 - 65.1]	90	[87.3 - 92.3]	0.1
'F2TSHDT2'	'*Thermoactinomyces intermedius*' (GCA 013867715.1)	93	[90.2 - 95.0]	62.3	[59.4 - 65.1]	90.1	[87.4 - 92.3]	0.12

'*Thermoactinomyces vulgaris*' (GCA 001187615.2)	'*Thermoactinomyces intermedius*' (GCA 013867715.1)	92.8	[90.0 - 94.9]	61.7	[58.8 - 64.5]	89.8	[87.0 - 92.1]	0.11
'*Thermoactinomyces vulgaris*' (GCA 001187615.2)	'*Thermoactinomyces intermedius*' (GCA 016055025.1)	92.7	[89.9 - 94.8]	61.6	[58.7 - 64.4]	89.7	[87.0 - 92.0]	0.12
'F2MC65HDT15'	'*Thermoactinomyces intermedius*' (GCA 013867715.1)	92.8	[90.0 - 94.9]	61.6	[58.7 - 64.4]	89.8	[87.1 - 92.1]	0.13
'F2MC65HDT15'	'*Thermoactinomyces intermedius*' (GCA 016055025.1)	92.8	[90.0 - 94.9]	61.6	[58.7 - 64.4]	89.8	[87.0 - 92.0]	0.11
'F1NBHDT1'	'*Thermoactinomyces intermedius*' (GCA 016055025.1)	90.7	[87.5 - 93.1]	61.5	[58.6 - 64.3]	88	[85.0 - 90.4]	0.02
'F1NBHDT1'	'*Thermoactinomyces intermedius*' (GCA 013867715.1)	90.7	[87.6 - 93.1]	61.5	[58.7 - 64.3]	88	[85.1 - 90.5]	0.04

'F1MC60HDT27'	'*Thermoactinomyces intermedius*' (GCA 016055025.1)	92.7	[89.9 - 94.8]	61.4	[58.5 - 64.2]	89.7	[86.9 - 91.9]	0
'F1MC60HDT27'	'*Thermoactinomyces intermedius*' (GCA 013867715.1)	92.8	[90.0 - 94.9]	61.4	[58.5 - 64.2]	89.7	[87.0 - 92.0]	0.02
'F1MC65HDT1'	'*Thermoactinomyces intermedius*' (GCA 016055025.1)	92.8	[90.0 - 94.9]	61.3	[58.4 - 64.1]	89.7	[87.0 - 92.0]	0.05
'F2MC60HDT7'	'*Thermoactinomyces intermedius*' (GCA 016055025.1)	91.7	[88.7 - 93.9]	61.3	[58.5 - 64.1]	88.8	[85.9 - 91.1]	0.07
'F2MC60HDT51'	'*Thermoactinomyces vulgaris*' (GCA 001187615.2)	93	[90.2 - 95.0]	61.3	[58.4 - 64.0]	89.9	[87.1 - 92.1]	0.2
'F2MC60HDT7'	'*Thermoactinomyces intermedius*' (GCA 013867715.1)	91.7	[88.7 - 94.0]	61.3	[58.4 - 64.1]	88.8	[85.9 - 91.2]	0.09

'F1NPHDT35'	'*Thermoactinomyces intermedius*' (GCA 013867715.1)	91.6	[88.6 - 93.9]	61.2	[58.3 - 64.0]	88.7	[85.8 - 91.0]	0.4
'F1NPHDT35'	'*Thermoactinomyces intermedius*' (GCA 016055025.1)	91.5	[88.5 - 93.8]	61.2	[58.3 - 64.0]	88.6	[85.7 - 91.0]	0.38
'F2MC60HDT51'	'F2MC65HDT15'	88.3	[84.9 - 91.1]	61.2	[58.3 - 64.0]	85.9	[82.8 - 88.5]	0.04
'F1MC60HDT27'	'F2MC65HDT1'	92.8	[90.0 - 94.8]	61.2	[58.3 - 63.9]	89.7	[86.9 - 91.9]	0.09
'F2MC65HDT1'	'F2MC65HDT15'	88.3	[84.8 - 91.0]	61.2	[58.3 - 64.0]	85.9	[82.7 - 88.5]	0.02
'F2TWHDT18'	'*Thermoactinomyces vulgaris*' (GCA 001187615.2)	93	[90.2 - 95.0]	61.2	[58.3 - 64.0]	89.9	[87.1 - 92.1]	0.21
'F1MC65HDT1'	'*Thermoactinomyces intermedius*' (GCA 013867715.1)	92.9	[90.1 - 94.9]	61.2	[58.4 - 64.0]	89.8	[87.0 - 92.0]	0.03
'F1MC60HDT27'	'F2MC60HDT51'	92.8	[90.0 - 94.9]	61.2	[58.3 - 63.9]	89.7	[86.9 - 91.9]	0.08

'F2MC65HDT1'	'*Thermoactinomyces vulgaris*' (GCA 001187615.2)	92.9	[90.2 - 95.0]	61.2	[58.4 - 64.0]	89.8	[87.1 - 92.1]	0.21
'F2MC60HDT7'	'F2MC60HDT51'	92	[89.0 - 94.2]	61.1	[58.3 - 63.9]	89	[86.1 - 91.3]	0.01
'F2MC60HDT51'	'F2TPHDT3'	91.1	[88.0 - 93.4]	61.1	[58.2 - 63.9]	88.2	[85.3 - 90.6]	0.1
'F2MC65HDT15'	'F2TWHDT18'	88.3	[84.9 - 91.1]	61.1	[58.3 - 63.9]	85.9	[82.8 - 88.5]	0.02
'F2TPHDT3'	'*Thermoactinomyces intermedius*' (GCA 013867715.1)	91.9	[89.0 - 94.1]	61.1	[58.2 - 63.9]	88.9	[86.1 - 91.3]	0
'F2MC60HDT7'	'F2MC65HDT1'	91.9	[89.0 - 94.2]	61.1	[58.2 - 63.9]	88.9	[86.1 - 91.3]	0.02
'F2MC65HDT1'	'F2TPHDT3'	91	[87.9 - 93.4]	61.1	[58.2 - 63.8]	88.2	[85.2 - 90.6]	0.11
'F2TWHDT2'	'*Thermoactinomyces intermedius*' (GCA 013867715.1)	92.1	[89.2 - 94.3]	61.1	[58.2 - 63.8]	89.1	[86.2 - 91.4]	0

'F1MC65HDT1'	'F2MC65HDT1'	92.8	[90.0 - 94.9]	61.1	[58.3 - 63.9]	89.7	[86.9 - 92.0]	0.14
'F1MC65HDT1'	'F2MC60HDT51'	92.8	[90.0 - 94.9]	61.1	[58.3 - 63.9]	89.7	[86.9 - 92.0]	0.13
'F1MC60HDT27'	'F2TWHDT18'	92.8	[90.0 - 94.8]	61.1	[58.3 - 63.9]	89.7	[86.9 - 91.9]	0.09
'F1NSHDT14'	'*Thermoactinomyces intermedius*' (GCA 013867715.1)	92.4	[89.5 - 94.5]	61.1	[58.2 - 63.9]	89.3	[86.5 - 91.6]	0.14
'F1MC65HDT1'	'F2TWHDT18'	92.8	[90.0 - 94.9]	61.1	[58.2 - 63.9]	89.7	[86.9 - 91.9]	0.14
'F2TFHDT17'	'*Thermoactinomyces intermedius*' (GCA 016055025.1)	91.4	[88.4 - 93.7]	61.1	[58.2 - 63.8]	88.5	[85.6 - 90.9]	0.11
'F2MC60HDT7'	'F2TWHDT18'	92	[89.0 - 94.2]	61.1	[58.2 - 63.9]	89	[86.1 - 91.3]	0.02
'F2TPHDT3'	'*Thermoactinomyces intermedius*' (GCA 016055025.1)	91.9	[88.9 - 94.1]	61.1	[58.2 - 63.9]	88.9	[86.0 - 91.2]	0.02

'F1NSHDT14'	'*Thermoactinomyces intermedius*' (GCA 016055025.1)	92.4	[89.5 - 94.5]	61.1	[58.2 - 63.9]	89.3	[86.5 - 91.6]	0.13
'F2TWHDT2'	'*Thermoactinomyces intermedius*' (GCA 016055025.1)	92.1	[89.2 - 94.3]	61.1	[58.2 - 63.8]	89.1	[86.2 - 91.4]	0.02
'F2TFHDT17'	'*Thermoactinomyces intermedius*' (GCA 013867715.1)	91.5	[88.5 - 93.8]	61.1	[58.2 - 63.8]	88.6	[85.7 - 91.0]	0.12
'F2TWHDT2'	'F2TWHDT18'	91.3	[88.2 - 93.6]	61	[58.1 - 63.7]	88.3	[85.4 - 90.7]	0.11
'F1NSHDT14'	'F2MC65HDT1'	91.5	[88.4 - 93.7]	61	[58.1 - 63.8]	88.5	[85.6 - 90.9]	0.04
'F2TPHDT3'	'F2TWHDT18'	91.1	[88.0 - 93.4]	61	[58.2 - 63.8]	88.2	[85.2 - 90.6]	0.11
'F2MC65HDT1'	'F2TWHDT2'	91.2	[88.2 - 93.6]	61	[58.1 - 63.8]	88.3	[85.4 - 90.7]	0.11
'F2MC60HDT51'	'F2TWHDT2'	91.3	[88.2 - 93.6]	61	[58.1 - 63.8]	88.4	[85.4 - 90.8]	0.1

'F1NSHDT14'	'F2TWHDT18'	91.5	[88.5 - 93.8]	61	[58.1 - 63.8]	88.5	[85.6 - 90.9]	0.04
'F1NSHDT14'	'F2MC60HDT51'	91.5	[88.5 - 93.8]	61	[58.1 - 63.8]	88.5	[85.6 - 90.9]	0.05
'F1NPHDT35'	'F2MC65HDT1'	89.5	[86.1 - 92.1]	60.9	[58.1 - 63.7]	86.8	[83.7 - 89.4]	0.29
'F1NPHDT35'	'F2MC60HDT51'	89.5	[86.2 - 92.1]	60.9	[58.1 - 63.7]	86.8	[83.8 - 89.4]	0.3
'F1NBHDT1'	'F2TWHDT18'	89.2	[85.9 - 91.8]	60.9	[58.1 - 63.7]	86.6	[83.5 - 89.2]	0.07
'F1NBHDT1'	'F2MC60HDT51'	89.2	[85.9 - 91.8]	60.9	[58.1 - 63.7]	86.6	[83.5 - 89.2]	0.06
'F1NPHDT35'	'F2TWHDT18'	89.5	[86.2 - 92.1]	60.9	[58.0 - 63.7]	86.8	[83.8 - 89.4]	0.29
'F1NBHDT1'	'F2MC65HDT1'	89.2	[85.8 - 91.8]	60.9	[58.1 - 63.7]	86.6	[83.5 - 89.1]	0.07
'F2TFHDT17'	'F2TWHDT18'	91.4	[88.3 - 93.7]	60.7	[57.8 - 63.4]	88.3	[85.4 - 90.8]	0.02
'F2MC60HDT51'	'F2TFHDT17'	91.4	[88.4 - 93.7]	60.7	[57.8 - 63.5]	88.4	[85.4 - 90.8]	0.03

'F2MC65HDT1'	'F2TFHDT17'	91.4	[88.3 - 93.7]	60.7	[57.8 - 63.4]	88.3	[85.4 - 90.7]	0.02
'F1NWHDT6'	'F2MC65HDT1'	91.6	[88.6 - 93.9]	60.4	[57.5 - 63.2]	88.5	[85.6 - 90.9]	0.31
'F1NWHDT6'	'F2TWHDT18'	91.7	[88.7 - 93.9]	60.4	[57.5 - 63.2]	88.5	[85.6 - 90.9]	0.31
'F1NWHDT6'	'F2MC60HDT51'	91.6	[88.6 - 93.9]	60.4	[57.5 - 63.2]	88.5	[85.6 - 90.9]	0.32
'F1NWHDT6'	'*Thermoactinomyces intermedius*' (GCA 016055025.1)	89.5	[86.2 - 92.1]	60.4	[57.6 - 63.2]	86.7	[83.6 - 89.2]	0.4
'F1NWHDT6'	'*Thermoactinomyces intermedius*' (GCA 013867715.1)	89.5	[86.2 - 92.1]	60.4	[57.6 - 63.2]	86.7	[83.6 - 89.3]	0.42
'*Thermoactinomyces vulgaris*' (GCA 003688725.1)	'*Thermoactinomyces intermedius*' (GCA 013867715.1)	92.3	[89.4 - 94.4]	60.2	[57.4 - 63.0]	89	[86.1 - 91.3]	0.09
'*Thermoactinomyces vulgaris*' (GCA 003688725.1)	'*Thermoactinomyces intermedius*' (GCA 016055025.1)	92.3	[89.4 - 94.4]	60.2	[57.4 - 63.0]	89	[86.2 - 91.4]	0.08

'F2MC60HDT51'	'*Thermoactinomyces vulgaris*' (GCA 003688725.1)	92.3	[89.4 - 94.5]	60.2	[57.3 - 62.9]	89	[86.2 - 91.4]	0
'F2MC65HDT1'	'*Thermoactinomyces vulgaris*' (GCA 003688725.1)	92.4	[89.5 - 94.5]	60.2	[57.3 - 63.0]	89.1	[86.2 - 91.4]	0.01
'F2TWHDT18'	'*Thermoactinomyces vulgaris*' (GCA 003688725.1)	92.3	[89.4 - 94.5]	60.1	[57.3 - 62.9]	89	[86.2 - 91.4]	0.01
'*Bacillus subtilis*' (GCA 002055965.1)	'*Thermoactinomyces intermedius*' (GCA 016055025.1)	12.6	[10.0 - 15.9]	35.9	[33.5 - 38.4]	13	[10.7 - 15.8]	4.67
'F2MC60HDT51'	'*Bacillus subtilis*' (GCA 002055965.1)	12.7	[10.0 - 15.9]	34.9	[32.4 - 37.4]	13.1	[10.7 - 15.8]	4.59
'*Bacillus subtilis*' (GCA 002055965.1)	'*Thermoactinomyces intermedius*' (GCA 013867715.1)	12.6	[10.0 - 15.9]	34	[31.6 - 36.5]	13	[10.7 - 15.8]	4.68
'F2TWHDT18'	'*Bacillus subtilis*' (GCA 002055965.1)	12.6	[10.0 - 15.9]	34	[31.5 - 36.5]	13.1	[10.7 - 15.8]	4.58

'F2TFHDT17'	'*Bacillus subtilis*' (GCA 002055965.1)	12.7	[10.0 - 15.9]	33.9	[31.5 - 36.4]	13.1	[10.7 - 15.8]	4.56
'F2MC65HDT1'	'*Bacillus subtilis*' (GCA 002055965.1)	12.6	[10.0 - 15.9]	33.6	[31.1 - 36.1]	13.1	[10.7 - 15.8]	4.58
'F2MC65HDT15'	'*Bacillus subtilis*' (GCA 002055965.1)	12.6	[10.0 - 15.9]	33.6	[31.2 - 36.1]	13	[10.7 - 15.8]	4.55
'F1MC65HDT1'	'*Bacillus subtilis*' (GCA 002055965.1)	12.6	[10.0 - 15.9]	33.1	[30.7 - 35.6]	13	[10.7 - 15.8]	4.71
'F2TSHDT2'	'*Bacillus subtilis*' (GCA 002055965.1)	12.6	[10.0 - 15.9]	33.1	[30.7 - 35.6]	13.1	[10.7 - 15.8]	4.56
'F2MC60HDT7'	'*Bacillus subtilis*' (GCA 002055965.1)	12.6	[10.0 - 15.9]	33.1	[30.7 - 35.6]	13.1	[10.7 - 15.8]	4.6
'F1NSHDT14'	'*Bacillus subtilis*' (GCA 002055965.1)	12.6	[10.0 - 15.9]	32.9	[30.5 - 35.4]	13	[10.7 - 15.8]	4.54
'F1NPHDT35'	'*Bacillus subtilis*' (GCA 002055965.1)	12.6	[9.9 - 15.9]	32.9	[30.5 - 35.4]	13	[10.7 - 15.8]	4.29
'F1NBHDT1'	'*Bacillus subtilis*' (GCA 002055965.1)	12.6	[10.0 - 15.9]	32.8	[30.4 - 35.4]	13	[10.7 - 15.8]	4.65
'F1MC60HDT27'	'*Bacillus subtilis*' (GCA 002055965.1)	12.6	[10.0 - 15.9]	32.8	[30.4 - 35.3]	13	[10.7 - 15.8]	4.66

'*Thermoactinomyces vulgaris*' (GCA 001187615.2)	'*Bacillus subtilis*' (GCA 002055965.1)	12.7	[10.0 - 16.0]	32.5	[30.1 - 35.0]	13.1	[10.8 - 15.9]	4.79
'F1NWHDT6'	'*Bacillus subtilis*' (GCA 002055965.1)	12.6	[10.0 - 15.9]	32.4	[29.9 - 34.9]	13	[10.7 - 15.8]	4.27
'F2TWHDT2'	'*Bacillus subtilis*' (GCA 002055965.1)	12.6	[10.0 - 15.9]	32.1	[29.6 - 34.6]	13	[10.7 - 15.8]	4.68
'F2TPHDT3'	'*Bacillus subtilis*' (GCA 002055965.1)	12.6	[10.0 - 15.9]	31.7	[29.3 - 34.2]	13	[10.7 - 15.8]	4.69
'*Bacillus subtilis*' (GCA 002055965.1)	'*Thermoactinomyces vulgaris*' (GCA 003688725.1)	12.5	[9.9 - 15.8]	25.1	[22.7 - 27.5]	12.9	[10.6 - 15.7]	4.59

Reference List

Aagot, N., O. Nybroe, P. Nielsen, and K. Johnsen. 2001. An altered *Pseudomonas* diversity is recovered from soil by using nutrient-poor *Pseudomonas*-selective soil extract media. Appl. Environ. Microbiol. 67(11):5233-5239.

Abanda-Nkpwatt, D., U. Krimm, L. Schreiber, and W. Schwab. 2006. Dual antagonism of aldehydes and epiphytic bacteria from strawberry leaf surfaces against the pathogenic fungus *Botrytis cinerea* in vitro. Biocontrol 51(3):279-291.

Abd El-Rahman, H. A., D. Fritze, C. Spröer, and D. Claus. 2002. Two novel psychrotolerant species, *Bacillus psychrotolerans* sp. nov. and *Bacillus psychrodurans* sp. nov., which contain ornithine in their cell walls. Int. J. Syst. Evol. Microbiol. 52(6):2127-2133.

Abellan-Schneyder, I., A. Siebert, K. Hofmann, M. Wenning, and K. Neuhaus. 2021. Full-length SSU rRNA gene sequencing allows species-level detection of bacteria, archaea, and yeasts present in milk. Microorganisms 9(6):1251.

Abol Fotouh, D. M., R. A. Bayoumi, and M. A. Hassan. 2016. Production of thermoalkaliphilic lipase from *Geobacillus thermoleovorans* DA2 and application in leather industry. Enzyme Res. 2016:1-9.

Afrilasari, W., Widanarni, and A. Meryandini. 2016. Effect of probiotic *Bacillus megaterium* PTB 1.4 on the population of intestinal microflora, digestive enzyme activity and the growth of catfish (*Clarias* sp.). HAYATI J. Biosci. 23(4):168-172.

Afshari, A., A. Jamshidi, J. Razmyar, and M. Rad. 2016. Genomic diversity of Clostridium perfringens strains isolated from food and human sources. Iran J. Vet. Res. 17(3):160-164.

AgResearch Limited. 2021. Some environmental attributes of dairy sheep farming - https://www.agresearch.co.nz/assets/Uploads/Dairy-sheep-New-Zealand-environmental-attributes-2021.pdf.

Agrimonti, C., B. Bottari, M. L. S. Sardaro, and N. Marmiroli. 2019. Application of real-time PCR (qPCR) for characterization of microbial populations and type of milk in dairy food products. Crit. Rev. Food Sci. Nutr. 59(3):423-442.

Ahlborn, N., W. Young, J. Mullaney, and L. M. Samuelsson. 2020. In vitro fermentation of sheep and cow milk using infant fecal bacteria. Nutrients 12(6):1802.

Ahmad, S., R. K. Scopes, G. N. Rees, and B. K. Patel. 2000. *Saccharococcus caldoxylosilyticus* sp. nov., an obligately thermophilic, xylose-utilizing, endospore-forming bacterium. Int. J. Syst. Evol. Microbiol. 50(2):517-523.

Akhtar, N., N. Ilyas, H. Yasmin, R. Z. Sayyed, Z. Hasnain, E. A. Elsayed, and H. A. El Enshasy. 2021. Role of *Bacillus cereus* in improving the growth and phytoextractability of *Brassica nigra* (L.) K. Koch in chromium contaminated soil. Molecules 26(6):1569.

Albenzio, M., A. Santillo, M. Caroprese, F. D'Angelo, R. Marino, and A. Sevi. 2009. Role of endogenous enzymes in proteolysis of sheep milk. J. Dairy Sci. 92(1):79-86.

Alexopoulos, C., A. Karagiannidis, S. K. Kritas, C. Boscos, I. E. Georgoulakis, and S. C. Kyriakis. 2001. Field evaluation of a bioregulator containing live *Bacillus cereus* spores on health status and performance of sows and their litters. J. Vet. Med. 48(3):137-145.

Ali, Y. B., R. Verger, and A. Abousalham. 2012. Lipases or esterases: does it really matter? toward a new bio-physico-chemical classification. Pages 31-51. Humana Press.

Alles, A. A., M. Wiedmann, and N. H. Martin. 2018. Rapid detection and characterization of postpasteurization contaminants in pasteurized fluid milk. J. Dairy Sci. 101(9):7746-7756.

Allison, A. 1995. Importing a sheep which offers more - the East Friesian. Pages 321-323 in Proc. N.Z. Soc. Anim. Prod. Vol. 55. New Zealand Society of Animal Production.

Alquati, C., L. De Gioia, G. Santarossa, L. Alberghina, P. Fantucci, and M. Lotti. 2002. The cold-active lipase of *Pseudomonas fragi*. FEBS J. 269(13):3321-3328.

Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman. 1990. Basic local alignment search tool. JMB 215(3):403-410.

and, F. and Agriculture Organization of the United Nations. 1997. FAOSTAT statistical database. [Rome] : FAO, c1997-.

Anderson, R. C., E. N. Bermingham, W. C. McNabb, A. L. Cookson, M. H. Tavendale, K. M. Armstrong, S. O. Knowles, and N. C. Roy. 2010. Moderate levels of dietary sheep milk powder reduce experimentally induced colonic inflammation in rats. Anim. Prod. Sci. 50(7):714.

Antil, S., R. Kumar, D. V. Pathak, A. Kumar, A. Panwar, A. Kumari, and V. Kumar. 2021. On the potential of *Bacillus aryabhattai* KMT-4 against *Meloidogyne javanica*. Egypt. J. Biol. Pest Control. 31(1).

Arbor, A. 2017. *Bacillus subtilis* 168 genome sequence GCF_002055965.1 (unpublished).

Arcuri, E. F., P. D. L. D. Silva, M. A. V. P. Brito, J. R. F. Brito, C. C. Lange, and M. M. D. A. Magalhães. 2008. Contagem, isolamento e caracterização de bactérias psicrotróficas contaminantes de leite cru refrigerado. Cienc. Rural 38(8):2250-2255.

Argôlo-Filho, R. and L. Loguercio. 2013. *Bacillus thuringiensis* is an environmental pathogen and host-specificity has developed as an adaptation to human-generated ecological niches. Insects 5(1):62-91.

Arias, C., B. Oliete, S. Seseña, L. Jimenez, M. D. Pérez-Guzmán, and R. Arias. 2013. Importance of on-farm management practices on lactate-fermenting *Clostridium* spp. spore contamination of Manchega ewe milk: Determination of risk factors and characterization of *Clostridium* population. Small Rumin. Res. 111(1-3):120-128.

Arslan, S., A. Eyi, and F. Özdemir. 2011. Spoilage potentials and antimicrobial resistance of *Pseudomonas* spp. isolated from cheeses. J. Dairy Sci. 94(12):5851-5856.

Asgarani, E., T. Ghashghaei, M. R. Soudi, and N. Alimadadi. 2015. Enterobacterial repetitive intergenic consensus (ERIC) PCR based genetic diversity of *Xanthomonas* spp. and its relation to xanthan production. Iran J. Microbiol. 7(1):38-44.

Assis, F. G. D. V. D., C. L. D. S. Ávila, J. C. Pinto, and R. F. Schwan. 2014. New inoculants on maize silage fermentation. Rev. Bras. Zootec 43(8):395-403.

Ávila, M., N. Gómez-Torres, M. Hernández, and S. Garde. 2014. Inhibitory activity of reuterin, nisin, lysozyme and nitrite against vegetative cells and spores of dairy-related *Clostridium* species. Int. J. Food Microbiol. 172:70-75.

Aydogan, E. L., G. Moser, C. Müller, P. Kämpfer, and S. P. Glaeser. 2018. Long-term warming shifts the composition of bacterial communities in the phyllosphere of *Galium album* in a permanent grassland field-experiment. Front. Microbiol. 9.

Ayscue, P., C. Lanzas, R. Ivanek, and Y. T. Gröhn. 2009. Modeling on-farm *Escherichia coli* O157:H7 population dynamics. Foodborne Pathog. Dis. 6(4):461-470.

Bacques, C., N. Thi Cuc, M. Serusclat, A. Dorier, and J. P. Perret. 1983. Etude in vitro de l'activité lipasique de souches bactériennes isolées de cæcum de lapins sur la trioléine marquée au C14. Ann. Nutr. Metab. 27(2):145-152.

Balthazar, C. F., T. C. Pimentel, L. L. Ferrão, C. N. Almada, A. Santillo, M. Albenzio, N. Mollakhalili, A. M. Mortazavian, J. S. Nascimento, M. C. Silva, M. Q. Freitas, A. S. Sant'Ana, D. Granato, and A. G. Cruz. 2017. Sheep milk: Physicochemical characteristics and relevance for functional food development. Compr. Rev. Food Sci. Food Saf. 16(2):247-262.

Barash, J. R., J. K. Hsia, and S. S. Arnon. 2010. Presence of soil-dwelling clostridia in commercial powdered infant formulas. J. Pediatr. 156(3):402-408.

Bava, L., S. Colombini, M. Zucali, M. Decimo, S. Morandi, T. Silvetti, M. Brasca, A. Tamburini, G. M. Crovetto, and A. Sandrucci. 2017. Efficient milking hygiene reduces bacterial spore contamination in milk. J. Dairy Res. 84(3):322-328.

Bava, L., M. Zucali, A. Sandrucci, M. Brasca, L. Vanoni, L. Zanini, and A. Tamburini. 2011. Effect of cleaning procedure and hygienic condition of milking equipment on bacterial count of bulk tank milk. Journal of Dairy Research 78(2):211-219.

Beauvais, W., E. V. Gart, M. Bean, A. Blanco, J. Wilsey, K. McWhinney, L. Bryan, M. Krath, C.-Y. Yang, D. Manriquez Alvarez, S. Paudyal, K. Bryan, S. Stewart, P. W. Cook, G. Lahodny, K. Baumgarten, R. Gautam, K. Nightingale, S. D. Lawhon, P. Pinedo, and R. Ivanek. 2018. The prevalence of *Escherichia coli* O157:H7 fecal shedding in feedlot pens is affected by the water-to-cattle ratio: A randomized controlled trial. PLOS ONE 13(2):e0192149.

Beef+Lamb New Zealand. 2019. Milksolids price New Zealand - Nominal price - https://beeflambnz.com/sites/default/files/data/files/Annual%20dairy%20milk%20solids.pdf.

Behrendt, U., A. Ulrich, P. Schumann, W. Erler, J. Burghardt, and W. Seyfarth. 1999. A taxonomic study of bacteria isolated from grasses: a proposed new species *Pseudomonas graminis* sp. nov. Int. J. Syst. Evol. Microbiol. 49(1):297-308.

Bencini, R. and T. Murray. 2012. Exploring the Commercial Potential of Sheep Milk.

Bender, S. F., C. Wagg, and M. G. A. van der Heijden. 2016. An underground revolution: biodiversity and soil ecological engineering for agricultural sustainability. Trends Ecol. Evol. 31(6):440-452.

Bennett, G. M. and N. A. Moran. 2015. Heritable symbiosis: The advantages and perils of an evolutionary rabbit hole. Proceedings of the National Academy of Sciences of the United States of America 112(33):10169-10176.

Beno, S. M., R. A. Cheng, R. H. Orsi, D. R. Duncan, X. Guo, J. Kovac, L. M. Carroll, N. H. Martin, and M. Wiedmann. 2020. *Paenibacillus odorifer*, the predominant *Paenibacillus* species isolated from milk in the United States, demonstrates genetic and phenotypic conservation of psychrotolerance but clade-associated differences in nitrogen metabolic pathways. mSphere 5(1).

Berendsen, E. M., R. A. Koning, J. Boekhorst, A. de Jong, O. P. Kuipers, and M. H. J. Wells-Bennik. 2016. High-level heat resistance of spores of *Bacillus amyloliquefaciens* and *Bacillus licheniformis* results from the presence of a spoVA operon in a Tn1546 transposon. Front. Microbiol. 7.

Berge, O., M.-H. Guinebretière, W. Achouak, P. Normand, and T. Heulin. 2002. *Paenibacillus graminis* sp. nov. and *Paenibacillus odorifer* sp. nov., isolated from plant roots, soil and food. Int. J. Syst. Evol. Microbiol. 52(2):607-616.

Bergère, J. L., D. L. Bars, and J. Commissaire. 1969. La bactofugation du lait et l'élimination des spores de *Clostridium tyrobutyricum*. Lait 49:507-519.

Berings, M., A. Jult, H. Vermeulen, N. De Ruyck, L. Derycke, H. Ucar, P. Ghekiere, R. Temmerman, J. Ellis, C. Bachert, B. N. Lambrecht, M. Dullaers, and P. Gevaert. 2017. Probiotics-impregnated bedding covers for house dust mite allergic rhinitis: A pilot randomized clinical trial. Clin. Exp. Allergy 47(8):1092-1096.

Bermudez, J., M. J. Gonzalez, J. A. Olivera, J. A. Burgueno, P. Juliano, E. M. Fox, and S. M. Reginensi. 2016. Seasonal occurrence and molecular diversity of clostridia species spores along cheesemaking streams of 5 commercial dairy plants. J. Dairy Sci. 99(5):3358-3366.

Bezuidt, O. K. I., T. P. Makhalanyane, M. A. Gomri, K. Kharroub, and D. A. Cowan. 2015. Draft genome sequence of thermophilic *Geobacillus* sp. strain Sah69, isolated from Saharan soil, southeast Algeria. Genome Announc. 3(6):e01447-01415.

Bhandari, S., D. K. Poudel, R. Marahatha, S. Dawadi, K. Khadayat, S. Phuyal, S. Shrestha, S. Gaire, K. Basnet, U. Khadka, and N. Parajuli. 2021. Microbial enzymes used in bioremediation. J. Chem. 2021:1-17.

Bhatt, K. and D. K. Maheshwari. 2019. Decoding multifarious role of cow dung bacteria in mobilization of zinc fractions along with growth promotion of *C. annuum* L. Sci. Rep. 9(1).

Biçer, Y., A. E. Telli, G. Sönmez, N. Telli, and G. Uçar. 2021. Comparison of microbiota and volatile organic compounds in milk from different sheep breeds. J. Dairy Sci. 104(12):12303-12311.

Bills, D. D. and E. A. Day. 1964. Determination of the major free fatty acids of cheddar cheese. J. Dairy Sci. 47(7):733-738.

Blagoveshchenskii, V. A., A. K. Tikhaze, and Z. I. Borishpolets. 1972. Lipase and tweenase activities of *Clostridium perfringens* and *Cl. botulinum*. Biull. Eksp. Biol. Med. 74(8):74-77.

Blow, M. J., T. A. Clark, C. G. Daum, A. M. Deutschbauer, A. Fomenkov, R. Fries, J. Froula, D. D. Kang, R. R. Malmstrom, R. D. Morgan, J. Posfai, K. Singh, A. Visel, K. Wetmore, Z. Zhao, E. M. Rubin, J. Korlach, L. A. Pennacchio, and R. J. Roberts. 2016. The epigenomic landscape of prokaryotes. PLOS Genetics 12(2):e1005854.

Boddinghaus, B., J. Wolters, W. Heikens, and E. C. Bottger. 1990. Phylogenetic analysis and identification of different serovars of *Mycobacterium intracellulare* at the molecular level. FEMS Microbiol. Lett. 58(2):197-203.

Borreani, G., P. Dolci, E. Tabacco, and L. Cocolin. 2013. Aerobic deterioration stimulates outgrowth of spore-forming *Paenibacillus* in corn silage stored under oxygen-barrier or polyethylene films. J. Dairy Sci. 96(8):5206-5216.

Borreani, G., F. Ferrero, D. Nucera, M. Casale, S. Piano, and E. Tabacco. 2019. Dairy farm management practices and the risk of contamination of tank milk from *Clostridium* spp. and *Paenibacillus* spp. spores in silage, total mixed ration, dairy cow feces, and raw milk. J. Dairy Sci. 102(9):8273-8289.

Borreani, G. and E. Tabacco. 2010. The relationship of silage temperature with the microbiological status of the face of corn silage bunkers. J. Dairy Sci. 93(6):2620-2629.

Boubendir, A., D. I. Serrazanetti, M. A. Hamidechi, L. Vannini, and M. E. Guerzoni. 2016. Changes in bacterial populations in refrigerated raw milk collected from a semi-arid area of Algeria. Ann. Microbiol. 66(2):777-783.

Bowman, J. P. 2006. The genus *Psychrobacter*. Pages 920-930 in The Prokaryotes. Springer New York.

Bramanti, E., C. Sortino, M. Onor, F. Beni, and G. Raspi. 2003. Separation and determination of denatured αs1-, αs2-, β- and κ-caseins by hydrophobic interaction chromatography in cows', ewes' and goats' milk, milk mixtures and cheeses. Journal of Chromatography A 994(1):59-74.

Brand, E., M. Liaudat, R. Olt, and W. Linxweiler. 2000. Rapid determination of lipase in raw, pasteurised and UHT-milk. MILCAD 55(10):573-576.

Brändle, J., V. Fraberger, K. Schuller, U. Zitz, W. Kneifel, and K. J. Domig. 2017. A critical assessment of four most probable number procedures for routine enumeration of cheese-damaging clostridia in milk. Int. Dairy J. 73:109-115.

Bremer, P. J., S. Fillery, and A. J. McQuillan. 2006. Laboratory scale Clean-In-Place (CIP) studies on the effectiveness of different caustic and acid wash steps on the removal of dairy biofilms. Int. J. Food Microbiol. 106(3):254-262.

Brightwell, G. and K. M. Horváth. 2018. Molecular discrimination of New Zealand sourced meat spoilage associated psychrotolerant *Clostridium* species by ARDRA and its comparison with 16s RNA gene sequencing. Meat Sci. 138:23-27.

Brillard, J., C. M. Dupont, O. Berge, C. Dargaignaratz, S. Oriol-Gagnier, C. Doussan, V. Broussolle, M. Gillon, T. Clavel, and A. Berard. 2015. The water cycle, a potential source of the bacterial pathogen *Bacillus cereus*. BioMed Res. Int. 2015:356928.

Brinkmann, C. M., C. Neuman, M. Katouli, and D. I. Kurtboke. 2014. Detection of *Thermoactinomyces* species in selected agricultural substrates from Queensland. Microb. Ecol. 67(4):804-809.

Brod, D. L., K. K. Bolsen, and B. E. Brent. 1982. Effect of water temperature on rumen temperature, digestion and rumen fermentation in sheep. J. Anim. Sci. 54(1):179-182.

Broda, D. M., D. J. Saul, P. A. Lawson, R. G. Bell, and D. R. Musgrave. 2000. *Clostridium gasigenes* sp. nov., a psychrophile causing spoilage of vacuum-packed meat. Int. J. Syst. Evol. Microbiol. 50(1):107-118.

Brown, A. and R. Luke. 2010. Siderophore production and utilization by milk spoilage *Pseudomonas* species. J. Dairy Sci. 93(4):1355-1363.

Bruzaroski, S. R., R. P. d. Souza, P. d. S. Pasquim, R. Fagnani, and E. H. W. d. Santana. 2020. Influence of storage temperature on the population of microorganisms in raw sheep milk and its physical-chemical profile. Res., Soc. Dev. 9(12):e27691210796.

Buchfink, B., C. Xie, and D. H. Huson. 2015. Fast and sensitive protein alignment using DIAMOND. Nat. Methods 12(1):59-60.

Buehler, A. J., N. H. Martin, K. J. Boor, and M. Wiedmann. 2018. Psychrotolerant spore-former growth characterization for the development of a dairy spoilage predictive model. J. Dairy Sci. 101(8):6964-6981.

Buehner, K. P., S. Anand, G. D. Djira, and A. Garcia. 2014. Prevalence of thermoduric bacteria and spores on 10 Midwest dairy farms. J. Dairy Sci. 97(11):6777-6784.

Bunk, B., A. Schulz, S. Stammen, R. Münch, M. J. Warren, D. Jahn, and R. Biedendieck. 2010. A short story about a big magic bug. Bioeng. Bugs 1(2):85-91.

Burgess, S. 2016. Characterisation of dairy strains of *Geobacillus stearothermophilus* and a genomics insight into its growth and survival during dairy manufacture : a thesis presented in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Microbiology at Massey University, Palmerston North, New Zealand.

Burgess, S. A., S. H. Flint, and D. Lindsay. 2014. Characterization of thermophilic bacilli from a milk powder processing plant. J. Appl. Microbiol. 116(2):350-359.

Burgess, S. A., D. Lindsay, and S. H. Flint. 2010. Thermophilic bacilli and their importance in dairy processing. Int. J. Food Microbiol. 144(2):215-225.

Burke, C. M. and A. E. Darling. 2016. A method for high precision sequencing of near full-length 16S rRNA genes on an Illumina MiSeq. PeerJ 4:e2492.

Burrow, K., W. Young, N. Hammer, S. Safavi, M. Scholze, M. McConnell, A. Carne, D. Barr, M. Reid, and A. E.-D. Bekhit. 2020. The effect of the supplementation of a diet low in calcium and phosphorus with either sheep milk or cow milk on the physical and mechanical characteristics of bone using a rat model. Foods 9(8):1070.

Burtscher, J., L. Hobl, W. Kneifel, and K. J. Domig. 2020. Short communication: Clostridial spore counts in vat milk of Alpine dairies. J. Dairy Sci. 103(3):2111-2116.

Buzby, J., s. H. Well, and J. Hyman. 2014. The estimated amount, value, and calories of postharvest food losses at the retail and consumer levels in the United States. in USDA-ERS economic information bulletin. USDA-ERS, ed, Washington, DC.

Cantoni, C., G. Soncini, S. Milesi, L. Cocolin, L. Iacumin, and G. Comi. 2006. Additional data about some defects of cheeses: Discoloration and blowing. Ind. Aliment 45(456):276-281.

Capodifoglio, E., A. M. C. Vidal, J. A. S. Lima, F. Bortoletto, L. F. D'Abreu, A. C. S. Gonçalves, A. C. N. Vaz, J. C. D. C. Balieiro, and A. S. Netto. 2016. Lipolytic and proteolytic activity of *Pseudomonas* spp. isolated during milking and storage of refrigerated raw milk. J. Dairy Sci. 99(7):5214-5223.

Carrascosa, C., R. Martínez, E. Sanjuán, R. Millán, C. Del Rosario-Quintana, F. Acosta, A. García, and J. R. Jaber. 2021. Identification of the *Pseudomonas fluorescens* group as being responsible for blue pigment on fresh cheese. J. Dairy Sci. 104(6):6548-6558.

Caselli, E., M. D'Accolti, A. Vandini, L. Lanzoni, M. T. Camerada, M. Coccagna, A. Branchini, P. Antonioli, P. G. Balboni, D. Di Luca, and S. Mazzacane. 2016. Impact of a probiotic-based cleaning intervention on the microbiota ecosystem of the hospital surfaces: focus on the resistome remodulation. PLOS ONE 11(2):e0148857.

Cavello, I., M. S. Urbieta, S. Cavalitto, and E. Donati. 2020. *Bacillus cytotoxicus* isolated from a pristine natural geothermal area reveals high keratinolytic activity. Microorganisms 8(6):796.

Cestonaro, T., M. S. S. d. M. Costa, L. A. d. M. Costa, D. C. Pereira, M. A. T. Rozatti, and M. F. L. Martins. 2017. Addition of cattle manure to sheep bedding allows vermicomposting process and improves vermicompost quality. Waste Manage. 61:165-170.

Chambers, J. V. 2002. The microbiology of raw milk. Pages 39-90 in Dairy Microbiology Handbook. R. K. Robinson, ed. John Wiley & Sons, Inc.

Chandra, P., Enespa, R. Singh, and P. K. Arora. 2020. Microbial lipases and their industrial applications: a comprehensive review. Microb. Cell Factories 19(1).

Chauhan, K., R. Dhakal, R. B. Seale, H. C. Deeth, C. J. Pillidge, I. B. Powell, H. Craven, and M. S. Turner. 2013. Rapid identification of dairy mesophilic and thermophilic sporeforming bacteria

using DNA high resolution melt analysis of variable 16S rDNA regions. Int. J. Food Microbiol. 165(2):175-183.

Chaves, J. Q., E. P. de Paiva, L. Rabinovitch, and A. M. Vivoni. 2017. Molecular characterization and risk assessment of *Bacillus cereus sensu lato* isolated from ultrahigh-temperature and pasteurized milk marketed in Rio de Janeiro, Brazil. J. Food Prot. 80(7):1060-1065.

Chen, L., R. M. Daniel, and T. Coolbear. 2003. Detection and impact of protease and lipase activities in milk and milk powders. Int. Dairy J. 13(4):255-275.

Cheng, K. 2020. WGS of *Thermoactinomyces* spp. (unpublished) - https://www.ncbi.nlm.nih.gov/nuccore/JAECVW000000000.1/.

Chia, J., K. Burrow, A. Carne, M. McConnell, L. Samuelsson, L. Day, W. Young, and A. E.-D. A. Bekhit. 2017. Minerals in sheep milk. Pages 345-362 in Nutrients in Milk and Their Implications on Health and Disease. R. J. C. Ronald Watson, Victor Preedy, ed. Elsevier Publishing, London.

Chiaverini, A., F. Guidi, M. Torresi, V. A. Acciari, G. Centorotola, A. Cornacchia, P. Centorame, C. Marfoglia, G. Blasi, M. Di Domenico, G. Migliorati, S. Roussel, F. Pomilio, and Y. Sevellec. 2021. Phylogenetic analysis and genome-wide association study applied to an Italian *Listeria monocytogenes* outbreak. Front. Microbiol. 12.

Chiesa, F., S. Lomonaco, D. Nucera, D. Garoglio, A. Dalmasso, and T. Civera. 2014. Distribution of *Pseudomonas* species in a dairy plant affected by occasional blue discoloration. Ital. J. Food Saf. 3(4).

Chon, J.-W., K.-Y. Song, H. Kim, and K.-H. Seo. 2014. Comparison of 3 selective media for enumeration of *Bacillus cereus* in several food matrixes. J. Food Sci. 79(12):M2480-M2484.

Christiansson, A., J. Bertilsson, and B. Svensson. 1999. *Bacillus cereus* spores in raw milk: Factors affecting the contamination of milk during the grazing period. J. Dairy Sci. 82(2):305-314.

Chukwuma, O. B., M. Rafatullah, H. A. Tajarudin, and N. Ismail. 2021. Bacterial diversity and community structure of a municipal solid waste landfill: a source of lignocellulolytic potential. Life 11(6):493.

Cihan, A. C., C. Cokmus, M. Koc, and B. Ozcan. 2014. *Anoxybacillus calidus* sp. nov., a thermophilic bacterium isolated from soil near a thermal power plant. Int. J. Syst. Evol. Microbiol. 64(Pt_1):211-219.

Circella, E., A. Schiavone, R. Barrasso, A. Camarda, N. Pugliese, and G. Bozzo. 2020. *Pseudomonas azotoformans* belonging to *Pseudomonas fluorescens* group as causative agent of blue coloration in carcasses of slaughterhouse rabbits. Animals 10(2):256.

Coblentz, W. K. and R. E. Muck. 2012. Effects of natural and simulated rainfall on indicators of ensilability and nutritive value for wilting alfalfa forages sampled before preservation as silage. J. Dairy Sci. 95(11):6635-6653.

Coblentz, W. K., R. E. Muck, M. A. Borchardt, S. K. Spencer, W. E. Jokela, M. G. Bertram, and K. P. Coffey. 2014. Effects of dairy slurry on silage fermentation characteristics and nutritive value of alfalfa. J. Dairy Sci. 97(11):7197-7211.

Coil, D., G. Jospin, and A. E. Darling. 2015. A5-miseq: an updated pipeline to assemble microbial genomes from Illumina MiSeq data. Bioinformatics 31(4):587-589.

Cole, J. R., Q. Wang, J. A. Fish, B. Chai, D. M. McGarrell, C. T. B. Y. Sun, A. Porras-Alfaro, C. R. Kuske, and J. M. Tiedje. 2014. Ribosomal Database Project: data and tools for high throughput rRNA analysis. Nucleic Acids Res. 42:D633-D642.

Collins, Y. F., P. L. H. McSweeney, and M. G. Wilkinson. 2003. Lipolysis and free fatty acid catabolism in cheese: a review of current knowledge. Int. Dairy J. 13(11):841-866.

Cook, N. B. 2003. Prevalence of lameness among dairy cattle in Wisconsin as a function of housing type and stall surface. J. Am. Vet. Med. Assoc. 223(9):1324-1328.

Coorevits, A., V. De Jonghe, J. Vandroemme, R. Reekmans, J. Heyrman, W. Messens, P. De Vos, and M. Heyndrickx. 2008. Comparative analysis of the diversity of aerobic spore-forming bacteria in raw milk from organic and conventional dairy farms. Syst. Appl. Microbiol. 31(2):126-140.

Cope-Selby, N., A. Cookson, M. Squance, I. Donnison, R. Flavell, and K. Farrar. 2017. Endophytic bacteria in Miscanthus seed: implications for germination, vertical inheritance of endophytes, plant evolution and breeding. GCB Bioenergy 9(1):57-77.

Courthaudon, J.-L., J.-M. Girardet, S. Campagne, L.-M. Rouhier, S. Campagna, G. Linden, and D. Lorient. 1999. Surface active and emulsifying properties of casein micelles compared to those of sodium caseinate. Int. Dairy J. 9:411-412.

Cristobal-Carballo, O., M. A. Khan, F. W. Knol, S. J. Lewis, D. R. Stevens, R. A. Laven, and S. A. McCoard. 2019. Impact of weaning age on rumen development in artificially reared lambs. J. Anim. Sci. 97(8):3498-3510.

Cross, T. 1968. Thermophilic Actinomycetes. J. Appl. Bacteriol. 31(1):36-53.

Cross, T. and D. Johnston. 1971. *Thermoactinomyces vulgaris*. Distribution in natural habitats. Barker, Gould and Wolf (Editors), Spore Research:315-330.

Cude, W. N. and A. Buchan. 2013. Acyl-homoserine lactone-based quorum sensing in the *Roseobacter* clade: complex cell-to-cell communication controls multiple physiologies. Front. Microbiol. 4:336.

D'Incecco, P., L. Pellegrino, J. A. Hogenboom, P. S. Cocconcelli, and D. Bassi. 2018. The late blowing defect of hard cheeses: Behaviour of cells and spores of *Clostridium tyrobutyricum* throughout the cheese manufacturing and ripening. LWT 87:134-141.

D'Incecco, P., V. Rosi, M. G. Fortina, M. Sindaco, G. Ricci, and L. Pellegrino. 2022. Biochemical, microbiological, and structural evaluations to early detect age gelation of milk caused by proteolytic activity of *Pseudomonas fluorescens*. Eur. Food Res. Technol. 248(8):2097-2107.

Da Silva Duarte, V., L. Treu, C. Sartori, R. S. Dias, I. Da Silva Paes, M. S. Vieira, G. R. Santana, M. I. Marcondes, A. Giacomini, V. Corich, S. Campanaro, C. C. Da Silva, and S. O. De Paula. 2020. Milk microbial composition of Brazilian dairy cows entering the dry period and genomic comparison between *Staphylococcus aureus* strains susceptible to the bacteriophage vB_SauM-UFV_DC4. Sci. Rep. 10(1).

Da Silva, G. J., W. R. Rivadavea, J. D. De Lima, P. H. R. Monteiro, and F. B. Da Silva. 2021. Microbial enzymes and soil health. Pages 133-155 in Plant Growth-Promoting Microbes for Sustainable Biotic and Abiotic Stress Management. Springer International Publishing.

Dabboussi, F., M. Hamze, E. Singer, V. Geoffroy, J.-M. Meyer, and D. Izard. 2002. Pseudomonas mosselii sp. nov., a novel species isolated from clinical specimens. Int. J. Syst. Evol. Microbiol. 52(2):363-376.

Datta, N. and H. C. Deeth. 2001. Age gelation of UHT milk - A review. Food and Bioproducts Processing 79(4):197-210.

Davies, D. R., R. J. Merry, and E. L. Bakewell. 1996. The effect of timing of slurry application on the microflora of grass, and changes occurring during silage fermentation. Grass Forage Sci. 51:42-51.

de Garnica, M. L., B. Linage, J. A. Carriedo, L. F. De La Fuente, M. C. Garcia-Jimeno, J. A. Santos, and C. Gonzalo. 2013. Relationship among specific bacterial counts and total bacterial and somatic cell counts and factors influencing their variation in ovine bulk tank milk. J. Dairy Sci. 96(2):1021-1029.

de Garnica, M. L., J. A. Santos, and C. Gonzalo. 2011. Influence of storage and preservation on microbiological quality of silo ovine milk. J. Dairy Sci. 94(4):1922-1927.

De Jonghe, V., A. Coorevits, J. De Block, E. Van Coillie, K. Grijspeerdt, L. Herman, P. De Vos, and M. Heyndrickx. 2010. Toxinogenic and spoilage potential of aerobic spore-formers isolated from raw milk. Int. J. Food Sci. Technol. 136(3):318-325.

De Jonghe, V., A. Coorevits, K. Van Hoorde, W. Messens, A. Van Landschoot, P. De Vos, and M. Heyndrickx. 2011. Influence of storage conditions on the growth of *Pseudomonas* species in refrigerated raw milk. Appl. Environ. Microbiol. 77(2):460-470.

de Moura Aguiar, B., R. de Longhi, R. C. Poli-Frederico, R. Fagnani, and E. H. W. de Santana. 2019. Lipoproteolytic capacity and potential of *Pseudomonas* spp. isolated from cold raw milk. J. Dairy Res. 86(4):467-469.

De Vos, P., G. M. Garrity, D. Jones, N. R. Krieg, W. Ludwig, F. A. Rainey, K.-H. Schleifer, and W. B. Whitman. 2009. The Firmicutes. in Bergey's Manual of Systematic Bacteriology. Vol. 3. Springer, New York.

Delaunay, L., E. Cozien, P. Gehannin, N. Mouhali, S. Mace, F. Postollec, I. Leguerinel, and A.-G. Mathot. 2021. Occurrence and diversity of thermophilic sporeformers in French dairy powders. Int. Dairy J. 113:104889.

Demirci, A., A. L. Pometto, and K. E. Johnson. 1993. Evaluation of biofilm reactor solid support for mixed-culture lactic acid production. Appl. Microbiol. Biotechnol. 38(6):728-733.

Deng, B., L. Wang, Q. Ma, T. Yu, D. Liu, Y. Dai, and G. Zhao. 2021. Genomics analysis of *Bacillus megaterium* 1259 as a probiotic and its effects on performance in lactating dairy cows. Animals 11(2):397.

Derakhshani, H., K. B. Fehr, S. Sepehri, D. Francoz, J. De Buck, H. W. Barkema, J. C. Plaizier, and E. Khafipour. 2018. Invited review: Microbiota of the bovine udder: Contributing factors and potential implications for udder health and mastitis susceptibility. J. Dairy Sci. 101(12):10605-10625.

Devries, T. J., S. Dufour, and D. T. Scholl. 2010. Relationship between feeding strategy, lying behavior patterns, and incidence of intramammary infection in dairy cows. J. Dairy Sci. 93(5):1987-1997.

Dezfulian, M., L. M. McCroskey, C. L. Hatheway, and V. R. Dowell, Jr. 1981. Selective medium for isolation of *Clostridium botulinum* from human feces. J. Clin. Microbiol. 13(3):526-531.

Dice, L. R. 1945. Measures of the amount of ecologic association between species. Ecology 26(3):297-302.

Dietrich, R., N. Jessberger, M. Ehling-Schulz, E. Märtlbauer, and P. E. Granum. 2021. The food poisoning toxins of *Bacillus cereus*. Toxins 13(2):98.

Dignam, B. E. A. 2016. Ecological assessments of *Pseudomonas* communities in pastoral soils: implications for disease suppression and sustainable production in agricultural grasslands: a thesis presented in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Microbial Ecology at Lincoln University, Lincoln, New Zealand.

Dingle, T. C. and S. M. Butler-Wu. 2013. MALDI-TOF mass spectrometry for microorganism identification. Clin. Lab. Med. 33(3):589-609.

Dobrzanski, T., F. Gravina, B. Steckling, L. R. Olchanheski, R. F. Sprenger, B. C. Espírito Santo, C. W. Galvão, P. M. Reche, R. A. Prestes, S. A. V. Pileggi, F. R. Campos, R. A. Azevedo, M. J. Sadowsky, F. L. Beltrame, and M. Pileggi. 2018. *Bacillus megaterium* strains derived from water and soil exhibit differential responses to the herbicide mesotrione. PLOS ONE 13(4):e0196166.

Dogan, B. and K. J. Boor. 2003. Genetic diversity and spoilage potentials among *Pseudomonas* spp. isolated from fluid milk products and dairy processing plants. Appl. Environ. Microbiol. 69(1):130-138.

Doll, E. V., S. Scherer, and M. Wenning. 2017. Spoilage of microfiltered and pasteurized extended shelf life milk is mainly induced by psychrotolerant spore-forming bacteria that often originate from recontamination. Front. Microbiol. 8:135.

Donia, M. S., P. Cimermancic, C. J. Schulze, L. C. Wieland Brown, J. Martin, M. Mitreva, J. Clardy, R. G. Linington, and M. A. Fischbach. 2014. A systematic analysis of biosynthetic gene clusters in the human microbiome reveals a common family of antibiotics. Cell 158(6):1402-1414.

Dore, S., A. M. Ferrini, B. Appicciafuoco, M. R. Massaro, G. Sotgiu, M. Liciardi, and E. A. Cannas. 2019. Efficacy of a terpinen-4-ol based dipping for post-milking teat disinfection in the prevention of mastitis in dairy sheep. J. Essent. Oil Res. 31(1):19-26.

Dorneles, E. M. S., J. A. Santana, D. Ribeiro, F. A. Dorella, A. S. Guimarães, M. S. Moawad, S. A. Selim, A. L. M. Garaldi, A. Miyoshi, M. G. Ribeiro, A. M. G. Gouveia, V. Azevedo, M. B. Heinemann, and A. P. Lage. 2014. Evaluation of ERIC-PCR as genotyping method for *Corynebacterium pseudotuberculosis* isolates. PLoS ONE 9(6):e98758.

Doyle, C. J., D. Gleeson, P. W. O'Toole, and P. D. Cotter. 2017. Impacts of seasonal housing and teat preparation on raw milk microbiota: A high-throughput sequencing study. Appl. Environ. Microbiol. 83(2):e02694-02616.

Doyle, C. J., P. W. O'Toole, and P. D. Cotter. 2018. Genomic characterization of sulphite reducing bacteria isolated from the dairy production chain. Front. Microbiol. 9(1507).

Drean, P., C. M. McAuley, S. C. Moore, N. Fegan, and E. M. Fox. 2015. Characterization of the spore-forming *Bacillus cereus sensu lato* group and *Clostridium perfringens* bacteria isolated from the Australian dairy farm environment. BMC Microbiol. 15:38.

Dreier, M., M. Meola, H. Berthoud, N. Shani, D. Wechsler, and P. Junier. 2022. High-throughput qPCR and 16S rRNA gene amplicon sequencing as complementary methods for the investigation of the cheese microbiota. BMC Microbiol. 22(1).

Drewry, J. J., R. P. Littlejohn, and R. J. Paton. 2000. A survey of soil physical properties on sheep and dairy farms in southern New Zealand. New Zealand J. Agric. Res. 43(2):251-258.

Driehuis, F. 2013. Silage and the safety and quality of dairy foods: a review. Agric. Food Sci. 22:16-34.

Driehuis, F., J. Hoolwerf, and J. L. W. Rademaker. 2016. Concurrence of spores of *Clostridium tyrobutyricum*, *Clostridium beijerinckii* and *Paenibacillus polymyxa* in silage, dairy cow faeces and raw milk. Int. Dairy J. 63:70-77.

Driehuis, F., E. Lucas-van den Bos, and M. H. J. WellsBennik. 2014. Sporen van thermofiele aërobe sporenvormers in compost en andere beddingmaterialen bij melkveebedrijven met een vrijloop- of ligboxenstal. in NIZO-RAPPORT E2014/045. NIZO, Ede, The Netherlands.

Driehuis, F., J. M. Wilkinson, Y. Jiang, I. Ogunade, and A. T. Adesogan. 2018. Silage review: Animal and human health risks from silage. J. Dairy Sci. 101(5):4093-4110.

Drouin, P., J. Tremblay, and F. Chaucheyras-Durand. 2019. Dynamic succession of microbiota during ensiling of whole plant corn following inoculation with *Lactobacillus buchneri* and *Lactobacillus hilgardii* alone or in combination. Microorganisms 7(12):595.

Druce, R. G. and S. B. Thomas. 1970. An ecological study of the psychrotrophic bacteria of soil, water, grass and hay. J. Appl. Bacteriol. 33(2):420-435.

Duan, Y., Y. Zhang, H. Dong, Y. Wang, X. Zheng, and J. Zhang. 2017. Effect of dietary *Clostridium butyricum* on growth, intestine health status and resistance to ammonia stress in Pacific white shrimp *Litopenaeus vannamei*. Fish Shellfish Immunol. 65:25-33.

Duman, M., M. Mulet, S. Altun, I. B. Saticioglu, B. Ozdemir, N. Ajmi, J. Lalucat, and E. García-Valdés. 2021. The diversity of *Pseudomonas* species isolated from fish farms in Turkey. Aquaculture 535:736369.

Dunlap, C. A., S.-W. Kwon, A. P. Rooney, and S.-J. Kim. 2015. *Bacillus paralicheniformis* sp. nov., isolated from fermented soybean paste. Int. J. Syst. Evol. Microbiol. 65(Pt_10):3487-3492.

Ebrahimpour, A., R. N. Z. R. A. Rahman, N. H. A. Kamarudin, M. Basri, and A. B. Salleh. 2011. Lipase production and growth modeling of a novel thermophilic bacterium: *Aneurinibacillus thermoaerophilus* strain AFNA. Electron. J. Biotechnol. 14:6-6.

Edesi, L., M. Järvan, M. Noormets, E. Lauringson, A. Adamson, and E. Akk. 2012. The importance of solid cattle manure application on soil microorganisms in organic and conventional cultivation. Acta Agric. Scand. B. Anim. Sci. 62(7):583-594.

Ehling-Schulz, M., D. Lereclus, and T. M. Koehler. 2019. The *Bacillus* cereus group: *Bacillus* species with pathogenic potential. Microbiol. Spectr. 7(3).

Eijlander, R. T., R. van Hekezen, A. Bienvenue, V. Girard, E. Hoornstra, N. B. Johnson, R. Meyer, A. Wagendorp, D. C. Walker, and M. H. J. Wells-Bennik. 2019. Spores in dairy – new insights in detection, enumeration and risk assessment. Int. J. Dairy Technol. 72(2):303-315.

Elarabi, N. I., A. A. Abdelhadi, R. H. Ahmed, I. Saleh, I. A. Arif, G. Osman, and D. S. Ahmed. 2020. *Bacillus aryabhattai* FACU: A promising bacterial strain capable of manipulate the glyphosate herbicide residues. Saudi J. Biol. Sci. 27(9):2207-2214.

Elmoslemany, A. M., G. P. Keefe, I. R. Dohoo, and B. M. Jayarao. 2009a. Risk factors for bacteriological quality of bulk tank milk in Prince Edward Island dairy herds. Part 1: Overall risk factors. J. Dairy Sci. 92(6):2634-2643.

Elmoslemany, A. M., G. P. Keefe, I. R. Dohoo, and B. M. Jayarao. 2009b. Risk factors for bacteriological quality of bulk tank milk in Prince Edward Island dairy herds. Part 2: Bacteria count-specific risk factors. J. Dairy Sci. 92(6):2644-2652.

Elmoslemany, A. M., G. P. Keefe, I. R. Dohoo, J. J. Wichtel, H. Stryhn, and R. T. Dingwell. 2010. The association between bulk tank milk analysis for raw milk quality and on-farm management practices. Prev. Vet. Med. 95(1-2):32-40.

Elwan, S. H., S. A. Mostafa, A. A. Khodair, and O. Ali. 1978. Identity and lipase activity of an isolate of *Thermoactinomyces vulgaris*. Zentralbl. Bakteriol. Mikrobiol. Hyg. Ser. B. 133(7-8):713-722.

Escobar Diaz, P. A., R. M. dos Santos, N. C. Baron, O. J. A. Gil, and E. C. Rigobelo. 2021. Effect of *Aspergillus* and *Bacillus* concentration on cotton growth promotion. Front. Microbiol. 12(2734).

Esteban-Blanco, C., B. Gutiérrez-Gil, F. Puente-Sánchez, H. Marina, J. Tamames, A. Acedo, and J. J. Arranz. 2020. Microbiota characterization of sheep milk and its association with somatic cell count using 16s rRNA gene sequencing. Journal of Animal Breeding and Genetics 137(1):73-83.

European Parliament. 2004. Regulation (EC) No. 853/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific hygiene rules for food of animal origin. Pages 55-205.

Evanowski, R. L., D. J. Kent, M. Wiedmann, and N. H. Martin. 2020. Milking time hygiene interventions on dairy farms reduce spore counts in raw milk. J. Dairy Sci. 103(5):4088-4099.

Evanowski, R. L., S. J. Reichler, D. J. Kent, N. H. Martin, K. J. Boor, and M. Wiedmann. 2017. Short communication: *Pseudomonas azotoformans* causes gray discoloration in HTST fluid milk. J. Dairy Sci. 100(10):7906-7909.

Fakhry, S., I. Sorrentini, E. Ricca, M. De Felice, and L. Baccigalupi. 2008. Characterization of spore forming bacilli isolated from the human gastrointestinal tract. J. Appl. Microbiol. 105(6):2178-2186.

Falardeau, J., K. Keeney, A. Trmčić, D. Kitts, and S. Wang. 2019. Farm-to-fork profiling of bacterial communities associated with an artisan cheese production facility. Food Microbiol. 83:48-58.

Falkowski, J. 1979. [Lipolytic activity from milk isolated strains of the species *Thermoactinomyces vulgaris*, Tsiklinsky 1899 (author's translation)]. Zentralblatt für Bakteriologie 168(3-4):361-366.

Falkowski, J. and K. Janda. 2003. Wystepowanie termofilnych promieniowców w przetworach mleczarskich [The appearance of the thermophilic actinomycetes in dairy products]. Rocz. Panstw. Zakl. Hig. 54(1):83-86.

Fantuz, F., E. Salimei, and P. Papademas. 2016. Chapter 9 - Macro- and micronutrients in non-cow milk and products and their impact on human health. Pages 209-261 in Non-Bovine Milk and Milk Products. E. Tsakalidou and K. Papadimitriou, ed. Academic Press, San Diego.

Farmer, J. J. and D. Brenner. 2015. *Obesumbacterium*. in Bergey's Manual of Systematics of Archaea and Bacteria. John Wiley & Sons, Inc.

Farooq, S., R. Nazir, S. A. Ganai, and B. A. Ganai. 2021. Isolation and characterization of a new cold-active protease from psychrotrophic bacteria of Western Himalayan glacial soil. Sci. Rep. 11(1).

Farris, J. S. 1972. Estimating phylogenetic trees from distance matrices. The American Naturalist 106(951):645-668.

Feldmann, M., A. Zimmermann, and M. Hoedemaker. 2006. [Influence of milking technique, milking hygiene and environmental hygiene parameters on the microbial contamination of milking machines]. Dtsch Tierarztl Wochenschr 113(7):274-281.

Feng, H. 2020. *Thermoactinomyces intermedius* strain s-9, whole genome shotgun sequencing project (unpublished) - https://www.ncbi.nlm.nih.gov/nuccore/JACEIR000000000.1.

Fenicia, L., L. Da Dalt, F. Anniballi, G. Franciosa, S. Zanconato, and P. Aureli. 2002. A case of infant botulism due to neurotoxigenic *Clostridium butyricum* type E associated with *Clostridium difficile* colitis. Eur. J. Clin. Microbiol. Infect. Dis. 21(10):736-738.

Fetrow, J. 2000. Mastitis: An economic consideration. National Mastitis Council Annual Meeting Proceedings:3-47.

Filippidou, S., T. Junier, T. Wunderlin, C.-C. Lo, P.-E. Li, P. S. Chain, and P. Junier. 2015. Under-detection of endospore-forming *Firmicutes* in metagenomic data. Comput. Struct. Biotechnol. J. 13:299-306.

Flint, S. and N. Hartley. 1996. A modified selective medium for the detection of *Pseudomonas* species that cause spoilage of milk and dairy products. Int. Dairy J. 6(2):223-230.

Flint, S. H., P. J. Bremer, and J. D. Brooks. 1997. Biofilms in dairy manufacturing plant-description, current concerns and methods of control. Biofouling 11(1):81-97.

Flossdorf, D. 2021. Analysis of dairy cattle feed as source of heat resistant bacterial spores in milk contamination and evaluation of consequences for milk quality. Vol. Doctor of Philosophy (Ph.D.). Massey University, Under embargo.

Fortina, M. G., D. Mora, P. Schumann, C. Parini, P. L. Manachini, and E. Stackebrandt. 2001. Reclassification of *Saccharococcus caldoxylosilyticus* as *Geobacillus caldoxylosilyticus* (Ahmad et al. 2000) comb. nov. Int. J. Syst. Evol. Microbiol. 51(6):2063-2071.

Frank, A. and K. Haselwandter. 1982. [Microbial sources of antigens in the environment of farmer's lung patients (author's translation)]. Wien. Med. Wochenschr. 132(4):87-90.

Frank, J. F. and A. E. Yousef. 2004. Tests for groups of microorganisms. In Standard Methods for the Examination of Dairy Products. American Public Health Association:227-247.

French Ministry of Agriculture and Food. 2017. Arrêté du 12 septembre 2017 relatif à la modification du cahier des charges de l'appellation d'origine protégée « Roquefort » AGRT1723071A.

Furet, J.-P., P. Quénée, and P. Tailliez. 2004. Molecular quantification of lactic acid bacteria in fermented milk products using real-time quantitative PCR. Int. J. Food Microbiol. 97(2):197-207.

Gallier, S., D. Gragson, R. Jiménez-Flores, and D. Everett. 2010. Using confocal laser scanning microscopy to probe the milk fat globule membrane and associated proteins. J. Agric. Food Chem. 58(7):4250-4257.

Gantner, V., M. Pero, M. Baban, Z. Skrtic, and A. Turalija. 2015. The overall and fat composition of milk of various species. Mljekarstvo / Dairy 65(4):223-231.

Garbeva, P., J. A. van Veen, and J. D. van Elsas. 2003. Predominant *Bacillus* spp. in agricultural soil under different management regimes detected via PCR-DGGE. Microb. Ecol. 45(3):302-316.

Garde, S., R. Arias, P. Gaya, and M. Nuñez. 2011. Occurrence of *Clostridium* spp. in ovine milk and Manchego cheese with late blowing defect: Identification and characterization of isolates. Int. Dairy J. 21(4):272-278.

Garrido-Sanz, D., J. P. Meier-Kolthoff, M. Göker, M. Martín, R. Rivilla, and M. Redondo-Nieto. 2016. Genomic and genetic diversity within the *Pseudomonas fluorescens* complex. PLoS ONE 11(2):e0150183.

Gaw, S. and G. McBride. 2010. Sheep dip factsheet n°3: Arsenic. in Sheep Dip. Vol. 3. University of Canterbury, Tasman District Council, New Zealand.

George, W. L., V. L. Sutter, D. Citron, and S. M. Finegold. 1979. Selective and differential medium for isolation of *Clostridium difficile*. J. Clin. Microbiol. 9(2):214-219.

Giacometti, F., L. Bardasi, G. Merialdi, M. Morbarigazzi, S. Federici, S. Piva, and A. Serraino. 2016. Shelf life of donkey milk subjected to different treatment and storage conditions. J. Dairy Sci. 99(6):4291-4299.

Giambra, I. J., Y. Jahan, T. Yin, P. Engel, C. Weimann, K. Brügemann, and S. König. 2021. Identification of thermophilic aerobic sporeformers in bedding material of compost-bedded dairy cows using microbial and molecular methods. Animals 11(10):2890.

Gibson, H., L. A. Sinclair, C. M. Brizuela, H. L. Worton, and R. G. Protheroe. 2008. Effectiveness of selected premilking teat-cleaning regimes in reducing teat microbial load on commercial dairy farms. Lett. Appl. Microbiol. 46(3):295-300.

Gill, D. M. 1982. Bacterial toxins: a table of lethal amounts. Microbiol Rev. 46(1):86-94.

Gillings, M. and M. Holley. 1997. Repetitive element PCR fingerprinting (rep-PCR) using enterobacterial repetitive intergenic consensus (ERIC) primers is not necessarily directed at ERIC elements. Lett. Appl. Microbiol. 25(1):17-21.

Glaser, R. W. 1914. The bacterial diseases of caterpillars. Psyche 21:026528.

Gleeson, D., J. Flynn, and B. O. Brien. 2018. Effect of pre-milking teat disinfection on new mastitis infection rates of dairy cows. Ir. Vet. J. 71(1).

Gleeson, D., A. O'Connell, and K. Jordan. 2013. Review of potential sources and control of thermoduric bacteria in bulk-tank milk. Irish J. Agric. Food Res. 52(2):217-227.

Godden, S., R. Bey, K. Lorch, R. Farnsworth, and P. Rapnicki. 2008. Ability of organic and inorganic bedding materials to promote growth of environmental bacteria. J. Dairy Sci. 91(1):151-159.

Goeker, M., M. Huntemann, A. Clum, M. Pillay, K. Palaniappan, N. Varghese, N. Mikhailova, D. Stamatis, T. Reddy, C. Daum, N. Shapiro, N. Ivanova, N. Kyrpides, and T. Woyke. 2018. *Thermoactinomyces vulgaris* strain DSM 43016, whole genome shotgun sequencing project (unpublished) - https://www.ncbi.nlm.nih.gov/nuccore/REFP00000000.1/.

Goh, K. M., H. M. Gan, K.-G. Chan, G. F. Chan, S. Shahar, C. S. Chong, U. M. Kahar, and K. P. Chai. 2014. Analysis of *Anoxybacillus* genomes from the aspects of lifestyle adaptations, prophage diversity, and carbohydrate metabolism. PLoS ONE 9(3):e90549.

Gómez-Torres, N., S. Garde, Á. Peirotén, and M. Ávila. 2015. Impact of *Clostridium* spp. on cheese characteristics: microbiology, color, formation of volatile compounds and off-flavors. Food Control 56:186-194.

Gomila, M., A. Peña, M. Mulet, J. Lalucat, and E. García-Valdés. 2015. Phylogenomics and systematics in *Pseudomonas*. Front. Microbiol. 6.

Gopal, N., C. Hill, P. R. Ross, T. P. Beresford, M. A. Fenelon, and P. D. Cotter. 2015. The prevalence and control of *Bacillus* and related spore-forming bacteria in the dairy industry. Front. Microbiol. 6:1418.

Gotz, S., J. M. Garcia-Gomez, J. Terol, T. D. Williams, S. H. Nagaraj, M. J. Nueda, M. Robles, M. Talon, J. Dopazo, and A. Conesa. 2008. High-throughput functional annotation and data mining with the Blast2GO suite. Nucleic Acids Res. 36(10):3420-3435.

Goudkov, A. V. and M. E. Sharpe. 1965. Clostridia in dairying. J. Appl. Bacteriol. 28(1):63-73.

Gould, W. D., C. Hagedorn, T. R. Bardinelli, and R. M. Zablotowicz. 1985. New selective media for enumeration and recovery of fluorescent pseudomonads from various habitats. Appl. Environ. Microbiol. 49(1):28-32.

Gounot, A. M. 1986. Psychrophilic and psychrotrophic microorganisms. Experientia 42(11-12):1192-1197.

Goverde, M., J. Willrodt, and A. Staerk. 2017. Evaluation of the recovery rate of different swabs for microbial environmental monitoring. J. Pharm. Sci. Technol. 71(1):33-42.

Grady, E. N., J. Macdonald, L. Liu, A. Richman, and Z.-C. Yuan. 2016. Current knowledge and perspectives of *Paenibacillus*: a review. Microb. Cell Factories 15(1).

Grady, K. L., J. W. Sorensen, N. Stopnisek, J. Guittar, and A. Shade. 2019. Assembly and seasonality of core phyllosphere microbiota on perennial biofuel crops. Nat. Commun. 10(1).

Gram, L., L. Ravn, M. Rasch, J. B. Bruhn, A. B. Christensen, and M. Givskov. 2002. Food spoilage—interactions between food spoilage bacteria. Int. J. Food Microbiol. 78(1):79-97.

Grass, J. E., L. H. Gould, and B. E. Mahon. 2013. Epidemiology of foodborne disease outbreaks caused by *Clostridium perfringens*, United States, 1998–2010. Foodborne Pathog. Dis. 10(2):131-136.

Green, M. J., Leach, K. A., Breen J. E. , Ohnstad, I., Tuer, S., Archer, S. C. , and A. J. Bradley. 2014. Recycled manure solids as bedding for dairy cattle: A scoping study. Cattle Practice 22.

Gregory, N. G. 1995. The role of shelterbelts in protecting livestock: A review. New Zealand J. Agric. Res. 38(4):423-450.

Grimont, F. and P. A. Grimont. 1986. Ribosomal ribonucleic acid gene restriction patterns as potential taxonomic tools. Ann. Inst. Pasteur Microbiol. 137b(2):165-175.

Groenewald, W. H., P. A. Gouws, and R. C. Witthuhn. 2009. Isolation, identification and typification of *Alicyclobacillus acidoterrestris* and *Alicyclobacillus acidocaldarius* strains from orchard soil and the fruit processing environment in South Africa. Food Microbiol. 26(1):71-76.

Guo, P., K. Zhang, X. Ma, and P. He. 2020. *Clostridium* species as probiotics: potentials and challenges. J. Anim. Sci. Biotechnol. 11(1).

Gupta, R. S., S. Patel, N. Saini, and S. Chen. 2020a. Robust demarcation of 17 distinct *Bacillus* species clades, proposed as novel Bacillaceae genera, by phylogenomics and comparative genomic analyses: description of *Robertmurraya kyonggiensis* sp. nov. and proposal for an emended genus *Bacillus* limiting it only to the members of the Subtilis and Cereus clades of species. Int. J. Syst. Evol. Microbiol. 70(11):5753-5798.

Gupta, T. B. and G. Brightwell. 2017. Farm level survey of spore-forming bacteria on four dairy farms in the Waikato region of New Zealand. MicrobiologyOpen 6(4):e00457.

Gupta, T. B., R. Jauregui, A. N. Risson, G. Brightwell, and P. Maclean. 2020b. Draft genome sequence of *Pseudomonas nitrititolerans* strain AGROB37, isolated from a sheep dairy farm in New Zealand. Microbiol Resour Announc 9(28).

Gupta, T. B., P. Maclean, R. Jauregui, A. N. Risson, and G. Brightwell. 2020c. Draft genome sequence of *Clostridium cochlearium* strain AGROS13, isolated from a sheep dairy farm in New Zealand. Microbiol. Resour. Announc. 9(26):e00593-00520.

Gupta, T. B., A. N. Risson, R. Jauregui, P. Maclean, and G. Brightwell. 2021. Draft genome sequence of *Pseudomonas saudiphocaensis* strain AGROB56, isolated from a dairy farm in New Zealand. Microbiol Resour Announc 10(1).

Gutiérrez-Peña, R., C. Avilés, H. Galán-Soldevilla, O. Polvillo, P. Ruiz Pérez-Cacho, J. L. Guzmán, A. Horcada, and M. Delgado-Pertíñez. 2021. Physicochemical composition, antioxidant status, fatty acid profile, and volatile compounds of milk and fresh and ripened ewes' cheese from a sustainable part-time grazing system. Foods 10(1):80.

Hagemann, M., K. Gärtner, M. Scharnagl, P. Bolay, S. Lott, J. Fuss, B. Huettel, R. Reinhardt, S. Klähn, and W. Hess. 2018. Identification of the DNA methyltransferases establishing the methylome of the cyanobacterium *Synechocystis* sp. PCC 6803. DNA res. 25.

Hahne, J., D. Isele, J. Berning, and A. Lipski. 2019. The contribution of fast growing, psychrotrophic microorganisms on biodiversity of refrigerated raw cow's milk with high bacterial counts and their food spoilage potential. Food Microbiol. 79:11-19.

Haldar, L. and D. N. Gandhi. 2016. Effect of oral administration of *Bacillus coagulans* B37 and *Bacillus pumilus* B9 strains on fecal coliforms, *Lactobacillus* and *Bacillus* spp. in rat animal model. Vet. World 9(7):766-772.

Hale, A., J. E. Kirby, and M. Albrecht. 2016. Fatal spontaneous *Clostridium bifermentans* necrotizing endometritis: A case report and literature review of the pathogen. Open Forum Infect. Dis. 3(2):ofw095.

Hale, W. B., M. W. Van Der Woude, and D. A. Low. 1994. Analysis of nonmethylated GATC sites in the *Escherichia coli* chromosome and identification of sites that are differentially methylated in response to environmental stimuli. J. Bacteriol. 176(11):3438-3441.

Han, M. M., L. Z. Mu, X. P. Liu, J. Zhao, X. F. Liu, and H. Liu. 2013. ERIC-PCR genotyping of *Pseudomonas aeruginosa* isolates from haemorrhagic pneumonia cases in mink. Vet. Rec. Open 1(1):e000043.

Hantsis-Zacharov, E. and M. Halpern. 2007. Culturable psychrotrophic bacterial communities in raw milk and their proteolytic and lipolytic traits. Appl. Environ. Microbiol. 73(22):7162-7168.

Harrison, L., Virden. 1980. Sheep production: Intensive systems, innovative techniques boost yields. United States Department of Agriculture - Agricultural Economic Report n°452.

Hatheway, C., D. Whaley, and V. Dowell Jr. 1980. Epidemiological aspects of *Clostridium perfringens* foodborne illness. Food Technol.

Hayer, J. J., C. Heinemann, B. G. Schulze-Dieckhoff, and J. Steinhoff-Wagner. 2022. A risk-oriented evaluation of biofilm and other influencing factors on biological quality of drinking water for dairy cows. J. Anim. Sci. 100(5).

He, H., J. Dong, C. N. Lee, and Y. Li. 2009. Molecular analysis of spoilage-related bacteria in pasteurized milk during refrigeration by PCR and denaturing gradient gel electrophoresis. J. Food Prot. 72(3):572-577.

He, X. S., R. Brückner, and R. H. Doi. 1991. The protease genes of *Bacillus subtilis*. Res. Microbiol. 142(7):797-803.

Healy, W. B. 1968. Ingested soil and animal nutrition. in Proc. NZ Grassland Association.

Healy, W. B. and T. G. Ludwig. 1965. Ingestion of soil by sheep in New Zealand in relation to wear of teeth. Nature Lett. 208(5012):806-807.

Henry, C. H. 1918. An investigation of the cultural reactions of certain anaerobes found in wounds. J. Pathol. Bacteriol. 21:344-385.

Hentschel, U., M. Steinert, and J. Hacker. 2000. Common molecular mechanisms of symbiosis and pathogenesis. Trends Microbiol. 8(5):226-231.

Henz, S. R., D. H. Huson, A. F. Auch, K. Nieselt-Struwe, and S. C. Schuster. 2005. Whole-genome prokaryotic phylogeny. Bioinformatics 21(10):2329-2335.

Heyndrickx, M. 2011. Dispersal of aerobic endospore-forming bacteria from soil and agricultural activities to food and feed. Pages 135-156 in Endospore-forming Soil Bacteria. Springer Berlin Heidelberg.

Heyndrickx, M., L. Lebbe, M. Vancanneyt, K. Kersters, P. De Vos, N. A. Logan, G. Forsyth, S. Nazli, N. Ali, and R. C. W. Berkeley. 1997. A polyphasic reassessment of the genus *Aneurinibacillus*, reclassification of *Bacillus thermoaerophilus* (Meier-Stauffer et al. 1996) as *Aneurinibacillus thermoaerophilus* comb, nov., and emended descriptions of *A. aneurinilyticus* corrig., *A. migulanus*, and *A. thermoaerophilus* Int. J. Syst. Evol. Microbiol. 47(3):808-817.

Heyndrickx, M., N. A. Logan, L. Lebbe, M. Rodríguez-Díaz, G. Forsyth, J. Goris, P. Scheldeman, and P. De Vos. 2004. *Bacillus galactosidilyticus* sp. nov., an alkali-tolerant β-galactosidase producer. Int. J. Syst. Evol. Microbiol. 54(2):617-621.

Hill, B., S. Hasell, and D. Schumacher. 2003. Report to the Technical Consultative Committee (TCC) – Recommended Product Safety Limits for the NZ Dairy Industry - https://www.mpi.govt.nz/dmsdocument/20453-Report-to-the-Technical-Consultative-Committee-TCC-Recommended-Product-Safety-Limits-for-the-NZ-Dairy-Industry. New Zealand Food Safety Authority, ed.

Hill, G. B. and S. Osterhout. 1972. Experimental effects of hyperbaric oxygen on selected clostridial species. II. In-vitro studies in mice. J. Infect. Dis. 125(1):26-35.

Hogenboom, J. A., L. Pellegrino, A. Sandrucci, V. Rosi, and P. D'Incecco. 2019. Invited review: Hygienic quality, composition, and technological performance of raw milk obtained by robotic milking of cows. J. Dairy Sci. 102(9):7640-7654.

Hong, H. A., E. To, S. Fakhry, L. Baccigalupi, E. Ricca, and S. M. Cutting. 2009. Defining the natural habitat of *Bacillus* spore-formers. Res. Microbiol. 160(6):375-379.

Horn, M. A., J. Ihssen, C. Matthies, A. Schramm, G. Acker, and H. L. Drake. 2005. *Dechloromonas denitrificans* sp. nov., *Flavobacterium denitrificans* sp. nov., *Paenibacillus anaericanus* sp. nov. and *Paenibacillus terrae* strain MH72, N2O-producing bacteria isolated from the gut of the earthworm *Aporrectodea caliginosa*. Int. J. Syst. Evol. Microbiol. 55(3):1255-1265.

Huck, J. R., B. H. Hammond, S. C. Murphy, N. H. Woodcock, and K. J. Boor. 2007a. Tracking spore-forming bacterial contaminants in fluid milk-processing systems. J. Dairy Sci. 90(10):4872-4883.

Huck, J. R., M. Sonnen, and K. J. Boor. 2008. Tracking heat-resistant, cold-thriving fluid milk spoilage bacteria from farm to packaged product. J. Dairy Sci. 91(3):1218-1228.

Huck, J. R., N. H. Woodcock, R. D. Ralyea, and K. J. Boor. 2007b. Molecular subtyping and characterization of psychrotolerant endospore-forming bacteria in two new york state fluid milk processing systems. J. Food Prot. 70(10):2354-2364.

Huerta-Cepas, J., D. Szklarczyk, D. Heller, A. Hernández-Plaza, S. K. Forslund, H. Cook, D. R. Mende, I. Letunic, T. Rattei, Lars, Christian, and P. Bork. 2019. eggNOG 5.0: a hierarchical, functionally and phylogenetically annotated orthology resource based on 5090 organisms and 2502 viruses. Nucleic Acids Res. 47(D1):D309-D314.

Hyatt, D., G.-L. Chen, P. F. Locascio, M. L. Land, F. W. Larimer, and L. J. Hauser. 2010. Prodigal: prokaryotic gene recognition and translation initiation site identification. BMC Bioinformatics 11(1):119.

Ichihashi, Y., Y. Date, A. Shino, T. Shimizu, A. Shibata, K. Kumaishi, F. Funahashi, K. Wakayama, K. Yamazaki, and A. Umezawa. 2020. Multi-omics analysis on an agroecosystem reveals the significant role of organic nitrogen to increase agricultural crop yield. Proceedings of the National Academy of Sciences of the United States of America 117(25):14552-14560.

Ioannou, N., R. Schneider, and R. Grogan. 1977. Effect of flooding on the soil gas composition and the production of microsclerotia by *Verticillium dahliae* in the field. Phytopathology 67:651-656.

Ivy, R. A., M. L. Ranieri, N. H. Martin, H. C. den Bakker, B. M. Xavier, M. Wiedmann, and K. J. Boor. 2012. Identification and characterization of psychrotolerant sporeformers associated with fluid milk production and processing. Appl. Environ. Microbiol. 78(6):1853-1864.

Jaborek, J., G. Lowe, and F. Fluharty. 2016. Effects of pen flooring type and bedding on lamb growth and carcass characteristics. Small Rumin. Res. 144:28-34.

Jackson, A. M., P. R. Poulton, and A. S. Ball. 1997. Importance of farming practice on the isolation frequency of *Thermoactinomyces* species. Soil Biol. Biochem. 29(2):207-210.

Jackson, T. A., J. F. Pearson, S. D. Young, J. Armstrong, and M. O'Callaghan. 2002. Abundance and distribution of microbial populations in sheep fleece. New Zealand J. Agric. Res. 45(1):49-55.

Jafari, M., M. Alebouyeh, A. M. Mortazavian, H. Hosseini, K. Ghanati, Z. Amiri, and M. R. Zali. 2016. Influence of heat shock temperatures and fast freezing on viability of probiotic sporeformers and the issue of spore plate count versus true numbers. Food Nutr. Res. 3(1):35-42.

Jiménez-Sobrino, L., A. Garzón-Sigler, M. D. Pérez-Guzmán Palomares, A. García-Martínez, and R. Arias-Sánchez. 2018. Calidad microbiológica diferencial de la leche de oveja procedente de tanque. Revista Científica, FCV-LUZ 28(1):11-18.

Jin, H., H. Wang, Y. Zhang, T. Hu, Z. Lin, B. Liu, J. Ma, X. Wang, Q. Liu, X. Lin, and Z. Xie. 2020. Description of *Azotobacter chroococcum* subsp. *isscasi* subsp. nov. isolated from paddy soil and establishment of *Azotobacter chroococcum* subsp. *chroococcum* subsp. nov. Int. J. Syst. Evol. Microbiol. 70(3):2124-2131.

Jolley, K. A., J. E. Bray, and M. C. J. Maiden. 2018. Open-access bacterial population genomics: BIGSdb software, the PubMLST.org website and their applications. Wellcome Open Research 3:124.

Jolley, K. A. and M. C. Maiden. 2010. BIGSdb: Scalable analysis of bacterial genome variation at the population level. BMC Bioinformatics 11(1):595.

Jones, P., D. Binns, H. Y. Chang, M. Fraser, W. Li, C. McAnulla, H. McWilliam, J. Maslen, A. Mitchell, G. Nuka, S. Pesseat, A. F. Quinn, A. Sangrador-Vegas, M. Scheremetjew, S. Y. Yong, R. Lopez, and S. Hunter. 2014. InterProScan 5: genome-scale protein function classification. Bioinformatics 30(9):1236-1240.

Juffs, H. 1975. Proteolysis detection in milk: III. Relationships between bacterial populations, tyrosine value and organoleptic quality during extended cold storage of milk and cream. J. Dairy Res. 42(1):31-41.

Juffs, H. S. and H. Deeth. 2007. Scientific evaluation of pasteurisation for pathogen reduction in milk and milk products - https://www.foodstandards.gov.au/code/proposals/documents/scientific%20evaluation.pdf. FSANZ Canberra.

Julien, M. C., P. Dion, C. Lafreniere, H. Antoun, and P. Drouin. 2008. Sources of clostridia in raw milk on farms. Appl. Environ. Microbiol. 74(20):6348-6357.

Kable, M. E., Y. Srisengfa, Z. Xue, L. C. Coates, and M. L. Marco. 2019. Viable and total bacterial populations undergo equipment- and time-dependent shifts during milk processing. Appl. Environ. Microbiol. 85(13).

Kalaycı Kara, A., Ö. Fakıoğlu, R. Kotan, M. Atamanalp, and G. Alak. 2021. The investigation of bioremediation potential of *Bacillus subtilis* and *B. thuringiensis* isolates under controlled conditions in freshwater. Arch. Microbiol. 203(5):2075-2085.

Kalogridou-Vassiliadou, D. 1992. Biochemical activities of *Bacillus* species isolated from flat sour evaporated milk. J. Dairy Sci. 75(10):2681-2686.

Kang, D. D., F. Li, E. Kirton, A. Thomas, R. Egan, H. An, and Z. Wang. 2019. MetaBAT 2: an adaptive binning algorithm for robust and efficient genome reconstruction from metagenome assemblies. PeerJ 7:e7359.

Karaca, B., S. Buzrul, and A. Coleri Cihan. 2019. *Anoxybacillus* and *Geobacillus* biofilms in the dairy industry: effects of surface material, incubation temperature and milk type. Biofouling 35(5):551-560.

Karczewski, K. J. and M. P. Snyder. 2018. Integrative omics for health and disease. Nat. Rev. Genet. 19(5):299-310.

Karmakar, K., R. Bhattacharya, A. Sharma, K. Parmar, U. Nath, K. N. Nataraja, E. N, G. Sharma, and D. Chakravortty. 2022. *Lysinibacillus macroides*-mediated control of cellulose-producing morphotype of *Salmonella*. J. Sci. Food Agric.

Karst, S. M., R. M. Ziels, R. H. Kirkegaard, E. A. Sørensen, D. McDonald, Q. Zhu, R. Knight, and M. Albertsen. 2021. High-accuracy long-read amplicon sequences using unique molecular identifiers with Nanopore or PacBio sequencing. Nat. Methods 18(2):165-169.

Katara, J., R. Deshmukh, N. K. Singh, and S. Kaur. 2012. Molecular typing of native *Bacillus thuringiensis* isolates from diverse habitats in India using REP-PCR and ERIC-PCR analysis. J. Gen. Appl. Microbiol. 58(2):83-94.

Kavita, K., V. K. Singh, A. Mishra, and B. Jha. 2014. Characterisation and anti-biofilm activity of extracellular polymeric substances from *Oceanobacillus iheyensis*. Carbohydr. Polym. 101:29-35.

Keenleyside, C. and G. Tucker. 2010. Farmland abandonment in the EU: An assessment of trends and prospects. Report prepared for WWF.

Kent, D. J., K. Chauhan, K. J. Boor, M. Wiedmann, and N. H. Martin. 2016. Spore test parameters matter: Mesophilic and thermophilic spore counts detected in raw milk and dairy powders differ significantly by test method. J. Dairy Sci. 99(7):5180-5191.

Keshri, J., Y. Chen, R. Pinto, Y. Kroupitski, Z. G. Weinberg, and S. Sela Saldinger. 2019. Bacterial dynamics of wheat silage. Front. Microbiol. 10:1532.

Keyburn, A. L., J. D. Boyce, P. Vaz, T. L. Bannam, M. E. Ford, D. Parker, A. Di Rubbo, J. I. Rood, and R. J. Moore. 2008. NetB, a new toxin that is associated with avian necrotic enteritis caused by *Clostridium perfringens*. PLoS Pathog. 4(2):e26.

Kikue, H., M. Chihaya, M. Nobuhito, M. Hidetoshi, and Y. Isao. 2019. Isolation and identification of bacteria from high-temperature compost at temperatures exceeding 90°C. Afr. J. Microbiol. Res. 13(7):134-144.

KIm, K. H., Y. L. Seo, J. H. Baek, H. M. Jin, and C. O. Jeon. 2021. *Paenibacillus agri* sp. nov., isolated from soil. Int. J. Syst. Evol. Microbiol. 71(8).

King, M. T. M., R. E. Crossley, and T. J. Devries. 2016. Impact of timing of feed delivery on the behavior and productivity of dairy cows. J. Dairy Sci. 99(2):1471-1482.

King, W., V. Burggraaf, N. Mapp, E. Ganche, K. Hutchinson, and M. Johnson. 2018. The role of pasture and silage in supporting milk production in dairy goats in New Zealand – a case study approach. N.Z. J. Anim. Sci. Prod. 78:111-115.

Kiselev, K. V., A. S. Dubrovina, and A. P. Tyunin. 2015. The methylation status of plant genomic DNA influences PCR efficiency. J. Plant. Physiol. 175:59-67.

Kleine, R. 1982. Properties of thermitase, a thermostable serine protease from *Thermoactinomyces vulgaris*. Acta Biol. Med. Ger. 41(1):89-102.

Klijn, N., F. F. Nieuwenhof, J. D. Hoolwerf, C. B. van der Waals, and A. H. Weerkamp. 1995. Identification of *Clostridium tyrobutyricum* as the causative agent of late blowing in cheese by species-specific PCR amplification. Appl. Environ. Microbiol. 61(8):2919-2924.

Knüpfer, M., P. Braun, K. Baumann, A. Rehn, M. Antwerpen, G. Grass, Wölfel, and Roman. 2020. Evaluation of a highly efficient DNA extraction method for *Bacillus anthracis* endospores. Microorganisms 8(5):763.

Kobayashi, H., Y. Tanizawa, M. Sakamoto, Y. Nakamura, M. Ohkuma, and M. Tohno. 2019. Reclassification of *Paenibacillus thermophilus* Zhou et al. 2013 as a later heterotypic synonym of *Paenibacillus macerans* (Schardinger 1905) Ash et al. 1994. Int. J. Syst. Evol. Microbiol. 69(2):417-421.

Koc, M., C. Cokmus, and A. C. Cihan. 2015. The genotypic diversity and lipase production of some thermophilic bacilli from different genera. Braz. J. Microbiol. 46(4):1065-1076.

Kohlmann, K. L., S. S. Nielsen, L. R. Steenson, and M. R. Ladisch. 1991. Production of proteases by psychrotrophic microorganisms. J. Dairy Sci. 74(10):3275-3283.

Kotimaa, M. H., L. Oksanen, and P. Koskela. 1991. Feeding and bedding materials as sources of microbial exposure on dairy farms. Scand. J. Work Environ. Health 17(2):117-122.

Kouker, G. and K. E. Jaeger. 1987. Specific and sensitive plate assay for bacterial lipases. Appl. Environ. Microbiol. 53(1):211-213.

Kreft, Ł., A. Botzki, F. Coppens, K. Vandepoele, and M. Van Bel. 2017. PhyD3: a phylogenetic tree viewer with extended phyloXML support for functional genomics data visualization. Bioinformatics 33(18):2946-2947.

Krishnamurthi, S., T. Chakrabarti, and E. Stackebrandt. 2009. Re-examination of the taxonomic position of *Bacillus silvestris (*Rheims et al. 1999) and proposal to transfer it to *Solibacillus* gen. nov. as *Solibacillus silvestris* comb. nov. Int. J. Syst. Evol. Microbiol. 59(5):1054-1058.

Krishnamurthi, S., A. Ruckmani, R. Pukall, and T. Chakrabarti. 2010. *Psychrobacillus* gen. nov. and proposal for reclassification of *Bacillus insolitus* (Larkin & Stokes, 1967,) *B. psychrotolerans* (Abd-El Rahman et al., 2002) and *B. psychrodurans* (Abd-El Rahman et al., 2002) as *Psychrobacillus insolitus* comb. nov., *Psychrobacillus psychrotolerans* comb. nov. and *Psychrobacillus psychrodurans* comb. nov. Syst. Appl. Microbiol. 33(7):367-373.

Kritas, S. K., A. Govaris, G. Christodoulopoulos, and A. R. Burriel. 2006. Effect of *Bacillus licheniformis* and *Bacillus subtilis* supplementation of ewe's feed on sheep milk production and young lamb mortality. J. Vet. Med. 53:170-173.

Kumar, H., L. Franzetti, A. Kaushal, and D. Kumar. 2019. *Pseudomonas fluorescens*: a potential food spoiler and challenges and advances in its detection. Ann. Microbiol. 69(9):873-883.

Kung, L. and R. Shaver. 2001. Interpretation and use of silage fermentation analysis reports - https://fyi.extension.wisc.edu/forage/interpretation-and-use-of-silage-fermentation-analysis-reports/.

Kung, L., R. D. Shaver, R. J. Grant, and R. J. Schmidt. 2018. Silage review: Interpretation of chemical, microbial, and organoleptic components of silages. J. Dairy Sci. 101(5):4020-4033.

Kung, L. J. 2011. Silage temperatures: how hot is too hot? - https://cdn.canr.udel.edu/wp-content/uploads/2014/02/HowHotisTooHot-2011.pdf. University of Delaware, Dept. of Animal & Food Sciences.

Kupferschmied, P., M. Maurhofer, and C. Keel. 2013. Promise for plant pest control: root-associated pseudomonads with insecticidal activities. Front. Plant Sci. 4.

Kurup, V., G. Hollick, and E. Pagan. 1980. *Thermoactinomyces intermedius*, a new species of amylase negative thermophilic actinomycetes. Bol. Cienc. Natur. 7:104-108.

Lagesen, K., P. Hallin, E. A. Rødland, H.-H. Stærfeldt, T. Rognes, and D. W. Ussery. 2007. RNAmmer: consistent and rapid annotation of ribosomal RNA genes. Nucleic Acids Res. 35(9):3100-3108.

Lagier, J.-C., G. Dubourg, M. Million, F. Cadoret, M. Bilen, F. Fenollar, A. Levasseur, J.-M. Rolain, P.-E. Fournier, and D. Raoult. 2018. Culturing the human microbiota and culturomics. Nat. Rev. Microbiol. 16(9):540-550.

Lagier, J. C., F. Armougom, M. Million, P. Hugon, I. Pagnier, C. Robert, F. Bittar, G. Fournous, G. Gimenez, M. Maraninchi, J. F. Trape, E. V. Koonin, B. La Scola, and D. Raoult. 2012. Microbial culturomics: paradigm shift in the human gut microbiome study. Clin. Microbiol. Infect. 18(12):1185-1193.

Lallemand Animal Nutrition. 2021. Microbial management of bedding and manure - https://lallemandanimalnutrition.com/en/europe/whats-new/microbial-management-of-bedding-and-manure/.

Lane, D. J. 1991. 16S/23S rRNA sequencing. in Nucleic acid techniques in bacterial systematics. E. Stackebrandt and M. Goodfellow, ed. John Wiley & Sons, New York.

Lardy, G., V. Anderson, and C. Dahlen. 2018. Alternative feeds for ruminants - https://www.ndsu.edu/agriculture/sites/default/files/2022-03/as1182_0.pdf. North Dakota State University. Fargo, North Dakota.

Larkin, J. M. and J. L. Stokes. 1967. Taxonomy of psychrophilic strains of *Bacillus*. J. Bacteriol. 94(4):889-895.

Lasprilla-Mantilla, M. I., V. Wagner, J. Pena, A. Frechette, K. Thivierge, S. Dufour, and C. Fernandez-Prada. 2019. Effects of recycled manure solids bedding on the spread of gastrointestinal parasites in the environment of dairies and milk. J. Dairy Sci. 102(12):11308-11316.

Le Bourhis, A.-G., J. Doré, J.-P. Carlier, J.-F. Chamba, M.-R. Popoff, and J.-L. Tholozan. 2007. Contribution of *C. beijerinckii* and *C. sporogenes* in association with *C. tyrobutyricum* to the butyric fermentation in Emmental type cheese. Int. J. Food Microbiol. 113(2):154-163.

Lee, H. S., M. Kwon, S. Heo, M. G. Kim, and G.-B. Kim. 2017a. Characterization of the biodiversity of the spoilage microbiota in chicken meat using next generation sequencing and culture dependent approach. Korean J. Food Sci. Anim. Resour. 37(4):535-541.

Lee, I., M. Chalita, S.-M. Ha, S.-I. Na, S.-H. Yoon, and J. Chun. 2017b. ContEst16S: an algorithm that identifies contaminated prokaryotic genomes using 16S RNA gene sequences. Int. J. Syst. Evol. Microbiol. 67(6):2053-2057.

Lee, N. K., W. S. Kim, and H. D. Paik. 2019. *Bacillus* strains as human probiotics: characterization, safety, microbiome, and probiotic carrier. Food Sci. Biotechnol. 28(5):1297-1305.

Lefort, V., R. Desper, and O. Gascuel. 2015. FastME 2.0: A Comprehensive, Accurate, and Fast Distance-Based Phylogeny Inference Program. Mol. Biol. Evol. 32(10):2798-2800.

LeJeune, J. T., T. E. Besser, N. L. Merrill, D. H. Rice, and D. D. Hancock. 2001. Livestock drinking water microbiology and the factors influencing the quality of drinking water offered to cattle. J. Dairy Sci. 84(8):1856-1862.

Leriche, F. and K. Fayolle. 2012. No seasonal effect on culturable pseudomonads in fresh milks from cattle herds. J. Dairy Sci. 95(5):2299-2306.

Leso, L., M. Barbari, M. A. Lopes, F. A. Damasceno, P. Galama, J. L. Taraba, and A. Kuipers. 2020. Invited review: Compost-bedded pack barns for dairy cows. J. Dairy Sci. 103(2):1072-1099.

Li, B., F. Liu, Y. Ren, Y. Ding, Y. Li, X.-F. Tang, and B. Tang. 2019. Complete genome sequence of *Thermoactinomyces vulgaris* strain CDF, a thermophilic bacterium capable of degrading chicken feathers. Microbiol. Resour. Announc. 8(28):e00530-00519.

Li, H., D. Liu, and J. Gao. 2011. Differentiation between *Bacillus thuringiensis* and *Bacillus cereus* by 16S rDNA-PCR and ERIC-PCR. J. Northeast Agric. Univ. 18(3):12-15.

Li, L., W. Abu Al-Soud, L. Bergmark, L. Riber, L. H. Hansen, J. Magid, and S. J. Sørensen. 2013. Investigating the diversity of *Pseudomonas* spp. In soil using culture dependent and independent techniques. Curr. Microbiol. 67(4):423-430.

Li, R., D. Jiang, M. Zheng, P. Tian, M. Zheng, and C. Xu. 2020. Microbial community dynamics during alfalfa silage with or without clostridial fermentation. Sci. Rep. 10(1).

Li, X., X. Wu, Y. Xu, and Y. Liu. 2021. First report of bacteremia caused by *Clostridium cadaveris* in China. Infect. Drug Resist. Volume 14:5411-5415.

Liang, L., P. Wang, X. Zhao, L. He, T. Qu, and Y. Chen. 2022. Single-molecule real-time sequencing reveals differences in bacterial diversity in raw milk in different regions and seasons in China. J. Dairy Sci. 105.

Lin, H., M. Shavezipur, A. Yousef, and F. Maleky. 2016. Prediction of growth of *Pseudomonas fluorescens* in milk during storage under fluctuating temperature. J. Dairy Sci. 99(3):1822-1830.

Liu, Y., Q. Lai, C. Dong, F. Sun, L. Wang, G. Li, and Z. Shao. 2013. Phylogenetic diversity of the *Bacillus pumilus* group and the marine ecotype revealed by multilocus sequence analysis. PLoS ONE 8(11):e80097.

Liu, Y., Q. Lai, J. Du, and Z. Shao. 2016. *Bacillus zhangzhouensis* sp. nov. and *Bacillus australimaris* sp. nov. Int. J. Syst. Evol. Microbiol. 66(3):1193-1199.

Liu, Z., Y. Wei, J. Li, and G.-C. Ding. 2022. Integrating 16S rRNA amplicon metagenomics and selective culture for developing thermophilic bacterial inoculants to enhance manure composting. Waste Manage. 144:357-365.

Lopetuso, L. R., F. Scaldaferri, F. Franceschi, and A. Gasbarrini. 2016. *Bacillus clausii* and gut homeostasis: state of the art and future perspectives. Expert Rev. Gastroenterol. Hepatol. 10(8):943-948.

Lucking, G., M. Stoeckel, Z. Atamer, J. Hinrichs, and M. Ehling-Schulz. 2013. Characterization of aerobic spore-forming bacteria associated with industrial dairy processing environments and product spoilage. Int. J. Food Microbiol. 166(2):270-279.

Lúquez, C., L. A. Joseph, and S. E. Maslanka. 2015. Molecular subtyping of *Clostridium botulinum* by pulsed-field gel electrophoresis. Methods Mol. Biol. 1301:103-113.

Lutgendorff, F., L. M. A. Akkermans, and J. D. Soderholm. 2008. The role of microbiota and probiotics in stress-induced gastrointestinal damage. Curr. Mol. Med. 8(4):282-298.

Ma, Y., D. M. Barbano, and M. Santos. 2003. Effect of CO2 addition to raw milk on proteolysis and lipolysis at 4°C. J. Dairy Sci. 86(5):1616-1631.

Maes, S., T. Vackier, S. Nguyen Huu, M. Heyndrickx, H. Steenackers, I. Sampers, K. Raes, A. Verplaetse, and K. De Reu. 2019. Occurrence and characterisation of biofilms in drinking water systems of broiler houses. BMC Microbiol. 19(1).

Magnusson, M., A. Christiansson, and B. Svensson. 2007a. *Bacillus cereus* spores during housing of dairy cows: Factors affecting contamination of raw milk. J. Dairy Sci. 90(6):2745-2754.

Magnusson, M., A. Christiansson, B. Svensson, and C. Kolstrup. 2006. Effect of different premilking manual teat-cleaning methods on bacterial spores in milk. J. Dairy Sci. 89:3866-3875.

Magnusson, M., B. Svensson, C. Kolstrup, and A. Christiansson. 2007b. *Bacillus cereus* in free-stall bedding. J. Dairy Sci. 90(12):5473-5482.

Mah, J. H., D. H. Kang, and J. Tang. 2009. Comparison of viability and heat resistance of *Clostridium sporogenes* stored at different temperatures. J. Food Sci. 74(1):M23-M27.

Mahamat Abdelrahim, A., N. Radomski, S. Delannoy, S. Djellal, M. Le Négrate, K. Hadjab, P. Fach, J.-A. Hennekinne, M.-Y. Mistou, and O. Firmesse. 2019. Large-scale genomic analyses and toxinotyping of *Clostridium perfringens* implicated in foodborne outbreaks in France. Front. Microbiol. 10.

Makhzoum, A., J. S. Knapp, and R. K. Owusu. 1995. Factors affecting growth and extracelluar lipase production by *Pseudomonas fluorescens* 2D. Food Microbiol. 12:277-290.

Malissiova, E., T. Papadopoulos, A. Kyriazi, M. Mparda, C. Sakorafa, A. Katsioulis, A. Katsiaflaka, M. Kyritsi, A. Zdragas, and C. Hadjichristodoulou. 2017. Differences in sheep and goats milk microbiological profile between conventional and organic farming systems in Greece. J. Dairy Res. 84(2):206-213.

Malta, D. S., E. M. Dalmina, M. N. Schmeier, B. Seguenka, J. Steffens, A. E. Bianchi, A. A. Lima Tribst, D. Cavalheiro, and E. Rigo. 2021. Impact of the preservation methods of sheep milk on the characteristics of Requeijão cremoso processed cheese. Int. Dairy J. 121:105101.

Mani, S., O. A. Aiyegoro, and M. A. Adeleke. 2021. Characterization of rumen microbiota of two sheep breeds supplemented with direct-fed lactic acid bacteria. Front. Vet. Sci. 7.

Manik, P., Z.-Y. Dong, G.-H. Liu, L. Li, M. Xiao, and W.-J. Li. 2019. Reclassification of *Bacillus aryabhattai* Shivaji *et al.* 2009 as a later heterotypic synonym of B*acillus megaterium* de Bary 1884 (Approved Lists 1980). FEMS Microbiol. Lett. 366(22).

Mankai, H., B. P. Anton, V. Wu, T. Vincze, R. J. Roberts, F. Limam, and A. Fomenkov. 2019. Complete genome sequence and methylome analysis of *Thermoactinomyces vulgaris* 2h. Microbiol. Resour. Announc. 8(32):e00657-00619.

Marchand, S., B. Duquenne, M. Heyndrickx, K. Coudijzer, and J. De Block. 2017. Destabilization and off-flavors generated by *Pseudomonas* proteases during or after UHT-processing of milk. Int. J. Food Contam. 4(1).

Marchand, S., K. Heylen, W. Messens, K. Coudijzer, P. De Vos, K. Dewettinck, L. Herman, J. De Block, and M. Heyndrickx. 2009. Seasonal influence on heat-resistant proteolytic capacity of *Pseudomonas lundensis* and *Pseudomonas fragi*, predominant milk spoilers isolated from Belgian raw milk samples. Environ. Microbiol. 11(2):467-482.

Marin, M., E. Pogurschi, and C. Nicolae. 2018. Use of volcanic tuff in dairy farms - solution for environmental protection. J. Environ. Prot. Ecol. 19(2):620-627.

Marston, C. K., C. Beesley, L. Helsel, and A. R. Hoffmaster. 2008. Evaluation of two selective media for the isolation of *Bacillus anthracis*. Lett. Appl. Microbiol. 47(1):25-30.

Martin, N. H., K. J. Boor, and M. Wiedmann. 2018. Symposium review: effect of post-pasteurization contamination on fluid milk quality. J. Dairy Sci. 101(1):861-870.

Martin, N. H., D. J. Kent, R. L. Evanowski, T. J. Zuber Hrobuchak, and M. Wiedmann. 2019. Bacterial spore levels in bulk tank raw milk are influenced by environmental and cow hygiene factors. J. Dairy Sci. 102(11):9689-9701.

Martin, N. H., S. C. Murphy, R. D. Ralyea, M. Wiedmann, and K. J. Boor. 2011. When cheese gets the blues: *Pseudomonas fluorescens* as the causative agent of cheese spoilage. J. Dairy Sci. 94(6):3176-3183.

Martin, N. H., P. Torres-Frenzel, and M. Wiedmann. 2021. Invited review: Controlling dairy product spoilage to reduce food loss and waste. J. Dairy Sci. 104(2):1251-1261.

Martinez, B. A., J. Stratton, and A. Bianchini. 2017. Isolation and genetic identification of spore-forming bacteria associated with concentrated-milk processing in Nebraska. J. Dairy Sci. 100(2):919-932.

Masiello, S. N., D. Kent, N. H. Martin, Y. H. Schukken, M. Wiedmann, and K. J. Boor. 2017. Longitudinal assessment of dairy farm management practices associated with the presence of psychrotolerant *Bacillales* spores in bulk tank milk on 10 New York State dairy farms. J. Dairy Sci. 100(11):8783-8795.

Maslanka, S. E., J. G. Kerr, G. Williams, J. M. Barbaree, L. A. Carson, J. M. Miller, and B. Swaminathan. 1999. Molecular subtyping of *Clostridium perfringens* by pulsed-field gel electrophoresis to facilitate food-borne-disease outbreak investigations. J. Clin. Microbiol. 37(7):2209-2214.

Matéos, A., M. Guyard-Nicodème, F. Baglinière, J. Jardin, F. Gaucheron, A. Dary, G. Humbert, and J. L. Gaillard. 2015. Proteolysis of milk proteins by AprX, an extracellular protease identified in *Pseudomonas* LBSA1 isolated from bulk raw milk, and implications for the stability of UHT milk. Int. Dairy J. 49:78-88.

Matselis, E. and I. G. Roussis. 1998. Proteinase and lipase production by Pseudomonas fluorescens. Proteolysis and lipolysis in thermized ewe's milk. Food Control 9(5):251-259.

Mattes, R. D. 2009. Oral detection of short-, medium-, and long-chain free fatty acids in humans. Chem. Senses 34(2):145-150.

McClane, B. A., F. A. Uzal, M. E. Fernandez Miyakawa, D. Lyerly, and T. Wilkins. 2006. The enterotoxic clostridia. Pages 698-752 in The Prokaryotes: Volume 4: Bacteria: *Firmicutes, Cyanobacteria*. M. Dworkin, S. Falkow, E. Rosenberg, K.-H. Schleifer, and E. Stackebrandt, ed. Springer US, New York, NY.

McCoard, S., D. Stevens, D. Selbie, L. Day, W. Young, A. E.-D. Bekhit, and L. Samuelsson. 2023. Supporting the growth of the dairy sheep industry in New Zealand–industry update and review of a programme linking industry and science. New Zealand J. Agric. Res.:1-35.

McGinnis, S. and T. L. Madden. 2004. BLAST: at the core of a powerful and diverse set of sequence analysis tools. Nucleic Acids Res 32(Web Server issue):W20-25.

McGuiggan, J. T. M., D. R. McCleery, A. Hannan, and A. Gilmour. 2002. Aerobic spore-forming bacteria in bulk raw milk: factors influencing the numbers of psychrotrophic, mesophilic and thermophilic *Bacillus* spores. Int. J. Dairy Technol. 55(2):100-107.

McHugh, A. J., C. Feehily, M. A. Fenelon, D. Gleeson, C. Hill, P. D. Cotter, and J. A. Gilbert. 2020. Tracking the dairy microbiota from farm bulk tank to skimmed milk powder. mSystems 5(2):e00226-00220.

McHugh, A. J., C. Feehily, C. Hill, and P. D. Cotter. 2017. Detection and Enumeration of Spore-Forming Bacteria in Powdered Dairy Products. Front Microbiol 8:109.

McHugh, A. J., C. Feehily, J. T. Tobin, M. A. Fenelon, C. Hill, and P. D. Cotter. 2018. Mesophilic sporeformers identified in whey powder by using shotgun metagenomic sequencing. Appl. Environ. Microbiol. 84(20).

McInnis, E. A., K. M. Kalanetra, D. A. Mills, and E. A. Maga. 2015. Analysis of raw goat milk microbiota: Impact of stage of lactation and lysozyme on microbial diversity. Food Microbiol. 46:121-131.

McKinnon, C. H. and G. L. Pettipher. 1983. A survey of sources of heat-resistant bacteria in milk with particular reference to psychrotrophic spore-forming bacteria. J. Dairy Res. 50(2):163-170.

Mead, G. C. and B. W. Adams. 1977. A selective medium for the rapid isolation of pseudomonads associated with poultry meat spoilage. Br. Poult. Sci. 18(6):661-670.

Mehta, D. 2018. The influence of spore-forming microorganisms on the quality and functionality of cultured dairy products. PhD thesis - https://openprairie.sdstate.edu/cgi/viewcontent.cgi?article=3972&context=etd. South Dakota State University

Mehta, D. S., L. E. Metzger, A. N. Hassan, B. K. Nelson, and H. A. Patel. 2019. The ability of spore formers to degrade milk proteins, fat, phospholipids, common stabilizers, and exopolysaccharides. J. Dairy Sci. 102(12):10799-10813.

Meier-Kolthoff, J. P., A. F. Auch, H.-P. Klenk, and M. Göker. 2013. Genome sequence-based species delimitation with confidence intervals and improved distance functions. BMC Bioinformatics 14(1):60.

Meier-Kolthoff, J. P., J. S. Carbasse, R. L. Peinado-Olarte, and M. Göker. 2022. TYGS and LPSN: a database tandem for fast and reliable genome-based classification and nomenclature of prokaryotes. Nucleic Acids Res. 50(D1):D801-D807.

Meier-Kolthoff, J. P. and M. Göker. 2019. TYGS is an automated high-throughput platform for state-of-the-art genome-based taxonomy. Nat. Commun. 10(1).

Meier-Kolthoff, J. P., R. L. Hahnke, J. Petersen, C. Scheuner, V. Michael, A. Fiebig, C. Rohde, M. Rohde, B. Fartmann, L. A. Goodwin, O. Chertkov, T. Reddy, A. Pati, N. N. Ivanova, V. Markowitz, N. C. Kyrpides, T. Woyke, M. Göker, and H.-P. Klenk. 2014. Complete genome sequence of DSM 30083T, the type strain (U5/41T) of *Escherichia coli*, and a proposal for delineating subspecies in microbial taxonomy. Stand. Genom. Sci. 9(1):2.

Meng, F., L. Ma, S. Ji, W. Yang, and B. Cao. 2014. Isolation and characterization of *Bacillus subtilis* strain BY-3, a thermophilic and efficient cellulase-producing bacterium on untreated plant biomass. Lett. Appl. Microbiol. 59(3):306-312.

Meng, L., Y. Zhang, H. Liu, S. Zhao, J. Wang, and N. Zheng. 2017. Characterization of *Pseudomonas* spp. And associated proteolytic properties in raw milk stored at low temperatures. Front. Microbiol. 8:2158.

Menzies, P., J. Jansen, C. Fitzpatrick, M. Foran, P. Wilman, and V. Taylor. 2013. A guide to udder health for dairy sheep. Section III: Milking management. Ontario Sheep Farmers - https://www.ontariosheep.org/uploads/userfiles/files/Dairy_Sheep_Udder_Health_Guide-2%20Section%20III(1).pdf. O. S. Farmers, ed.

Merieau, A., B. Gugi, J. F. Guespin-Michel, and N. Orange. 1993. Temperature regulation of lipase secretion by *Pseudomonas fluorescens* strain MFO. Appl. Microbiol. Biotechnol. 39(1):104-109.

Milani, C., G. Alessandri, M. Mangifesta, L. Mancabelli, G. A. Lugli, F. Fontana, G. Longhi, R. Anzalone, A. Viappiani, S. Duranti, F. Turroni, R. Costi, A. Annicchiarico, A. Morini, L. Sarli, M. C. Ossiprandi, D. v. Sinderen, and M. Ventura. 2020. Untangling species-level composition of complex bacterial communities through a novel metagenomic approach. mSystems 5(4):e00404-00420.

Miller, R. A., D. J. Kent, K. J. Boor, N. H. Martin, and M. Wiedmann. 2015a. Different management practices are associated with mesophilic and thermophilic spore levels in bulk tank raw milk. J. Dairy Sci. 98(7):4338-4351.

Miller, R. A., D. J. Kent, M. J. Watterson, K. J. Boor, N. H. Martin, and M. Wiedmann. 2015b. Spore populations among bulk tank raw milk and dairy powders are significantly different. J. Dairy Sci. 98(12):8492-8504.

Ministry for Primary Industries. 2022. Animal Product Notice: Production, Supply and Processing - https://www.mpi.govt.nz/dmsdocument/50182-Animal-Products-Notice-Production-Supply-and-Processing.

Ministry for Primary Industry Regulation and Assurance Branch. 2019. Independent Verification Programme: Dairy Products (July 2017 to June 2018) Microbiological Parameter (Food Safety and Process Hygiene) Results. Report n^{o}: 2018/15 - https://www.mpi.govt.nz/dmsdocument/32794/direct. in New Zealand Food Safety Technical Paper Ministry for Primary Industry, Wellington.

Moatsou, G. and L. Sakkas. 2019. Sheep milk components: Focus on nutritional advantages and biofunctional potential. Small Rumin. Res. 180:86-99.

Moeller, R., G. Horneck, R. Facius, and E. Stackebrandt. 2005. Role of pigmentation in protecting *Bacillus* sp. endospores against environmental UV radiation. FEMS Microbiol. Ecol. 51(2):231-236.

Mokrane, S., N. Bouras, A. Meklat, A. Lahoum, A. Zitouni, C. Verheecke, F. Mathieu, P. Schumann, C. Spröer, N. Sabaou, and H.-P. Klenk. 2016. *Thermoactinomyces khenchelensis* sp. nov., a filamentous bacterium isolated from soil sediment of a terrestrial hot spring. Antonie van Leeuwenhoek 109(2):311-317.

Mollica, M. P., G. Trinchese, F. Cimmino, E. Penna, G. Cavaliere, R. Tudisco, N. Musco, C. Manca, A. Catapano, M. Monda, P. Bergamo, S. Banni, F. Infascelli, P. Lombardi, and M. Crispino. 2021. Milk fatty acid profiles in different animal species: Focus on the potential effect of selected pufas on metabolism and brain functions. Nutrients 13(4):1111.

Monika, R. K. Singh, A. Shrivastava, A. Yadav, and A. K. Srivastava. 2020. 8 - Endophytic bacteria as a source of bioactive compounds. Pages 175-188 in Microbial Endophytes. A. Kumar and V. K. Singh, ed. Woodhead Publishing.

Morales, P., E. Fernández-García, and M. Nuñez. 2005. Volatile compounds produced in cheese by *Pseudomonas* strains of dairy origin belonging to six different species. J. Agric. Food Chem. 53(17):6835-6843.

Moreno, R. and F. Rojo. 2014. Features of pseudomonads growing at low temperatures: another facet of their versatility. Environ. Microbiol. Rep. 6(5):417-426.

Morrison, H. B. and B. W. Hammer. 1941. Distribution of *Pseudomonas fragi*. J. Dairy Sci. 24(1):9-18.

Morton, J. M., J. F. Penry, J. Malmo, and G. A. Mein. 2014. Premilking teat disinfection: Is it worthwhile in pasture-grazed dairy herds? J. Dairy Sci. 97(12):7525-7537.

Mottet, A., F. Teillard, P. Boettcher, G. De' Besi, and B. Besbes. 2018. Review: Domestic herbivores and food security: current contribution, trends and challenges for a sustainable development. Animal 12(s2):s188-s198.

Muck, R. E., E. M. G. Nadeau, T. A. McAllister, F. E. Contreras-Govea, M. C. Santos, and L. Kung, Jr. 2018. Silage review: Recent advances and future uses of silage additives. J. Dairy Sci. 101(5):3980-4000.

Mulcock, A. P. and P. E. Horn. 1965. Thermophilic bacteria from wool. New Zealand J. Agric. Res. 8(4):818-824.

Murphy, P. M., D. Lynch, and P. M. Kelly. 1999. Growth of thermophilic spore forming bacilli in milk during the manufacture of low heat powders. Int. J. Dairy Technol. 52(2):45-50.

Murphy, S. C., N. H. Martin, D. M. Barbano, and M. Wiedmann. 2016. Influence of raw milk quality on processed dairy products: How do raw milk quality test results relate to product quality and yield? J. Dairy Sci. 99(12):10128-10149.

Murphy, S. I., D. Kent, N. H. Martin, R. L. Evanowski, K. Patel, S. M. Godden, and M. Wiedmann. 2019. Bedding and bedding management practices are associated with mesophilic and thermophilic spore levels in bulk tank raw milk. J. Dairy Sci. 102(8):6885-6900.

Murphy, S. I., D. Kent, J. Skeens, M. Wiedmann, and N. H. Martin. 2021. A standard set of testing methods reliably enumerates spores across commercial milk powders. J. Dairy Sci. 104(3):2615-2631.

Mutschlechner, M., N. Lackner, R. Markt, W. Salvenmoser, C. A. Dunlap, and A. O. Wagner. 2021. Proposal of *Thermoactinomyces mirandus* sp. nov., a filamentous, anaerobic bacterium isolated from a biogas plant. Antonie van Leeuwenhoek 114(1):45-54.

Muylaert, A., M. Lebrun, J. N. Duprez, S. Labrozzo, H. Theys, B. Taminiau, and J. Mainil. 2010. Enterotoxaemia-like syndrome and *Clostridium perfringens* in veal calves. Vet. Rec. 167(2):64-65.

Nakamura, Y., G. Cochrane, and I. Karsch-Mizrachi. 2013. The International Nucleotide Sequence Database Collaboration. Nucleic Acids Res. 41(D1):D21-D24.

Necidová, L., Š. Bursová, A. Skočková, B. Janštová, P. Prachařová, Ž. Ševčíková, and B. Janštová. 2014. Growth and enterotoxin production of *Bacillus cereus* in cow, goat, and sheep milk. Acta Vet. Brno 83(10):S3-S8.

Neher, D. A., T. D. Andrews, T. R. Weicht, A. Hurd, and J. W. Barlow. 2022. Organic farm bedded pack system microbiomes: A case study with comparisons to similar and different bedded packs. Dairy 3(3):587-607.

Nelson, D. M., A. J. Glawe, D. P. Labeda, I. K. O. Cann, and R. I. Mackie. 2009. *Paenibacillus tundrae* sp. nov. and *Paenibacillus xylanexedens* sp. nov., psychrotolerant, xylan-degrading bacteria from Alaskan tundra. Int. J. Syst. Evol. Microbiol. 59(7):1708-1714.

Nickerson, K. W. and L. A. Bulla, Jr. 1974. Physiology of sporeforming bacteria associated with insects: minimal nutritional requirements for growth, sporulation, and parasporal crystal formation of *Bacillus thuringiensis*. Appl. Microbiol. 28(1):124-128.

Nie, Z. N., G. N. Ward, and A. T. Michael. 2001. Impact of pugging by dairy cows on pastures and indicators of pugging damage to pasture soil in south-western Victoria. Aust. J. Agric. Res. 52(1):37.

Nielsen, K. M., J. L. Ray, and P. J. Johnsen. 2009. Horizontal gene transfer: Uptake of extracellular DNA by bacteria. Pages 587-596 in Encyclopedia of Microbiology (Third Edition). M. Schaechter, ed. Academic Press, Oxford.

Nieper, B. A., A. Khan, S. Ganesh, F. W. Knol, S. Peterson, K. Stafford, D. Stevens, and S. A. McCoard. 2017. The effects of early access to meal on the behaviour of artificially reared dairy lambs - https://www.nzsap.org/system/files/proceedings/35%20Nieper.pdf. Proc. N.Z. Soc. Anim. Prod. 77.

Nilsson, M. and I. Renberg. 1990. Viable endospores of *Thermoactinomyces vulgaris* in lake sediments as indicators of agricultural history. Appl. Environ. Microbiol. 56(7):2025-2028.

Nishimori, E., K. Kita-Tsukamoto, and H. Wakabayashi. 2000. *Pseudomonas plecoglossicida* sp. nov., the causative agent of bacterial haemorrhagic ascites of ayu, *Plecoglossus altivelis*. Int. J. Syst. Evol. Microbiol. 50(1):83-89.

Niu, C., Z. Fan, F. Zheng, Y. Li, C. Liu, J. Wang, and Q. Li. 2018. Isolation and identification of gas-producing spoilage microbes in fermented broad bean paste. Food Control 84:8-16.

Niu, C., Y. Xue, C. Liu, F. Zheng, J. Wang, and Q. Li. 2020. Identification of gas-forming spoilage bacteria in chili sauce and its control using nisin and salt. LWT 118:108658.

Norring, M., E. Manninen, A. M. de Passillé, J. Rushen, L. Munksgaard, and H. Saloniemi. 2008. Effects of sand and straw bedding on the lying behavior, cleanliness, and hoof and hock injuries of dairy cows. J. Dairy Sci. 91(2):570-576.

Nunez, J. A., F. J. Chavarri, and M. Nure. 1984. Psychrotrophic bacterial flora of raw ewes' milk, with particular reference to Gram negative rods. J. Appl. Bacteriol. 57.

Obeidat, M., H. khyami-horani, A. Al-Zoubi, and I. Otri. 2012. Isolation, characterization, and hydrolytic activities of *Geobacillus* species from Jordanian hot springs. African Journal of Biotechnology 11.

Ogunade, I. M., Y. Jiang, A. A. Pech Cervantes, D. H. Kim, A. S. Oliveira, D. Vyas, Z. G. Weinberg, K. C. Jeong, and A. T. Adesogan. 2018. Bacterial diversity and composition of alfalfa silage as analyzed by Illumina MiSeq sequencing: Effects of *Escherichia coli* O157:H7 and silage additives. J. Dairy Sci. 101(3):2048-2059.

Oguntimehin, M. O. and B. S. Adeleke. 2022. Microbiological, physical, and chemical assessment of palm oil under ginger extracts and sterilization treatment. DYSONA Appl. Sci. 3(2):33-45.

Ohkubo, Y., K. Uchida, H. Motoshima, and N. Katano. 2019. Simple membrane filtration method for estimating numbers of *Paenibacillus* spp. spores in raw milk, using β-galactosidase activity as a selection criterion. Int. Dairy J. 96:10-14.

Oikonomou, G., M. F. Addis, C. Chassard, M. E. F. Nader-Macias, I. Grant, C. Delbès, C. I. Bogni, Y. Le Loir, and S. Even. 2020. Milk microbiota: What are we exactly talking about? Front. Microbiol. 11.

Okinaka, R. T. and P. Keim. 2016. The Phylogeny of *Bacillus cereus sensu lato*. Microbiol. Spectr. 4(1).

Oksanen, J., F. Blanchet, M. Friendly, R. Kindt, P. Legendre, D. McGlinn, P. Minchin, R. O'Hara, G. Simpson, P. Solymos, M. Stevens, E. Szoecs, and H. Wagner. 2020. vegan: Community Ecology Package - https://cran.r-project.org/web/packages/vegan/vegan.pdf. 2.5-7 ed.

Oliver, S. P., M. J. Lewis, T. L. Ingle, B. E. Gillespie, and K. R. Matthews. 1993. Prevention of Bovine Mastitis by a Premilking Teat Disinfectant Containing Chlorous Acid and Chlorine Dioxide. J. Dairy Sci. 76(1):287-292.

Oren, A. and G. M. Garrity. 2021. Valid publication of the names of forty-two phyla of prokaryotes. Int. J. Syst. Evol. Microbiol. 71(10).

Ortíz-Castro, R., E. Valencia-Cantero, and J. López-Bucio. 2008. Plant growth promotion by *Bacillus megaterium* involves cytokinin signaling. Plant Signal. Behav. 3(4):263-265.

Ostrov, I., N. Sela, E. Belausov, D. Steinberg, and M. Shemesh. 2019. Adaptation of *Bacillus* species to dairy associated environment facilitates their biofilm forming ability. Food Microbiol. 82:316-324.

Pahalagedara, A. S. N. W., S. Flint, J. Palmer, A. Subbaraj, G. Brightwell, and T. B. Gupta. 2020. Antimicrobial activity of soil *Clostridium* enriched conditioned media against *Bacillus mycoides*, *Bacillus cereus*, and *Pseudomonas aeruginosa*. Front. Microbiol. 11(3113).

Pahalagedara, A. S. N. W., R. Jauregui, P. Maclean, E. Altermann, S. Flint, J. Palmer, G. Brightwell, and T. B. Gupta. 2021. Culture and genome-based analysis of four soil *Clostridium* isolates reveal their potential for antimicrobial production. BMC Genomics 22(1).

Pahlow, G., R. E. Muck, F. Driehuis, S. J. W. H. O. Elferink, and S. F. Spoelstra. 2003. Microbiology of ensiling. Pages 31-93 in Silage Science and Technology.

Palmer, J. S., R. L. Hough, H. M. West, and L. M. Avery. 2019. A review of the abundance, behaviour and detection of clostridial pathogens in agricultural soils. Eur. J. Soil Sci. 70(4):911-929.

Paludetti, L. F., A. L. Kelly, B. O'Brien, K. Jordan, and D. Gleeson. 2019. Microbiological quality of milk from farms to milk powder manufacture: an industrial case study. J. Dairy Res. 86(2):242-247.

Pancza, B., M. Szathmáry, I. Gyurján, B. Bánkuti, Z. Tudós, S. Szathmary, L. Stipkovits, Z. Sipos-Kozma, B. Ásványi, L. Varga, K. Szenthe, and F. Bánáti. 2021. A rapid and efficient DNA isolation method for qPCR-based detection of pathogenic and spoilage bacteria in milk. Food Control 130:108236.

Parente, E., A. Ricciardi, and T. Zotta. 2020. The microbiota of dairy milk: A review. Int. Dairy J. 107:104714.

Park, Y. W., M. Juárez, M. Ramos, and G. F. W. Haenlein. 2007. Physico-chemical characteristics of goat and sheep milk. Small Rumin. Res. 68(1):88-113.

Partanen, P., J. Hultman, L. Paulin, P. Auvinen, and M. Romantschuk. 2010. Bacterial diversity at different stages of the composting process. BMC Microbiol. 10(1):94.

Pasteur, L., inventor. 1865. La pasteurisation. France Pat. No. 67,000.

Patel, H. K., P. Ferrante, M. Xianfa, S. G. Javvadi, S. Subramoni, M. Scortichini, and V. Venturi. 2017. Identification of loci of *Pseudomonas syringae* pv. *actinidiae* involved in lipolytic activity and their role in colonization of kiwifruit leaves. Phytopathology 107(6):645-653.

Patel, K., S. M. Godden, E. Royster, B. A. Crooker, J. Timmerman, and L. Fox. 2019. Relationships among bedding materials, bedding bacteria counts, udder hygiene, milk quality, and udder health in US dairy herds. J. Dairy Sci. 102(11):10213-10234.

Patel, S. and R. S. Gupta. 2020. A phylogenomic and comparative genomic framework for resolving the polyphyly of the genus *Bacillus*: Proposal for six new genera of *Bacillus* species, *Peribacillus* gen. nov., *Cytobacillus* gen. nov., *Mesobacillus* gen. nov., *Neobacillus* gen. nov., *Metabacillus g*en. nov. and *Alkalihalobacillus* gen. nov. Int. J. Syst. Evol. Microbiol. 70(1):406-438.

Paula, William, K. Mruz, and Thomas. 2001. Clinical features of clostridial bacteremia: A review from a rural area. Clin. Infect. Dis. 33(3):349-353.

Pavel, A. B. and C. I. Vasile. 2012. PyElph - a software tool for gel images analysis and phylogenetics. BMC Bioinformatics 13(1):9.

Pedroso, A., A. Hurley-Bacon, A. Zedek, T. Kwan, A. Jordan, G. Avellaneda, C. Hofacre, B. Oakley, S. Collett, J. Maurer, and M. Lee. 2013. Can probiotics improve the environmental microbiome and resistome of commercial poultry production? Int. J. Environ. Res. Public Health 10(10):4534-4559.

Peterson, S. W. and C. Prichard. 2015. The sheep dairy industry in New Zealand: a review - https://www.nzsap.org/system/files/proceedings/73-Peterson.pdf. Proc. N.Z. Soc. Anim. Prod. 75:119-126.

Petit, L., M. Gibert, and M. R. Popoff. 1999. *Clostridium perfringens*: toxinotype and genotype. Trends Microbiol. 7(3):104-110.

Phillips, J. D. and M. W. Griffiths. 1986. Factors contributing to the seasonal variation of *Bacillus* spp. in pasteurized dairy products. J. Appl. Bacteriol. 61(4):275-285.

Pietrzak-Fiećko, R. and A. M. Kamelska-Sadowska. 2020. The comparison of nutritional value of human milk with other mammals' milk. Nutrients 12(5):1404.

Pirisi, A., A. Lauret, and J. P. Dubeuf. 2007. Basic and incentive payments for goat and sheep milk in relation to quality. Small Rumin. Res. 68(1-2):167-178.

Pitt, A., J. Schmidt, E. Lang, W. B. Whitman, T. Woyke, and M. W. Hahn. 2018. Polynucleobacter meluiroseus sp. nov., a bacterium isolated from a lake located in the mountains of the Mediterranean island of Corsica. Int. J. Syst. Evol. Microbiol. 68(6):1975-1985.

Pomiès, D., M. Barbet, M. Fages, S. Hulin, A. L. Jacquot, and B. Blay. 2014. Milk production without wet forage for Saint-Nectaire PDO cheese. Pages 385-389 in Forage resources and ecosystem services provided by Mountain and Mediterranean grasslands and rangelands Vol. 109. R. Baumont, P. Carrère, M. Jouven, G. Lombardi, A. López-Francos, A. Peeters, C. Porqueddu, and B. Martin, ed. Zaragoza : CIHEAM / INRA / FAO / VetAgro Sup Clermont-Ferrand / Montpellier SupAgro - https://om.ciheam.org/om/pdf/a109/00007747.pdf.

Popoff, M. R. 1984. Selective medium for isolation of *Clostridium butyricum* from human feces. J. Clin. Microbiol. 20(3):417-420.

Postollec, F., A.-G. Mathot, M. Bernard, M.-L. Divanac'H, S. Pavan, and D. Sohier. 2012. Tracking spore-forming bacteria in food: From natural biodiversity to selection by processes. Int. J. Food Microbiol. 158(1):1-8.

Pourshafie, M., P. Vahdani, and M. Popoff. 2005. Genotyping *Clostridium botulinum* toxinotype A isolates from patients using amplified rDNA restriction analysis. J. Med. Microbiol. 54(10):933-936.

Prabhakaran, M., M. B. Couger, C. A. Jackson, T. Weirick, and B. Z. Fathepure. 2015. Genome sequences of the lignin-degrading *Pseudomonas* sp. strain ys-1p and *Rhizobium* sp. strain ys-1r isolated from decaying wood. Genome Announc. 3(2):e00019-00015.

Pramularsih, I., E. O. Kyere, S. N. Md Zain, and S. Flint. 2022. Testing for total bacteria in dairy powder - Comparison of test incubation temperatures (a case study). Int. Dairy J. 134:105452.

Priest, F. G. 1977. Extracellular enzyme synthesis in the genus *Bacillus*. Bacteriol. Rev. 41(3):711-753.

Pringsheim, E. G. and C. F. Robinow. 1947. Observations on two very large bacteria, *Caryophanon latum* Peshkoff and *Lineola longa* (nomenprovisorium). J. Gen. Microbiol. (1):267-278.

Pruesse, E., C. Quast, K. Knittel, B. M. Fuchs, W. Ludwig, J. Peplies, and F. O. Glöckner. 2007. SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. Nucleic Acids Res. 35(21):7188-7196.

Pulina, G., M. J. Milan, M. P. Lavin, A. Theodoridis, E. Morin, J. Capote, D. L. Thomas, A. H. D. Francesconi, and G. Caja. 2018. Invited review: Current production trends, farm structures, and economics of the dairy sheep and goat sectors. J. Dairy Sci. 101(8):6715-6729.

Purahong, W., L. Orrù, I. Donati, G. Perpetuini, A. Cellini, A. Lamontanara, V. Michelotti, G. Tacconi, and F. Spinelli. 2018. Plant microbiome and its link to plant health: Host species, organs and *Pseudomonas syringae* pv. *actinidiae* infection shaping bacterial phyllosphere communities of kiwifruit plants. Front. Plant Sci. 9.

Quigley, L., D. J. O'Sullivan, D. Daly, O. O'Sullivan, Z. Burdikova, R. Vana, T. P. Beresford, R. P. Ross, G. F. Fitzgerald, P. L. McSweeney, L. Giblin, J. J. Sheehan, and P. D. Cotter. 2016. *Thermus* and the pink discoloration defect in cheese. mSystems 1(3).

Quigley, L., O. O'Sullivan, C. Stanton, T. P. Beresford, R. P. Ross, G. F. Fitzgerald, and P. D. Cotter. 2013. The complex microbiota of raw milk. FEMS Microbiol Reviews 37(5):664-698.

Quintieri, L., L. Caputo, M. Brasca, and F. Fanelli. 2021. Recent advances in the mechanisms and regulation of QS in dairy spoilage by *Pseudomonas* spp. Foods 10(12):3088.

Quintieri, L., F. Fanelli, and L. Caputo. 2019a. Antibiotic resistant *Pseudomonas* spp. spoilers in fresh dairy products: An underestimated risk and the control strategies. Foods 8(9):372.

Quintieri, L., D. Zühlke, F. Fanelli, L. Caputo, V. C. Liuzzi, A. F. Logrieco, C. Hirschfeld, D. Becher, and K. Riedel. 2019b. Proteomic analysis of the food spoiler *Pseudomonas fluorescens*

ITEM 17298 reveals the antibiofilm activity of the pepsin-digested bovine lactoferrin. Food Microbiol. 82:177-193.

R Core Team. 2022. R: A language and environment for statistical computing - http://www.R-project.org/. R Foundation for Statistical Computing, Vienna, Austria.

Rahman, R., M. Masomian, A. Salleh, and M. Basri. 2009. A new thermostable lipase by *Aneurinibacillus thermoaerophilus* strain HZ : Nutritional studies. Ann. Microbiol. 59:133-139.

Rahmati, A., M. Gal, G. Northey, and J. S. Brazier. 2005. Subtyping of *Clostridium difficile* polymerase chain reaction (PCR) ribotype 001 by repetitive extragenic palindromic PCR genomic fingerprinting. J. Hosp. Infect. 60(1):56-60.

Rajmohan, S., C. E. R. Dodd, and W. M. Waites. 2002. Enzymes from isolates of *Pseudomonas fluorescens* involved in food spoilage. J. Appl. Microbiol. 93:205-213.

Ram Mohan, N., M. S. Fullmer, A. M. Makkay, R. Wheeler, A. Ventosa, A. Naor, J. P. Gogarten, and R. T. Papke. 2014. Evidence from phylogenetic and genome fingerprinting analyses suggests rapidly changing variation in *Halorubrum* and *Haloarcula* populations. Front. Microbiol. 5.

Ranieri, M. L. and K. J. Boor. 2009. Short communication: bacterial ecology of high-temperature, short-time pasteurized milk processed in the United States. J. Dairy Sci. 92(10):4833-4840.

Ranieri, M. L., R. A. Ivy, W. R. Mitchell, E. Call, S. N. Masiello, M. Wiedmann, and K. J. Boor. 2012. Real-time pcr detection of *Paenibacillus* spp. In raw milk to predict shelf life performance of pasteurized fluid milk products. Appl. Environ. Microbiol. 78(16):5855-5863.

Ranjbar, R., P. Pezeshknejad, F. Khamesipour, K. Amini, and R. Kheiri. 2017. Genomic fingerprints of *Escherichia coli* strains isolated from surface water in Alborz province, Iran. BMC Research Notes 10(1).

Rao, M. B., A. M. Tanksale, M. S. Ghatge, and V. V. Deshpande. 1998. Molecular and biotechnological aspects of microbial proteases. Microbiol Mol Biol Rev 62(3):597-635.

Rasolofo, E. A., D. St-Gelais, G. LaPointe, and D. Roy. 2010. Molecular analysis of bacterial population structure and dynamics during cold storage of untreated and treated milk. Int. J. Food Microbiol. 138(1-2):108-118.

Rauscher, B. and I. Lewandowski. 2016. Miscanthus Horse Bedding Compares Well to Alternatives. Pages 297-305 in Perennial Biomass Crops for a Resource-Constrained World. Springer International Publishing.

Ray, T., T. N. Gaire, C. J. Dean, S. Rowe, S. M. Godden, and N. R. Noyes. 2022. The microbiome of common bedding materials before and after use on commercial dairy farms. Animal Microbiome 4(1).

Reichler, S. J., N. H. Martin, R. L. Evanowski, J. Kovac, M. Wiedmann, and R. H. Orsi. 2019. A century of gray: A genomic locus found in 2 distinct *Pseudomonas* spp. is associated with historical and contemporary color defects in dairy products worldwide. J. Dairy Sci. 102(7):5979-6000.

Reichler, S. J., S. I. Murphy, A. W. Erickson, N. H. Martin, A. B. Snyder, and M. Wiedmann. 2020. Interventions designed to control postpasteurization contamination in high-temperature, short-time-pasteurized fluid milk processing facilities: A case study on the effect of employee training, clean-in-place chemical modification, and preventive maintenance. J. Dairy Sci. 103(8):7569-7584.

Reichler, S. J., S. I. Murphy, N. H. Martin, and M. Wiedmann. 2021. Identification, subtyping, and tracking of dairy spoilage-associated *Pseudomonas* by sequencing the *ileS* gene. J. Dairy Sci. 104(3):2668-2683.

Reichler, S. J., A. Trmčić, N. H. Martin, K. J. Boor, and M. Wiedmann. 2018. *Pseudomonas fluorescens* group bacterial strains are responsible for repeat and sporadic postpasteurization contamination and reduced fluid milk shelf life. J. Dairy Sci. 101(9):7780-7800.

Reindl, A., M. Dzieciol, I. Hein, M. Wagner, and P. Zangerl. 2014. Enumeration of clostridia in goat milk using an optimized membrane filtration technique. J. Dairy Sci. 97(10):6036-6045.

Reinemann, D., G. Wolters, and M. Rasmussen. 2003. Review of practices for cleaning and sanitation of milking machines - https://www.oxidationtech.com/downloads/Tech/Milk%20machine%20disinfection%20practices%20non-O3.pdf. Int. Dairy Fed. Bul. 381.

Repaci, G. 1910. Contribution à l'étude de la flore bactérienne anaérobie des gangrènes pulmonaires. Un streptobacille anaérobie. CR Seances Soc. Biol. Fil. 68:410-412.

Reponen, T. A., S. V. Gazenko, S. A. Grinshpun, K. Willeke, and E. C. Cole. 1998. Characteristics of airborne actinomycete spores. Appl. Environ. Microbiol. 64(10):3807-3812.

Ribeiro Junior, J. C., A. M. de Oliveira, F. G. Silva, R. Tamanini, A. L. M. de Oliveira, and V. Beloti. 2018. The main spoilage-related psychrotrophic bacteria in refrigerated raw milk. J. Dairy Sci. 101(1):75-83.

Ribeiro Júnior, J. C., B. Kussumoto de Alcântara, and V. Beloti. 2016. Spoilage potential of *Paenibacillus* sp. in Brazilian raw milk. Cienc. Rural 46(4):637-640.

Roberts, M. C., G. Garland-Lewis, S. Trufan, S. J. Meschke, H. Fowler, R. C. Shean, A. L. Greninger, and P. M. Rabinowitz. 2018. Distribution of *Staphylococcus* species in dairy cows, workers and shared farm environments. FEMS Microbiol. Lett. 365(15).

Robertson, A. H. 1927. Thermophilic and thermoduric microorganisms, with special reference to species isolated from milk. in The thermal resistance of microorganisms. II. N.Y. (Geneva).

Rodríguez, M., J. C. Reina, V. Béjar, and I. Llamas. 2020. *Psychrobacillus vulpis* sp. nov., a new species isolated from faeces of a red fox in Spain. Int. J. Syst. Evol. Microbiol. 70(2):882-888.

Rood, J. I., V. Adams, J. Lacey, D. Lyras, B. A. McClane, S. B. Melville, R. J. Moore, M. R. Popoff, M. R. Sarker, J. G. Songer, F. A. Uzal, and F. Van Immerseel. 2018. Expansion of the *Clostridium perfringens* toxin-based typing scheme. Anaerobe 53:5-10.

Rothman, R. E., M. D. Majmudar, G. D. Kelen, G. Madico, C. A. Gaydos, T. Walker, and T. C. Quinn. 2002. Detection of bacteremia in emergency department patients at risk for infective

endocarditis using universal 16S rRNA primers in a decontaminated polymerase chain reaction assay. J. Infect. Dis. 186(11):1677-1681.

Rowbotham, R. F. and P. L. Ruegg. 2015. Association of bedding types with management practices and indicators of milk quality on larger Wisconsin dairy farms. J. Dairy Sci. 98(11):7865-7885.

Roy, D., P. J. Moughan, A. Ye, S. M. Hodgkinson, N. Stroebinger, S. Li, A. C. Dave, C. A. Montoya, and H. Singh. 2022. Structural changes in milk from different species during gastric digestion in piglets. J. Dairy Sci. 105(5):3810-3831.

Roy, D., A. Ye, P. J. Moughan, and H. Singh. 2020. Composition, structure, and digestive dynamics of milk from different species—a review. Front. Nutr. 7.

Roy, D., A. Ye, P. J. Moughan, and H. Singh. 2021. Structural changes in cow, goat, and sheep skim milk during dynamic in vitro gastric digestion. J. Dairy Sci. 104(2):1394-1411.

Rueckert, A., R. S. Ronimus, and H. W. Morgan. 2004. A RAPD-based survey of thermophilic bacilli in milk powders from different countries. Int. J. Food Microbiol. 96(3):263-272.

Rueckert, A., R. S. Ronimus, and H. W. Morgan. 2005a. Development of a rapid detection and enumeration method for thermophilic bacilli in milk powders. J. Microbiol. Methods 60(2):155-167.

Rueckert, A., R. S. Ronimus, and H. W. Morgan. 2005b. Rapid differentiation and enumeration of the total, viable vegetative cell and spore content of thermophilic bacilli in milk powders with reference to *Anoxybacillus flavithermus*. J. Appl. Microbiol. 99(5):1246-1255.

Ruscoe, H. L., R. G. Taketani, I. M. Clark, G. Lund, D. Hughes, I. C. Dodd, P. R. Hirsch, and T. H. Mauchline. 2021. Land management legacy affects abundance and function of the acds gene in wheat root associated pseudomonads. Front. Microbiol. 12.

Ryan, R. P., S. Monchy, M. Cardinale, S. Taghavi, L. Crossman, M. B. Avison, G. Berg, D. Van Der Lelie, and J. M. Dow. 2009. The versatility and adaptation of bacteria from the genus *Stenotrophomonas*. Nat. Rev. Microbiol. 7(7):514-525.

Sadiq, F. A., S. Flint, and G. He. 2018. Microbiota of milk powders and the heat resistance and spoilage potential of aerobic spore-forming bacteria. Int. Dairy J. 85:159-168.

Sadiq, F. A., Y. Li, T. Liu, S. Flint, G. Zhang, and G. He. 2016a. A RAPD based study revealing a previously unreported wide range of mesophilic and thermophilic spore formers associated with milk powders in China. Int. J. Food Microbiol. 217:200-208.

Sadiq, F. A., Y. Li, T. Liu, S. Flint, G. Zhang, L. Yuan, Z. Pei, and G. He. 2016b. The heat resistance and spoilage potential of aerobic mesophilic and thermophilic spore forming bacteria isolated from Chinese milk powders. Int. J. Food Microbiol. 238:193-201.

Saito, A., S. Ikeda, H. Ezura, and K. Minamisawa. 2007. Microbial community analysis of the phytosphere using culture-independent methodologies. Microbes Environ. 22(2):93-105.

Salmerón, J., C. de Vega, F. J. Pérez-Elortondo, M. Albisu, and L. J. R. Barrón. 2002. Effect of pasteurization and seasonal variations in the microflora of ewe's milk for cheesemaking. Food Microbiol. 19(2):167-174.

Santos, J. A., C. J. González, T. M. López-Díaz, M. L. García-López, and A. Otero. 1996. Extracellular protease production by dairy strains of *Aeromonas hydrophila* as affected by growth media and incubation temperature. Food Microbiol. 13(1):47-51.

Santos, M., J. J. Jiménez, B. Bartolomé, C. Gómez-Cordovés, and M. J. del Nozal. 2003a. Variability of brewer's spent grain within a brewery. Food Chem. 80(1):17-21.

Santos, M. V., Y. Ma, Z. Caplan, and D. M. Barbano. 2003b. Sensory threshold of off-flavors caused by proteolysis and lipolysis in milk. J. Dairy Sci. 86(5):1601-1607.

Scheldeman, P., L. Herman, S. Foster, and M. Heyndrickx. 2006. *Bacillus sporothermodurans* and other highly heat-resistant spore formers in milk. J. Appl. Microbiol. 101(3):542-555.

Scheldeman, P., A. Pil, L. Herman, P. De Vos, and M. Heyndrickx. 2005. Incidence and diversity of potentially highly heat-resistant spores isolated at dairy farms. Appl. Environ. Microbiol. 71(3):1480-1494.

Schneider, R. M., J. H. Harrison, and K. A. Loney. 1995. The effects of bacterial inoculants, beet pulp, and propionic acid on ensiled wet brewers grains. J. Dairy Sci. 78(5):1096-1105.

Scott, S. A., J. D. Brooks, J. Rakonjac, K. M. R. Walker, and S. H. Flint. 2007. The formation of thermophilic spores during the manufacture of whole milk powder. Int. J. Dairy Technol. 60(2):109-117.

Seale, R. B., R. Dhakal, K. Chauhan, H. M. Craven, H. C. Deeth, C. J. Pillidge, I. B. Powell, and M. S. Turner. 2012. Genotyping of Present-Day and Historical Geobacillus Species Isolates from Milk Powders by High-Resolution Melt Analysis of Multiple Variable-Number Tandem-Repeat Loci. Appl. Environ. Microbiol. 78(19):7090-7097.

Seeliger, H. P. and H. Werner. 1963. [Qualitative and quantitative research on the human intestinal flora]. Ann. Inst. Pasteur 105:911-936.

Seemann, T. 2014. Prokka: rapid prokaryotic genome annotation. Bioinformatics 30(14):2068-2069.

Seghal Kiran, G., A. Nishanth Lipton, J. Kennedy, A. D. Dobson, and J. Selvin. 2014. A halotolerant thermostable lipase from the marine bacterium *Oceanobacillus* sp. PUMB02 with an ability to disrupt bacterial biofilms. Bioengineered 5(5):305-318.

Sellami-Kamoun, A., A. Haddar, N. E.-H. Ali, B. Ghorbel-Frikha, S. Kanoun, and M. Nasri. 2008. Stability of thermostable alkaline protease from *Bacillus licheniformis* RP1 in commercial solid laundry detergent formulations. Microbiol. Res. 163(3):299-306.

Seol, D., J. S. Lim, S. Sung, Y. H. Lee, M. Jeong, S. Cho, W. Kwak, and H. Kim. 2022. Microbial identification using rRNA operon region: Database and tool for metataxonomics with long-read sequence. Microbiol. Spectr. 10(2):e02017-02021.

Setlow, P. 2006. Spores of *Bacillus subtilis*: their resistance to and killing by radiation, heat and chemicals. J. Appl. Microbiol. 101(3):514-525.

Shabana, I. I., N. N. Albakri, and N. A. Bouqellah. 2021. Metagenomic investigation of faecal microbiota in sheep and goats of the same ages. J. Taibah Univ. Sci. 15(1):1-9.

Shahidi, S. A. and A. R. Ferguson. 1971. New quantitative, qualitative, and confirmatory media for rapid analysis of food for *Clostridium perfringens*. Appl. Microbiol. 21(3):500-506.

Shangkuan, Y. H., J. F. Yang, H. C. Lin, and M. F. Shaio. 2000. Comparison of PCR-RFLP, ribotyping and ERIC-PCR for typing *Bacillus anthracis* and *Bacillus cereus* strains. J. Appl. Microbiol. 89(3):452-462.

Shen, Y., Y. Fu, Y. Yu, J. Zhao, J. Li, Y. Li, X. Wang, J. Zhang, and W. Xiang. 2017. *Psychrobacillus lasiicapitis* sp. nov., isolated from the head of an ant (*Lasius fuliginosu*s). Int. J. Syst. Evol. Microbiol. 67(11):4462-4467.

Shipe, W. F., R. Bassette, D. D. Deane, W. L. Dunkley, E. G. Hammond, W. J. Harper, D. H. Kleyn, M. E. Morgan, J. H. Nelson, and R. A. Scanlan. 1978. Off flavors of milk: nomenclature, standards, and bibliography. J. Dairy Sci. 61(7):855-869.

Shivaji, S., P. Chaturvedi, K. Suresh, G. S. Reddy, C. B. Dutt, M. Wainwright, J. V. Narlikar, and P. M. Bhargava. 2006. *Bacillus aerius* sp. nov., *Bacillus aerophilus* sp. nov., *Bacillus stratosphericus* sp. nov. and *Bacillus altitudinis* sp. nov., isolated from cryogenic tubes used for collecting air samples from high altitudes. Int. J. Syst. Evol. Microbiol. 56(Pt 7):1465-1473.

Shrestha, A., L. M. Samuelsson, P. Sharma, L. Day, D. Cameron-Smith, and A. M. Milan. 2021. Comparing response of sheep and cow milk on acute digestive comfort and lactose malabsorption: A randomized controlled trial in female dairy avoiders. Front. Nutr. 8.

Siala, A., I. R. Hill, and T. R. G. Gray. 2000. Populations of spore-forming bacteria in an acid forest soil, with special reference to *Bacillus subtilis*. Microbiology 81(1):183-190.

Sikolenko, M. A. and L. N. Valentovich. 2022. RiboGrove: a database of full-length prokaryotic 16S rRNA genes derived from completely assembled genomes. Res. Microbiol. 173(4):103936.

Silby, M. W., C. Winstanley, S. A. C. Godfrey, S. B. Levy, and R. W. Jackson. 2011. *Pseudomonas* genomes: diverse and adaptable. FEMS Microbiol. Rev. 35(4):652-680.

Simão, F. A., R. M. Waterhouse, P. Ioannidis, E. V. Kriventseva, and E. M. Zdobnov. 2015. BUSCO: assessing genome assembly and annotation completeness with single-copy orthologs. Bioinformatics 31(19):3210-3212.

Simões, M., L. C. Simões, M. O. Pereira, and M. J. Vieira. 2008. Antagonism between *Bacillus cereus* and *Pseudomonas fluorescens* in planktonic systems and in biofilms. Biofouling 24(5):339-349.

Singh, A., T. Kumari, M. Rajput, A. Baishya, N. Bhatt, and S. Roy. 2020. A review: Effect of bedding material on production, reproduction and health and behavior of dairy animals. Int. J. Livest. Res. 10:11-20.

Skeie, S. B., M. Haland, I. M. Thorsen, J. Narvhus, and D. Porcellato. 2019. Bulk tank raw milk microbiota differs within and between farms: A moving goalpost challenging quality control. J. Dairy Sci. 102(3):1959-1971.

Soler-Rivas, C., S. Jolivet, N. Arpin, J. M. Olivier, and H. J. Wichers. 1999. Biochemical and physiological aspects of brown blotch disease of *Agaricus bisporus*. FEMS Microbiol. Rev. 23(5):591-614.

Soler, L. 2003. Comparison of three molecular methods for typing *Aeromonas popoffii* isolates. Antonie van Leeuwenhoek 83(4):341-349.

Songer, J. G. 1996. Clostridial enteric diseases of domestic animals. Clin. Microbiol. Rev. 9(2):216-234.

Sorhaug, T. and L. Stepaniak. 1997. Psychrotrophs and their enzymes in milk and dairy products: Quality aspects. Trends Food Sci. Technol. 8.

Soufiane, B. and J.-C. Côté. 2013. *Bacillus weihenstephanensis* characteristics are present in *Bacillus cereus* and *Bacillus mycoides* strains. FEMS Microbiol. Lett. 341(2):127-137.

Souza, V. L., N. M. Lopes, O. F. Zacaroni, V. A. Silveira, R. A. N. Pereira, J. A. Freitas, R. Almeida, G. G. S. Salvati, and M. N. Pereira. 2017. Lactation performance and diet digestibility of dairy cows in response to the supplementation of *Bacillus subtilis* spores. Livestock Science 200:35-39.

Spigaglia, P., F. Barbanti, F. Marocchi, M. Mastroleo, M. Baretta, P. Ferrante, E. Caboni, S. Lucioli, and M. Scortichini. 2020. *Clostridium bifermentans* and *C. subterminale* are associated with kiwifruit vine decline, known as moria, in Italy. Plant Pathology 69(4):765-774.

Stadhouders, J. and K. Jørgensen. 1990. Prevention of the contamination of raw milk by a hygienic milk production. Bul. Int. Dairy Fed. (251):32-36.

Stanford, K., T. Reuter, B. H. Gilroyed, and T. A. McAllister. 2015. Impacts of sporulation temperature, exposure to compost matrix and temperature on survival of *Bacillus cereus* spores during livestock mortality composting. J. Appl. Microbiol. 118(4):989-997.

StatsNZ. 2020. Agricultural production statistics: Variable by total. New Zealand (Annual-Jun) (Final) 2020 - http://infoshare.stats.govt.nz/.

Stelma, G. N., Jr. 2018. Use of bacterial spores in monitoring water quality and treatment. J. Water Health 16(4):491-500.

Stock, I., T. Grueger, and B. Wiedemann. 2003. Natural antibiotic susceptibility of strains of *Serratia marcescens* and the *S. liquefaciens* complex: *S. liquefaciens sensu stricto, S. proteamaculans* and *S. grimesii*. Int. J. Antimicrob. Agents 22(1):35-47.

Stoeckel, M., G. Lücking, M. Ehling-Schulz, Z. Atamer, and J. Hinrichs. 2016. Bacterial spores isolated from ingredients, intermediate and final products obtained from dairies: thermal resistance in milk. Dairy Sci. Technol. 96(4):569-577.

Su, X., M. Tortorice, S. Ryo, X. Li, K. Waterman, A. Hagen, and Y. Yin. 2020. Sensory lexicons and formation pathways of off-aromas in dairy ingredients: A review. Molecules 25(3):569.

Suchodolski, J. S., M. E. Markel, J. F. Garcia-Mazcorro, S. Unterer, R. M. Heilmann, S. E. Dowd, P. Kachroo, I. Ivanov, Y. Minamoto, and E. M. Dillman. 2012. The fecal microbiome in dogs with acute diarrhea and idiopathic inflammatory bowel disease. PloS one 7(12):e51907.

Sun, L., Å. Lundh, A. Höjer, G. Bernes, D. Nilsson, M. Johansson, M. Hetta, A. H. Gustafsson, K. H. Saedén, and J. Dicksved. 2022. Milking system and premilking routines have a strong effect on the microbial community in bulk tank milk. J. Dairy Sci. 105(1):123-139.

Sutherland, A. D. and R. Murdoch. 1994. Seasonal occurence of psychrotrophic *Bacillus* species in raw milk and studies on the interactions with mesophilic *Bacillus* sp. Int. J. Food Technol. 21(4):279-292.

Suzuki, Y., T. Kishigami, K. Inoue, Y. Mizoguchi, N. Eto, M. Takagi, and S. Abe. 1983. *Bacillus thermoglucosidasius* sp. nov., a new species of obligately thermophilic bacilli. Syst. Appl. Microbiol. 4(4):487-495.

Swaminathan, B., T. J. Barrett, S. B. Hunter, and R. V. Tauxe. 2001. PulseNet: the molecular subtyping network for foodborne bacterial disease surveillance, United States. Emerg. Infect. Dis. 7(3):382-389.

te Giffel, M. C., A. Wagendorp, A. Herrewegh, and F. Driehuis. 2002. Bacterial spores in silage and raw milk. Antonie van Leeuwenhoek 81(1/4):625-630.

Teh, K. H., D. Lindsay, J. Palmer, P. Andrewes, P. Bremer, and S. Flint. 2013. Lipolysis within single culture and co-culture biofilms of dairy origin. Int. J. Food Microbiol. 163(2):129-135.

Teixeira, P., Z. Lopes, J. Azeredo, R. Oliveira, and M. J. Vieira. 2005. Physico-chemical surface characterization of a bacterial population isolated from a milking machine. Food Microbiol. 22(2):247-251.

Teng, F., M. G. Reis, Y. Ma, and L. Day. 2018. Effects of season and industrial processes on volatile 4-alkyl-branched chain fatty acids in sheep milk. Food Chem. 260:327-335.

Thomas, D. L., Y. M. Berger, B. C. McKusick, and C. M. Mikolayunas. 2014. Dairy sheep production research at the University of Wisconsin-Madison, USA – a review. J. Anim. Sci. Biotechnol. 5(1):22.

Timmis, K., R. Cavicchioli, J. L. Garcia, B. Nogales, M. Chavarría, L. Stein, T. J. McGenity, N. Webster, B. K. Singh, J. Handelsman, V. Lorenzo, C. Pruzzo, J. Timmis, J. L. R. Martín, W. Verstraete, M. Jetten, A. Danchin, W. Huang, J. Gilbert, R. Lal, H. Santos, S. Y. Lee, A. Sessitsch, P. Bonfante, L. Gram, R. T. P. Lin, E. Ron, Z. C. Karahan, J. R. Meer, S. Artunkal, D. Jahn, and L. Harper. 2019. The urgent need for microbiology literacy in society. Environ. Microbiol. 21(5):1513-1528.

Tindall, B. J., R. Rosselló-Móra, H. J. Busse, W. Ludwig, and P. Kämpfer. 2010. Notes on the characterization of prokaryote strains for taxonomic purposes. Int. J. Syst. Evol. Microbiol. 60(1):249-266.

Tjaberg, T. B. 1974. Proteases of *Clostridium botulinum*. Acta Veterinaria Scandinavica 15(4):487-506.

Tonamo, A., I. Komlósi, L. Varga, L. Czeglédi, and F. Peles. 2020. Bacteriological quality of raw ovine milk from different sheep farms. Animals 10(7):1163.

Tremonte, P., L. Tipaldi, M. Succi, G. Pannella, L. Falasca, V. Capilongo, R. Coppola, and E. Sorrentino. 2014. Raw milk from vending machines: Effects of boiling, microwave treatment, and refrigeration on microbiological quality. J. Dairy Sci. 97(6):3314-3320.

Tribst, A. A. L., L. T. P. Falcade, and M. M. De Oliveira. 2019. Strategies for raw sheep milk storage in smallholdings: Effect of freezing or long-term refrigerated storage on microbial growth. J. Dairy Sci. 102(6):4960-4971.

Tryfinopoulou, P., E. H. Drosinos, and G. J. E. Nychas. 2001. Performance of *Pseudomonas* CFC-selective medium in the fish storage ecosystems. J. Microbiol. Methods 47(2):243-247.

Tsilinsky, P. 1899. On the thermophilic moulds. Ann. Inst. Pasteur 13:500-505.

Turchi, B., S. Pero, B. Torracca, F. Fratini, S. Mancini, A. Galiero, F. Pedonese, R. Nuvoloni, and D. Cerri. 2016. Occurrence of *Clostridium* spp. in ewe's milk: enumeration and identification of isolates. Dairy Sci. Technol. 96(5):693-701.

Tyler, K. D., G. Wang, S. D. Tyler, and W. M. Johnson. 1997. Factors affecting reliability and reproducibility of amplification-based DNA fingerprinting of representative bacterial pathogens. J. Clin. Microbiol. 35(2):339-346.

Unsworth, B. A., T. Cross, M. R. Seaward, and R. E. Sims. 1977. The longevity of thermoactinomycete endospores in natural substrates. J. Appl. Bacteriol. 42(1):45-52.

Urbina, P., M. I. Collado, A. Alonso, F. M. Goñi, M. Flores-Díaz, A. Alape-Girón, J.-M. Ruysschaert, and M. F. Lensink. 2011. Unexpected wide substrate specificity of *C. perfringens* α-toxin phospholipase C. Biochim. Biophys. Acta - Biomembr. 1808(10):2618-2627.

Uzal, F. and J. Songer. 2008. Diagnosis of *Clostridium perfringens* intestinal infections in sheep and goats. J. Vet. Diagn. Invest. 20(3):253-265.

Vacheyrou, M., A. C. Normand, P. Guyot, C. Cassagne, R. Piarroux, and Y. Bouton. 2011. Cultivable microbial communities in raw cow milk and potential transfers from stables of sixteen French farms. Int. J. Food Microbiol. 146(3):253-262.

Vaishampayan, P., A. Probst, S. Krishnamurthi, S. Ghosh, S. Osman, A. McDowall, A. Ruckmani, S. Mayilraj, and K. Venkateswaran. 2010. *Bacillus horneckiae* sp. nov., isolated from a spacecraft-assembly clean room. Int. J. Syst. Evol. Microbiol. 60(5):1031-1037.

Valerio, F., P. De Bellis, M. Di Biase, S. L. Lonigro, B. Giussani, A. Visconti, P. Lavermicocca, and A. Sisto. 2012. Diversity of spore-forming bacteria and identification of *Bacillus amyloliquefaciens* as a species frequently associated with the ropy spoilage of bread. Int. J. Food Microbiol. 156(3):278-285.

Van Den Bogart, H. G. G., G. Van Den Ende, P. C. C. Van Loon, and L. J. L. D. Van Griensven. 1993. Mushroom worker's lung: Serologic reactions to thermophilic actinomycetes present in the air of compost tunnels. Mycopathologia 122(1):21-28.

Van Eenige, M. J., G. H. Counotte, and J. P. Noordhuizen. 2013. Drinking water for dairy cattle: always a benefit or a microbiological risk? - https://www.academia.edu/es/33946342/Drinking_water_for_dairy_cattle_always_a_benefit_or_a_microbiological_risk. Tijdschr Diergeneeskd 138(2):86-97, 99.

Van Tassell, J. A., N. H. Martin, S. C. Murphy, M. Wiedmann, K. J. Boor, and R. A. Ivy. 2012. Evaluation of various selective media for the detection of *Pseudomonas* species in pasteurized milk. J. Dairy Sci. 95(3):1568-1574.

Van Waasbergen, L. G., D. L. Balkwill, F. H. Crocker, B. N. Bjornstad, and R. V. Miller. 2000. Genetic diversity among *Arthrobacter* species collected across a heterogeneous series of terrestrial deep-subsurface sediments as determined on the basis of 16s rrna and *recA* gene sequences. Appl. Environ. Microbiol. 66(8):3454-3463.

VanderKelen, J. J., R. D. Mitchell, A. Laubscher, M. W. Black, A. L. Goodman, A. K. Montana, A. M. Dekhtyar, R. Jimenez-Flores, and C. L. Kitts. 2016. Short communication: Typing and tracking *Bacillaceae* in raw milk and milk powder using pyroprinting. J. Dairy Sci. 99(1):146-151.

Vangay, P., E. B. Fugett, Q. Sun, and M. Wiedmann. 2013. Food microbe tracker: a web-based tool for storage and comparison of food-associated microbes. J. Food Prot. 76(2):283-294.

Venkateswaran, K., M. Kempf, F. Chen, M. Satomi, W. Nicholson, and R. Kern. 2003. *Bacillus nealsonii* sp. nov., isolated from a spacecraft-assembly facility, whose spores are gamma-radiation resistant. Int. J. Syst. Evol. Microbiol. 53(Pt 1):165-172.

Verdier-Metz, I., V. Michel, C. Delbès, and M.-C. Montel. 2009. Do milking practices influence the bacterial diversity of raw milk? Food Microbiol. 26(3):305-310.

Verhegghe, M., J. De Block, S. Van Weyenberg, L. Herman, M. Heyndrickx, and E. Van Coillie. 2019. Effect of a pre-milking teat foam and a liner disinfectant on the presence of mesophilic and (proteolytic) psychrotrophic bacteria prior to milking. J. Dairy Res. 86(4):432-435.

Versalovic, J., F. J. De Bruijn, and J. R. Lupski. 1998. Repetitive sequence-based PCR (rep-PCR) DNA fingerprinting of bacterial genomes. Pages 437-454 in Bacterial Genomes. Springer US.

Versalovic, J., T. Koeuth, and J. R. Lupski. 1991. Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. Nucleic Acids Res. 19(24):6823-6831.

Vidal, A. M. C., A. Saran Netto, A. C. N. Vaz, E. Capodifóglio, A. C. S. Gonçalves, G. A. M. Rossi, A. S. Figueiredo, and V. L. A. Ruiz. 2017. *Pseudomonas* spp.: contamination sources in bulk tanks of dairy farms. Pesqui. Vet. Bras. 37(9):941-948.

Vilar, M. J., J. L. Rodríguez-Otero, M. L. Sanjuán, F. J. Diéguez, M. Varela, and E. Yus. 2012. Implementation of HACCP to control the influence of milking equipment and cooling tank on the milk quality. Trends Food Sci. Technol. 23(1):4-12.

Vissers, M. M., F. Driehuis, M. C. Te Giffel, P. De Jong, and J. M. Lankveld. 2007a. Concentrations of butyric acid bacteria spores in silage and relationships with aerobic deterioration. J. Dairy Sci. 90:928–936.

Vissers, M. M., F. Driehuis, M. C. Te Giffel, P. De Jong, and J. M. Lankveld. 2007b. Minimizing the level of butyric acid bacteria spores in farm tank milk. J. Dairy Sci. 90(7):3278-3285.

Vissers, M. M., F. Driehuis, M. C. Te Giffel, P. De Jong, and J. M. Lankveld. 2007c. Short communication: Quantification of the transmission of microorganisms to milk via dirt attached to the exterior of teats. J. Dairy Sci. 90(8):3579-3582.

Vissers, M. M., M. C. Te Giffel, F. Driehuis, P. De Jong, and J. M. Lankveld. 2007d. Minimizing the level of *Bacillus cereus* spores in farm tank milk. J. Dairy Sci. 90(7):3286-3293.

Vissers, M. M. M., F. Driehuis, M. C. Te Giffel, P. De Jong, and J. M. G. Lankveld. 2006. Improving farm management by modeling the contamination of farm tank milk with butyric acid bacteria. J. Dairy Sci. 89(3):850-858.

Vissers, M. M. M., M. C. Te Giffel, F. Driehuis, P. De Jong, and J. M. G. Lankveld. 2007e. Predictive modeling of *Bacillus cereus* spores in farm tank milk during grazing and housing periods. J. Dairy Sci. 90(1):281-292.

Vithanage, N. R., J. Bhongir, S. R. Jadhav, C. S. Ranadheera, E. A. Palombo, T. R. Yeager, and N. Datta. 2017. Species-level discrimination of psychrotrophic pathogenic and spoilage gram-negative raw milk isolates using a combined MALDI-TOF MS proteomics–bioinformatics-based approach. J. Proteome Res. 16(6):2188-2203.

Vojdani, A., C. Turnpaugh, and E. Vojdani. 2018. Immune reactivity against a variety of mammalian milks and plant-based milk substitutes. J. Dairy Res. 85(3):358-365.

von Neubeck, M., C. Baur, M. Krewinkel, M. Stoeckel, B. Kranz, T. Stressler, L. Fischer, J. Hinrichs, S. Scherer, and M. Wenning. 2015. Biodiversity of refrigerated raw milk microbiota and their enzymatic spoilage potential. Int. J. Food Microbiol. 211:57-65.

von Neubeck, M., C. Huptas, C. Gluck, M. Krewinkel, M. Stoeckel, T. Stressler, L. Fischer, J. Hinrichs, S. Scherer, and M. Wenning. 2016. *Pseudomonas helleri* sp. nov. and *Pseudomonas weihenstephanensis* sp. nov., isolated from raw cow's milk. Int. J. Syst. Evol. Microbiol. 66(3):1163-1173.

Voronina, E. Y., L. V. Lysak, and Y. A. Zagryadskaya. 2011. The quantity and structure of the saprotrophic bacterial complex of the mycorhizosphere and hyphosphere of symbiotrophic basidiomycetes. Biol. Bull. 38(6):622-628.

Vyssotski, M., K. Lagutin, S. Bloor, A. MacKenzie, D. Scott, M. Broadhurst, L. Day, and L. Samuelsson. 2017. Lipid profile of New Zealand sheep milk. Food New Zealand 17.

Walker, V. K., G. R. Palmer, and G. Voordouw. 2006. Freeze-thaw tolerance and clues to the winter survival of a soil community. Appl. Environ. Microbiol. 72(3):1784-1792.

Walsh, A. M., F. Crispie, K. Daari, O. O'Sullivan, J. C. Martin, C. T. Arthur, M. J. Claesson, K. P. Scott, and P. D. Cotter. 2017. Strain-level metagenomic analysis of the fermented dairy beverage nunu highlights potential food safety risks. Appl. Environ. Microbiol. 83(16):e01144-01117.

Wang, B., Y. Luo, K. H. Myung, and J. X. Liu. 2014. Effects of Storage Duration and Temperature on the Chemical Composition, Microorganism Density, and In vitro Rumen Fermentation of Wet Brewers Grains. Asian-Australasian journal of animal sciences 27(6):832-840.

Watterson, M. J., D. J. Kent, K. J. Boor, M. Wiedmann, and N. H. Martin. 2014. Evaluation of dairy powder products implicates thermophilic sporeformers as the primary organisms of interest. J. Dairy Sci. 97(4):2487-2497.

Waturangi, D. E., I. Joanito, Yogiara, and S. Thomas. 2012. Use of REP- and ERIC-PCR to reveal genetic heterogeneity of *Vibrio cholerae* from edible ice in Jakarta, Indonesia. Gut Pathog. 4(1):2.

Webb, M. D., G. C. Barker, K. E. Goodburn, and M. W. Peck. 2019. Risk presented to minimally processed chilled foods by psychrotrophic *Bacillus cereus*. Trends Food Sci. Technol. 93:94-105.

Weber, M., J. Liedtke, S. Plattes, and A. Lipski. 2019. Bacterial community composition of biofilms in milking machines of two dairy farms assessed by a combination of culture-dependent and -independent methods. PLoS One 14(9):e0222238.

Weber, S., S. Stubner, and R. Conrad. 2001. Bacterial populations colonizing and degrading rice straw in anoxic paddy soil. Appl. Environ. Microbiol. 67(3):1318-1327.

Weenk, G., J. Van den Brink, C. Struijk, and D. Mossel. 1995. Modified methods for the enumeration of spores of mesophilic *Clostridium* species in dried foods. Int. J. Food Microbiol. 27(2-3):185-200.

Wehr, H. M., J. F. Frank, and A. P. H. Association. 2004. Standard methods for the examination of dairy products. American Public Health Association Washington.

Weijtens, M. J. B. M., R. D. Reinders, H. A. P. Urlings, and J. Van der Plas. 1999. *Campylobacter* infections in fattening pigs; excretion pattern and genetic diversity. J. Appl. Microbiol. 86(1):63-70.

Weingart, H. and B. Völksch. 1997. Genetic fingerprinting of *Pseudomonas syringae* pathovars using ERIC-, REP-, and IS50-PCR. J. Phytopathol. 145(8-9):339-345.

Wells-Bennik, M. H., F. Driehuis, and S. A. van Hijum. 2016. Prospects for improved control of dairy-relevant sporeformers using-omics technologies. Curr. Opin. Food Sci. 10:38-44.

Welsh, J. and M. McClelland. 1990. Fingerprinting genomes using PCR with arbitrary primers. Nucleic Acids Res. 18(24):7213-7218.

White, K. A. J. and S. J. G. Hall. 1998. Behaviour of lambs (*Ovis aries*) in relation to spatial patterns of defecation on a pasture. J. Zool. 245(1):111-117.

Wick, R. R., L. M. Judd, C. L. Gorrie, and K. E. Holt. 2017. Unicycler: Resolving bacterial genome assemblies from short and long sequencing reads. PLoS Comput. Biol. 13(6):e1005595.

Wickham, H. 2016. ggplot2: Elegant graphics for data analysis. Springer-Verlag New York.

Wilcock, R. J., R. M. Monaghan, B. S. Thorrold, A. S. Meredith, K. Betteridge, and M. J. Duncan. 2007. Land-water interactions in five contrasting dairying catchments: issues and solutions. Land Use Wat. Resourc. Res. 7(1732-2016-140272).

Wilderman, P. J., A. I. Vasil, Z. Johnson, M. J. Wilson, H. E. Cunliffe, I. L. Lamont, and M. L. Vasil. 2001. Characterization of an endoprotease (PrpL) encoded by a PvdS-regulated gene in *Pseudomonas aeruginosa*. Infection and immunity 69(9):5385-5394.

Wilkinson, J. M., M. R. F. Lee, M. J. Rivero, and A. T. Chamberlain. 2020. Some challenges and opportunities for grazing dairy cows on temperate pastures. Grass Forage Sci. 75(1):1-17.

Williams, J. G. K., A. R. Kubelik, K. J. Livak, J. A. Rafalski, and S. V. Tingey. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res. 18(22):6531-6535.

Williams, M., C. N. Davis, D. L. Jones, E. S. Davies, P. Vasina, D. Cutress, M. T. Rose, R. A. Jones, and H. W. Williams. 2021. Lying behaviour of housed and outdoor-managed pregnant sheep. Appl. Anim. Behav. Sci. 241:105370.

Witthuhn, M., G. Lücking, Z. Atamer, M. Ehling-Schulz, and J. Hinrichs. 2011. Thermal resistance of aerobic spore formers isolated from food products. Int. J. Dairy Technol. 64(4):486-493.

Wolf, A., A. Fritze, M. Hagemann, and G. Berg. 2002. *Stenotrophomonas rhizophila* sp. nov., a novel plant-associated bacterium with antifungal properties. Int. J. Syst. Evol. Microbiol. 52(6):1937-1944.

Wolfgang, W. J., A. Coorevits, J. A. Cole, P. De Vos, M. C. Dickinson, G. E. Hannett, R. Jose, E. J. Nazarian, P. Schumann, A. Van Landschoot, S. E. Wirth, and K. A. Musser. 2012. *Sporosarcina newyorkensis* sp. nov. from clinical specimens and raw cow's milk. Int. J. Syst. Evol. Microbiol. 62(Pt 2):322-329.

Wollum, A. G. 1994. Soil sampling for microbiological analysis. Pages 1-14 in Methods of Soil Analysis.

Woods, R. G., M. Burger, C. A. Beven, and I. R. Beacham. 2001. The *aprX-lipA* operon of *Pseudomonas fluorescens* B52: a molecular analysis of metalloprotease and lipase production. Microbiology (Reading) 147(Pt 2):345-354.

Wu, T., Z. Zhang, B. Liu, D. Hou, Y. Liang, J. Zhang, and P. Shi. 2013. Gut microbiota dysbiosis and bacterial community assembly associated with cholesterol gallstones in large-scale study. BMC Genomics 14(1):669.

Wu, X. Y., M. Walker, B. Vanselow, R. L. Chao, and J. Chin. 2007. Characterization of mesophilic bacilli in faeces of feedlot cattle. J. Appl. Microbiol. 102(3):872-879.

Xiao, D., X. Yuan, M. Wang, H. He, M. L. P. Essengue Samboukel, Y. Zhang, and E. Wang. 2020. Selective enrichment of C*lostridium* spp. By nutrition control from sihe coal geological microbial communities. Applied Biochemistry and Biotechnology 192(3):952-964.

Xiaoting, W., N. Chengcheng, J. Chunhui, L. Yan, L. Jing, M. Qingling, Q. Jun, W. Lixia, C. Kuojun, Z. Jinsheng, Z. Zaichao, Y. Weiwei, P. Yelong, and C. Xuepeng. 2021. Antimicrobial resistance profiling and molecular typing of ruminant-borne isolates of *Clostridium perfringens* from Xinjiang, China. J. Glob. Antimicrob. Resist. 27:41-45.

Yadav, A. N., S. G. Sachan, P. Verma, R. Kaushik, and A. K. Saxena. 2016. Cold active hydrolytic enzymes production by psychrotrophic bacilli isolated from three sub-glacial lakes of NW Indian Himalayas. J. Basic Microbiol. 56(3):294-307.

Yasawong, M., S. Areekit, A. Pakpitchareon, S. Santiwatanakul, and K. Chansiri. 2011. Characterization of Thermophilic Halotolerant *Aeribacillus pallidus* TD1 from Tao Dam Hot Spring, Thailand. Int. J. Mol. Sci. 12(8):5294-5303.

You, L., C. Yang, H. Jin, L.-Y. Kwok, Z. Sun, and H. Zhang. 2022. Metagenomic features of traditional fermented milk products. LWT 155:112945.

Yuan, D.-D., G.-C. Liu, D.-Y. Ren, D. Zhang, L. Zhao, C.-P. Kan, Y.-Z. Yang, W. Ma, Y. Li, and L.-B. Zhang. 2012. A survey on occurrence of thermophilic bacilli in commercial milk powders in China. Food Control 25(2):752-757.

Yuan, L., M. Burmølle, F. A. Sadiq, N. Wang, and G. He. 2018a. Interspecies variation in biofilm-forming capacity of psychrotrophic bacterial isolates from Chinese raw milk. Food Control 91:47-57.

Yuan, L., F. A. Sadiq, T. Liu, S. Flint, J. Chen, H. Yang, J. Gu, G. Zhang, and G. He. 2017. Psychrotrophic bacterial populations in Chinese raw dairy milk. LWT 84:409-418.

Yuan, L., F. A. Sadiq, T. J. Liu, Y. Li, J. S. Gu, H. Y. Yang, and G. Q. He. 2018b. Spoilage potential of psychrotrophic bacteria isolated from raw milk and the thermo-stability of their enzymes. J. Zhejiang Univ. Sci. B 19(8):630-642.

Yukimura, K., R. Nakai, S. Kohshima, J. Uetake, H. Kanda, and T. Naganuma. 2009. Spore-forming halophilic bacteria isolated from Arctic terrains: Implications for long-range transportation of microorganisms. Polar Sci. 3(3):163-169.

Yunitsyna, O., I. Sinelnikov, O. Kisil, K. Bolotova, A. Aksenov, and G. Simonsen. 2019. Isolation of thermophilic enzyme-producing *Parageobacillus* bacteria from chipped woody waste. BioResources 14(1):14.

Zabaleta, L., M. Albisu, and L. J. R. Barron. 2017. Volatile compounds associated with desirable flavour and off-flavour generation in ewe´s raw milk commercial cheeses. Eur. Food Res. Technol. 243(8):1405-1414.

Zain, S. N. M., S. H. Flint, R. Bennett, and H.-S. Tay. 2015. Characterisation and biofilm screening of the predominant bacteria isolated from whey protein concentrate 80. Dairy Sci. Technol. 96(3):285-295.

Zarei, M., H. Mohammadpour, D. Gharibi, and M. Pourmahdi Borujeni. 2020. Identification of *Pseudomonas jessenii* and *Pseudomonas gessardii* as the most proteolytic *Pseudomonas* isolates in

Iranian raw milk and their impact on stability of sterilized milk during storage. J. Dairy Res. 87(3):368-374.

Zarei, O., L. Shokoohizadeh, H. Hossainpour, and M. Y. Alikhani. 2018. Molecular analysis of *Pseudomonas aeruginosa* isolated from clinical, environmental and cockroach sources by ERIC-PCR. BMC Research Notes 11(1).

Zecconi, A., J. Hamanno, V. Bronzo, P. Moroni, G. Giovannini, and R. Piccinini. 2002. Relationship between teat tissue immune defences and intramammary infections. Pages 287-293 in Biology of the Mammary Gland. J. A. Mol and R. A. Clegg, ed. Springer US, Boston, MA.

Zhang, C., E. Bijl, B. Svensson, and K. Hettinga. 2019. The extracellular protease AprX from *Pseudomonas* and its spoilage potential for UHT milk: A review. Compr. Rev. Food Sci. Food Saf. 18(4):834-852.

Zhang, D. 2020. The effect of psychrotrophic bacteria on the quality of UHT milk : a thesis presented in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Food Microbiology, The School of Food and Advanced Technology, Massey University, Palmerston North, New Zealand - https://mro.massey.ac.nz/bitstream/handle/10179/15827/ZhangPhDThesis.pdf?sequence=1&isAllowed=y.

Zhang, G., X. Fang, G. Feng, Y. Li, and Y. Zhang. 2020. Silage fermentation, bacterial community, and aerobic stability of total mixed ration containing wet corn gluten feed and corn stover prepared with different additives. Animals 10(10):1775.

Zhao, Y., M. P. M. Caspers, K. I. Metselaar, P. De Boer, G. Roeselers, R. Moezelaar, M. Nierop Groot, R. C. Montijn, T. Abee, and R. Kort. 2013. Abiotic and microbiotic factors controlling biofilm formation by thermophilic sporeformers. Appl. Environ. Microbiol. 79(18):5652-5660.

Zheng, M., D. Niu, D. Jiang, R. Li, L. Meng, and C. Xu. 2020. Metagenome analyses reveal the role of *Clostridium perfringens* in alfalfa silage anaerobic deterioration. FEMS Microbiol. Lett. 367(8).

Zheng, M., D. Niu, S. Zuo, P. Mao, L. Meng, and C. Xu. 2018. The effect of cultivar, wilting and storage period on fermentation and the clostridial community of alfalfa silage. Ital. J. Anim. Sci. 17(2):336-346.

Zheng, M. L., D. Z. Niu, D. Jiang, S. S. Zuo, and C. C. Xu. 2017. Dynamics of microbial community during ensiling direct-cut alfalfa with and without LAB inoculant and sugar. J. Appl. Microbiol. 122(6):1456-1470.

Zhou, Y., W. Wei, Q. Che, Y. Xu, X. Wang, X. Huang, and R. Lai. 2008. *Bacillus pallidus* sp. nov., isolated from forest soil. Int. J. Syst. Evol. Microbiol. 58(12):2850-2854.

Zhuang, S., Y. Tan, H. Hong, D. Li, L. Zhang, and Y. Luo. 2022. Exploration of the roles of spoilage bacteria in degrading grass carp proteins during chilled storage: A combined metagenomic and metabolomic approach. Food Research International 152:110926.

Ziyaina, M., B. N. Govindan, B. Rasco, T. Coffey, and S. S. Sablani. 2018. Monitoring shelf life of pasteurized whole milk under refrigerated storage conditions: Predictive models for quality loss. J. Food Sci. 83(2):409-418.

Zweifel, C., J. E. Muehlherr, M. Ring, and R. Stephan. 2005. Influence of different factors in milk production on standard plate count of raw small ruminant's bulk-tank milk in Switzerland. Small Rumin. Res. 58(1):63-70.