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Analysis of dairy cattle feed as source of heat resistant bacterial spores in milk and evaluation of contamination consequences for milk quality.

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

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at

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Declaration

I hereby declare that the work presented in this thesis has not been submitted for any other degree or professional qualification, and that it is the result of my own independent work.

11.07.2021

Date

Abstract, highlights and limitations

Abstract

Spore-forming bacteria are resistant to heat treatments designed to control the growth of other bacteria and can impact the quality of milk. Bacterial spores in milk are hypothesised to originate from cattle feed, among other sources. In recent decades, supplementary feed usage and variability of used feed types has increased in New Zealand. To ensure low numbers of bacterial spores in dairy products, the number of spores in common New Zealand dairy cattle feed and milk were observed at psychotropic, mesophilic and thermophilic temperatures under aerobic and anaerobic conditions and the bacterial spore species found were identified. Differences were detected in the number of bacterial spores and variety of species present, between sample types. Tuber feed and palm kernel expeller (PKE) contained the greatest quantity of spores as well as the greatest diversity of bacterial spore species, while milk contained the lowest quantity and diversity. This supports a hypothesis that some cattle feeds are more likely to be a source of milk contamination than others. Almost all (96 %) of bacterial spore species found in milk were also found in cattle feed, indicating that spores may transfer from feed to milk.

To provide more definitive evidence of the transfer of bacterial spores from cattle feed to milk, the genomes of 109 bacterial isolates from milk and feed were sequenced and compared. Clusters of similar genomes were found between milk and feed isolates, however within the set criteria of the study, no match was found. A greater genomic diversity was observed in cattle feed compared to milk. A lower genomic diversity was observed among *Bacillus licheniformis* and *B. pumilus* isolates compared to isolates from *B. cereus, B. mycoides or B. thermoamylovorans*.

Not all spore-forming bacteria isolates cause issues in milk production therefore the potential of bacterial spores isolated from raw milk, to damage milk was determined. A

set of 20 bacterial spore isolates were screened for their ability to grow in milk, form biofilms, produce milk damaging enzymes and resist heat treatment. All isolates grew in milk, almost all had some ability to produce biofilm on stainless steel and produce milk degrading enzymes. Differences in biofilm production and heat resistance of spores were found between the isolates. Out of all isolates, two *B. licheniformis* isolates were found to possess the greatest potential to damage milk and whole genome data was used to investigate the genes responsible for differences in the milk damaging abilities. A set of gene alleles was identified which might affect the potential of an isolate to influence milk quality.

Highlights

- Determining the bacterial spore diversity in current New Zealand cattle feed and milk
- The potential of comparative genomics to trace bacterial spores between cattle feed and milk
- Characterisation of the milk damaging potential of common bacterial spore isolates from milk
- Identification of spore-forming bacteria genes responsible for a high milk damaging potential in isolates.

Limitations

- Only the most popular feeds were analysed.
- Culture dependant methods were used for most of the work.
- Tracking bacterial spores from feed to milk involved multiple species across multiple farms rather than a more intensive study of a single spore species on a single farm.
- Milk damaging potential was assessed for only of subset of the most common isolates of bacterial spores found in milk.

Conference presentations associated with this research

David Flossdorf, Steve Flint, Jon Palmer, Paul Jamieson, Denise Lindsay & Nicholas Sneddon; Spore-forming bacterial populations in New Zealand dairy feeds. Oral presentation. New Zealand Microbiological Society Conference (NZMS 2019), Palmerston north, 11.2019

David Flossdorf, Steve Flint, Jon Palmer, Paul Jamieson, Denise Lindsay & Nicholas Sneddon; Spore-forming bacterial populations in New Zealand dairy feeds. Poster presentation. Australian Society for Microbiology Conference (ASM 2019), Adelaide 07.2019

David Flossdorf, Steve Flint, Jon Palmer, Paul Jamieson, Denise Lindsay & Nicholas Sneddon; Spore-forming bacterial populations in New Zealand dairy feeds. Oral presentation. New Zealand Microbiological Society Conference (NZMS 2018), Dunedin 11.2018.

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Chapter 1: General introduction

1.1 Background and importance

Spore-forming bacteria in cow milk are potential spoilage microorganisms, which can lead to product loss (Lucking et al., 2013). Previous studies have indicated that the bacterial spores in raw milk can originate from what is fed to the lactating herd (Magnusson et al., 2007, Vissers et al., 2007a). However, few studies have extensively examined the bacterial spore populations in different dairy cattle feeds. With recent increases in the use of new supplementary feeds, such examinations are now increasingly important to ensure good milk quality (MPI, 2017). Isolating and identifying the bacterial spore populations of cattle feed and corresponding milk samples, could help predict which spores can potentially be transferred into milk This knowledge could allow for informed adjustments of dairy cow diet, to lower bacterial spore counts in milk when needed. So far evidence for the transfer of bacterial spores from feed to milk is mostly based on the identification of the same species in cattle feed and milk using traditional identification methods whilst evidence based on molecular biology is rare (Magnusson et al., 2007, Vissers et al., 2007b, Martin et al., 2019).

Not all bacterial spore isolates are equally damaging to milk quality, even if they belong to the same species (Huang et al., 2021). An isolate which can grow in milk, form a strong biofilm within a production plant quickly, survive heat treatment and secrete milk damaging enzymes will potentially affect milk quality more than an isolate lacking such abilities (Gopal et al., 2015). Identification of bacterial genotypes that are particularly undesirable in milk, can allow risk mitigation for final product (Porcellato et al., 2021, Mehta et al., 2019).

In this study, the bacterial spore population in common New Zealand cattle feeds as well as raw milk were analysed. Isolates were analysed for evidence of transfer from cattle feed to milk using whole genome sequencing. A selection of isolates were further

analysed for their ability to cause damage to milk during processing and gene alleles related to milk damage were analysed. This knowledge can help to categorise contaminating bacterial spores according to their milk damaging potential, supporting decision making and cleaning in dairy product manufacture.

1.2 Research questions

- What bacterial spores are present in New Zealand’s dairy cattle feeds and milk?
- What bacterial spores are transferred between cattle feed and raw milk?
- What impact do those spore-forming bacteria have on milk quality?

1.3 Hypotheses

- Different feeds will have different spore-forming populations, which will be present in the corresponding raw milk.
- Cattle feed influences the bacterial spore populations in the raw milk.
- The bacterial spores differ in their ability to impact milk quality.

1.4 Objectives

To understand the bacterial spore populations associated with cattle feeds and corresponding raw milk in the New Zealand context, and to investigate their transmission as well as their milk damaging potential to provide recommendations of farming practices to improve the quality of raw milk in terms of bacterial spore content.

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Chapter 2: Literature review

2.1 Introduction

Worldwide milk production reached 906 million tonnes in 2020 (FAO, 2021). Around 22 million of these are produced in New Zealand, making it the 9th largest producer in the world (behind India, the USA, Pakistan, China, Germany, Brazil, Russia and France) (FAO, 2021). The dairy sector makes up around $7.8 billion (3.5%) to New Zealand's total GDP (NZIER, 2017). To ensure the exported dairy products are of good quality, it is important to minimise microbial spoilage.

Raw milk contains few microorganisms, however, it is a rich medium for microbial growth due to its fat, protein and carbohydrate content (Jenness, 1988). This can lead to rapid spoilage of the milk if no effort is made to limit microbial growth (Ledenbach and Marshall, 2009). Bacterial spores can be of concern, as they can survive hurdle technologies like pasteurisation, reducing the quality of final products (Li et al., 2019). To reduce the risk of food waste and financial loss due to spore-forming bacteria, it is important to know their identity and origin. One potential source of these microorganisms could be cattle feeds, the subject of this study.

2.2 Bacterial spore populations in milk and their link to milk quality issues

2.2.1 Bacterial spore's importance to milk quality

Spore-forming bacteria are common in a wide range of habitats including milk and milk products (Doyle et al., 2015, Nicholson, 2002). Once spore-forming bacteria enter the product, they are challenging to remove since spores are highly resistant to extreme temperatures and other environmental factors like nutrient limitation, pressure or radiation (Gopal et al., 2015). The bacterial spores survive through processing and can

be found in dairy products like ultrahigh temperature treatment (UHT) milk, cheese or milk powder (Kmiha et al., 2017, Oliveira et al., 2016, Burgess et al., 2014). They gain their resistance through the process of sporulation, which transforms vegetative cells into spores (Hutchison et al., 2014). This process includes the reduction of water content and the development of protective structures like an exosporium, a coat and/or a cortex and the protection of the DNA through small acid-soluble protein (Setlow, 2006). The quantity of heat resistant bacteria in raw milk is important for downstream applications, leading to some food processors to have incentives which, reward farmers who produce (among other factors) milk with low counts of heat resistant bacteria (Murphy et al., 2016).

In the dairy industry, several spore species can be found to survive standard pasteurisation at 72.5 °C for 15 seconds and in certain circumstances even harsher heat treatments like UHT at 140 °C for 5 seconds, making them a concern for milk quality and shelf life (Scheldeman et al., 2006). The issue with spore-forming bacteria in milk processing is not just their survival but their ability to germinate post-pasteurisation. Once in the milk they can produce heat stable lipases or proteinases, as well as acids which alter milk quality (Chen et al., 2004, Kalogridou-Vassiliadou, 1992). Production of such spoilage enzymes can cause downstream quality degradation as these enzymes survive UHT even if all bacteria are killed (Zhang et al., 2020). Biofilm formation is another issue caused in the processing equipment by spore-forming bacteria, requiring more frequent cleaning and even temporary shutdown of the affected parts of manufacturing plants (Burgess et al., 2010, Flint et al., 1997). Biofilms are formed by cells which adhere to surfaces or other cells and start producing an extracellular matrix to protect the cells from external influences (Teh et al., 2014). These biofilms can act as a reservoir from which milk spoiling enzymes are secreted, and spores spread biofilms throughout a production plant, causing product and plant damage (Gupta and Anand, 2018, Teh et al., 2014, Marchand et al., 2012).

2.2.2 Bacterial spore population in milk

Spore-forming bacteria can be detected in the farm environment and in many stages of milk production or processing (Figure 2-1) (Oliveira et al., 2016). Different bacterial spore species can contaminate raw milk as they can originate from the cow's teat canal, skin, faeces, soil, bedding, feed and milking equipment (Figure 2-1) (Martin et al., 2019, Miller et al., 2015a, Vacheyrou et al., 2011, Wu et al., 2007, Gill et al., 2006, McKinnon et al., 1990). This leads to a wide variety of bacteria found in the raw milk at the bulk tank storage point, which differ depending on external factors like season, region or farm management practises (Feligini et al., 2015, Miller et al., 2015a, Giannino et al., 2009, Heck et al., 2009). The large number of potential factors which influence the bacterial spore population of milk leads to contradictory reports stating spores increase in summer while others report a decrease in summer (Kmiha et al., 2017, Kable et al., 2016, Buehner et al., 2014, Vissers et al., 2007a). The bacterial composition of the milk is further affected by transport or storage conditions, as well as processing steps, such as pasteurisation, or the manufacture of products such as milk powder (Kable et al., 2016, Miller et al., 2015b).

The exact species and number of spore-forming bacteria in milk in the literature is inconsistent due to different studies using different temperatures and times to select for spores (Kent et al., 2016). This makes the accumulation of knowledge on spores in milk complicated. However, culture dependent analysis has indicated that *Bacillus* and to a lesser extent *Paenibacillus* species, are the predominant spore-forming bacteria genus in raw milk with *B. circulans, B. licheniformis, B. pumilus, B. subtilis, B. weihenstephanensis* and *B. cereus* being some of the most common species (Masiello et al., 2014, Coorevits et al., 2008).

Bacterial growth in milk is controlled during storage, transport and processing by maintaining temperatures below 10 °C, which can lead to psychrotrophic bacteria making up a large portion of the milk microbiome (Zhang et al., 2019, Moyer et al., 2017). Psychrotrophic spore-forming bacteria are of concern since they survive many hurdle

technologies and proliferate in under refrigeration, making them a threat to milk quality even in low numbers, resulting in dairy product spoilage (Ledenbach and Marshall, 2009).

Common examples of psychrotrophic bacterial spore-formers that can be found in milk after pasteurisation are *B. cereus*, *B. coagulans, B. mycoides, B. licheniformis* or *B. subtilis* (Hanson et al., 2005, Matta and Punj, 1999, Meer et al., 1991). The issue of psychrotrophic spore-forming bacteria increases when they form biofilms in milk processing plants or milk tankers. The removal of these biofilms cannot always be accomplished by standard cleaning in place (CIP) procedures, as Bremer et al. (2006) showed for spore-forming bacilli and other Gram-positive bacteria.

A prominent psychrotrophic spore-forming bacterium which is often associated with milk spoilage is *B. cereus*, commonly found in milk and farm environments like soil, bedding, feeds and cow faeces (Vissers et al., 2007a). *Bacillus cereus* includes psychrotrophic and mesophilic strains able to produce proteases, lipases and lactase during various stages of milk processing (Porcellato et al., 2021, Mugadza and Buys, 2017, Lucking et al., 2013, Stone and Rowlands, 1952). Besides spoilage, *B. cereus* can pose a public health risk since it produces enterotoxins (Huang et al., 2021, Porcellato et al., 2021). While *B. cereus* is the most well-known pathogenic spore-former in milk, other potential toxin producers in milk, include *B. licheniformis*, *B. pseudomycoides* or *B. thuringiensis* as well as *Clostridium* species like *C. botulinum* or *C. perfringens* (Miller et al., 2018, Doyle et al., 2015, Nieminen et al., 2007). Bacteria which survive processing can cause disease outbreaks even in developed countries like the United States of America where between 1993 and 2006, 43 disease outbreaks were caused by milk or milk products which went through some kind of heat treatment prior to consumption (Langer et al., 2012). However, good sanitary standards, quality feed, rapid cooling of the raw milk and pasteurisation can limit the number of pathogens in raw milk and enable the safe consumption of pasteurised dairy products (Hill et al., 2012, Soomro et al., 2002, Sanaa et al., 1993, D’Aoust et al., 1988).

Bacillus thermoamylovorans, gained attention after it was increasingly found in milk and farm environments and was involved in dairy product spoilage (Flint et al., 2017, Scheldeman et al., 2005). The spores can be highly heat resistant, with the potential to survive UHT processing and it produces lipases and beta-galactosidases, which is why it is often associated with food spoilage (Berendsen et al., 2015, Lucking et al., 2013, Coorevits et al., 2011). *Bacillus thermoamylovorans* is capable of producing acids out of lactose or glycerol, as well as toxins, making it undesirable within milk products (Ryu et al., 2021, Coorevits et al., 2011).

In New Zealand, previous studies of the bacteria in raw milk were mostly focused on pathogenic bacterial or spores in milk powder but few studies on the bacterial spore composition in raw milk (Marshall et al., 2016, Burgess et al., 2014, Hill et al., 2012, Hill and Smythe, 2012, Scott et al., 2007). There have however been changes in cattle feeding practices in New Zealand in the last 20 years, as seen by a two and three fold increase in imported and harvested supplementary respectively, which could affect dairy product quality, if current trends continue (MPI, 2017, Hill and Smythe, 2012).

2.3 Farm management and its effect on the spore-forming bacteria in raw milk

2.3.1 Potential effects of New Zealand herd size, housing strategies and organic farming on the spore-forming bacteria in raw milk

Differences in geographical location and management of farms can result in different bacterial compositions of the raw milk (Feligini et al., 2015). The effect that New Zealand's location and farm management practices have on the spore-forming microorganisms in milk has not been thoroughly explored. There are several New Zealand specific management practices which may be associated with a change in bacterial spore levels. For example, New Zealand has a higher average herd size per farm (419 cows per farm 2015/16) than other large milk producers like the US (214 cows

per farm 2015) or the UK (142 cows per farm 2015), which was shown to be associated with a lower number of psychrotolerant, mesophilic and thermophilic spore-formers, in the USA (Barkema et al., 2015, Miller et al., 2015a, Masiello et al., 2014). However, this has yet to be determined as true for New Zealand's dairy industry. The connection between larger herd sizes and lower spore counts in the raw milk was also reported by Miller et al. (2015a), who also indicated that the bedding material influences the mesophilic or thermophilic spore counts. Different bedding material was reported as a reservoir for different quantities and species of spore-forming bacteria, potentially transferring spores from faeces to the udder skin (Huck et al., 2008). The contaminated bedding material is not just a potential vector but also a medium in which bacteria can proliferate, depending on the chosen bedding material and its maintenance (Magnusson et al., 2007, Zdanowicz et al., 2004). However, Bradley et al. (2018) showed that even though sand, sawdust and recycled manure solids bedding differ in their thermophilic spore and *Bacillus cereus* count, they do not seem to influence the level of those in the raw milk. A relationship between *B. cereus* spore concentrations in bedding and raw milk could not be established by Vissers et al. (2007a) either, leaving bedding materials potential to impact spore levels in milk unsettled. In New Zealand however, the use of cow barns and bedding material is uncommon contrary to most other big milk producing countries, and cows are generally managed solely on pasture during lactation (Holmes et al., 2002). The influence of pasture as bedding material on the bacteria in the milk is largely unknown. However, a study in France showed, that a freestall barn can lead to lower microbial contamination in the milk compared to a stanchion barn, concluding that greater physical separation of living space and milking parlour leads to lower spore counts in milk (Vacheyrou et al., 2011). This could indicate even lower contamination levels for cows managed solely on pasture. It may be possible that pasture bedding further results in lower chance of contact between faecal matter and the udder skin. This could result in udder skin exposure to pasture which contains between 10^3 to 10^4 CFU/g of mesophilic spores while faecal matter contains between 10^4 to 10^6 CFU/ml, but this

claim requires further investigation (Gupta and Brightwell, 2017, Stout, 1960). Contrary to that point, there is some evidence that the spore population in pasture has a higher percentage of psychrotrophic spores, likely due to their abundance in soil, compared to other bedding material (McKinnon and Pettipher, 1983). It was further shown that housing of cows correlates with reduced psychrotrophic spore contamination of raw milk, though the total amount of spores per gram was lower in pasture than bedding material (Slaghuis et al., 1997, McKinnon and Pettipher, 1983).

Organic farming can affect the bacterial spore levels in raw milk due to slightly decreased levels of thermotolerant spores compared with conventional farms (Coorevits et al., 2008). This was concluded to potentially arise from the feeding of larger quantities of feed concentrates, whose spore content is on average made up of 20 % thermotolerant spores (Vaerewijck et al., 2001). Europe has experienced an increase in organic farming systems with an increase in the area under organic cultivation of 21.1 % between 2010 to 2015 (DG-AGRI, 2016). This trend does not seem to extend to New Zealand, where the area under organic cultivation decreased by about 55 % between 2012 and 2016 (OANZ, 2016).

2.3.2 Potential effects of New Zealand dairy farming season and feed management on the spore-forming bacteria in raw milk

The growth rate of grass in different seasons incentivises New Zealand dairy farmers to use a seasonal spring-calving based production system to take advantage of the faster growth of pasture in spring and summer, in which cows are inseminated to give birth between early July to late August (LIC and DairyNZ, 2016, Holmes et al., 2002). In contrast to that, many countries in the northern hemisphere including European countries and the USA have a year round calving system (Cooley, 2017, AHDB, 2016). In New Zealand, during and after calving in spring, the cows are rotationally grazed on different paddocks (around 20-30 days to complete a grazing of each paddock on the

farm) and while pasture production is limited supplementary feeding can be used (DairyNZ, 2017). In summer, a greater pasture growth rates enables pasture-based farming with longer rotation lengths (30-40 days), where supplementary feed is scarcely used to even in dry periods in late summer (DairyNZ, 2015, Chapman et al., 2009, Holmes et al., 2002). In autumn, cows are dried off and in winter, cows are again rotated on paddocks due to a lower growth rate of pasture with more supplementary feeds being used and very long rotation lengths of up to 120 days (DairyNZ, 2017). These farm management practices result in a lower milk production in litres per cow (4,185 litres per year per cow 2015/16) compared to the UK (7,942 litres per year per cow 2015/16) or the USA (10,020 litres per year per cow 2016) (Cooley, 2017, AHDB, 2016, LIC and DairyNZ, 2016, Holmes et al., 2002). The consequences of these different farm management practises and milk yields on the bacterial spore counts in milk are largely unknown, however changes in feed usage can affect the bacterial spores in milk (Magnusson et al., 2007).

New Zealand's farm management differs from other big milk producing countries in cow feed management (Holmes et al., 2002). Pasture comprises over 80 % of the cow's dry matter intake with the majority of the remainder being pasture silage (MPI, 2017). This differs from other large milk producers, who usually use a greater percentage of concentrated feeds (USA or Russia with up to 40 % concentrates) or silages (France or Netherlands with more than 40 % from silage) in the cow's diet (FAO, 2014). A largely pasture-based diet can affect the microbiome of milk, as shown by Hagi et al. (2010) for cows which changed from inside feeding to an outside grazed diet. The change in feeding caused a change in the milk microbiota from predominantly *Lactobacillus* species to a microbiota that contained a large abundance of *Staphylococcus* species (Hagi et al., 2010). The diversity of bacterial spores present on New Zealand farms was reported by Gupta and Brightwell (2017). This study looked at the spore-forming bacteria in effluent on New Zealand's dairy farms and found 19 *Bacillus* spp., five *Paenibacillus* spp. and 17 *Clostridium* species. However they did not take into consideration, if the spores make it

into the milk. Further, New Zealand's farm management systems, like every other one worldwide, are subject to constant change. In recent years, an increased usage of non-pasture feeds has seen this fraction of the diet more than quadruple between 1991 (4.2 % of the diet) and 2015 (18 % of the diet), with an especially strong increase in the use of supplementary feed from 1.2 % to 8 % over the same period (MPI, 2017). The supplementary feed used in 2015 was largely (84 %) made up of palm kernel expeller (PKE) (36 %), maize silage (31 %) and fodder beet (17 %) (MPI, 2017). The microbiota of some of the increasingly used feeds is mostly unknown and thereby their potential effect on the raw milk microbiome is also unknown.

2.4 Methods to examine spore-forming bacteria from raw milk or cow feeds

2.4.1 Methods for the selection of bacterial spores

Bacterial spores were first discovered in a dairy product, by Ferdinand Cohn after he boiled cheese and recognised that it was still spoiling (Cohn, 1877). Even though technology has advanced in the past 140 years, the spoilage of dairy products despite heat treatment, due to bacterial spores, is still relevant (Lucking et al., 2013). Over the years however, Cohn's method of boiling to eliminate vegetative cells, was not developed into universally standardised methods in the literature (Kable et al., 2016). Temperatures used for the heat treatment, range from 72 °C to 106 °C for times of 5 to 30 min, followed by incubation at 30 °C, 37 °C or 55 °C on a variety of agar plates (Watterson et al., 2014, Vaerewijck et al., 2001, Christiansson et al., 1999). This leads to different assessments of spore content and can make the comparison of spore populations between publications challenging (Kent et al., 2016). There is some common ground when it comes to testing in the dairy industry. A standard method for dairy products is heat treatment of 80 °C for 12 min to eliminate all vegetative cells and selects for a general spore count according to "Standard Methods for the Examination of Dairy Products"

(Wehr and Frank, 2004). A heat treatment at 100 °C for 30 min will select for highly thermo-resistant spores and 106 °for 30 min to select for especially heat resistant spores (McHugh et al., 2017). As an alternative to the use of heat to select for spores, resistance to ethanol or pressure have also been used (Moerman, 2005, Koransky et al., 1978).

2.4.2 Culturing methods for the detection of bacterial spores

Once the spores are selected, a common detection method is incubation on agar plates that enables enumeration and the isolation of spore-forming bacteria for further studies (McHugh et al., 2017). This method can only enumerate bacteria that are capable of grow under the chosen incubation conditions but the advantage of this method is that culturable strains of the bacteria can be isolated and used for further testing (Stefani et al., 2015).

The agar on which bacteria are grown is usually chosen depending on the origin environment of the bacteria and the expected species. Some agars, like “Brain Heart Infusion Agar” (BHI) or “Plate Count Milk Agar” (MPCA), are designed to grow a wide variety of spore-forming microorganisms from an analysed environment (Coorevits et al., 2008, Giffel et al., 2002). Examples of agars for selecting specific spore-forming bacteria are “mannitol egg yolk polymyxin agar” for *B. cereus* or “Differential Reinforced Clostridial agar” (DRCA) for *Clostridium* species (Vissers et al., 2007a, Vissers et al., 2007b, Gibbs and Freame, 1965).

The incubation temperatures vary depending on the groups of spore-formers to be selected. Agar plates are incubated at 6 °C for the growth of psychrotrophic bacteria, 30 – 35 °C for mesophilic bacteria and 55 °C for thermophilic bacteria (McHugh et al., 2017, Watterson et al., 2014).The availability of oxygen is important. Anaerobic or aerobic incubation will often lead to very different microorganisms growing up on otherwise identically treated agar plates (Sizova et al., 2012). If a culture dependent method is to

be used for spore detection, the correct method needs to be chosen for the study to ensure optimal coverage of abundant diversity (Lagier et al., 2015).

Once isolated, bacterial identification can be carried out using specific culture, DNA based methods like sequencing or using matrix-assisted laser desorption/ionization time of flight mass spectrometer biotyper (MALDI-TOF) (Strejcek et al., 2018, Braga et al., 2013). MALDI-TOF finds wide usage in food assurance or medical applications due to its fast processing, low cost and easy implementation into routine workflows (Pavlovic et al., 2013, Caliendo et al., 2013). To identify a bacterial isolate using MALDI-TOF in brief, pure biological material or a protein extract of the isolate is spotted onto a metal target and co-crystallised with a matrix solution (Clark et al., 2013). After drying, the analyte is laser irradiated resulting in the evaporation and ionisation of parts of the analyte which are analysed by a mass spectrometer which reports a spectrum of mass-to-charge ratios for the analyte (Clark et al., 2013). The analytes spectra can then be compared to a reference database to identify the bacterium (Clark et al., 2013).

2.4.3 Culture independent methods for the detection of bacterial spores

Current culture dependent methods can’t reliably detect all milk damaging bacteria within a sample due to different bacteria requiring different growth conditions (Martin et al., 2011). Using culture independent methods can avoid that shortcoming. Early techniques in the area are based on Polymerase chain reaction (PCR) products including, PCR-temporal temperature gradient gel electrophoresis (PCR-TTGE) or PCR-denaturing gradient gel electrophoresis (PCR-DGGE), which work by separating PCR-amplicons based on their denaturation points due to temperature or chemical gradients (Jany and Barbier, 2008). Since the denaturation point is dependent on the DNA sequence and the denaturation status influences the mobility of an amplicon in an electrophorese gel, PCR-TTGE or PCR-DGGE, can identify microorganisms depending on the distance the PCR

products travel through the gel using a database (Jany and Barbier, 2008). For the creation of such databases, a known bacterial culture is however needed (Petersen et al., 2007). Denaturing high-performance liquid chromatography (DHPLC), works similar to DGGE by treating the PCR amplicons with chemicals to induce denaturation, however the separation is achieved by using High-performance liquid chromatography (HPLC) and not gel electrophoresis (Ercolini et al., 2008).

In recent years these PCR-based techniques are increasingly being replaced be more accurate next generation high throughput sequencing techniques (Ju and Zhang, 2015, Jany and Barbier, 2008). For bacterial biodiversity, a common approach is high throughput sequencing, for which DNA is directly extracted from an environmental sample followed of the sequencing of a marker region like the *rpoB* gene or variable regions of the 16S rRNA gene (Metabarcoding) (Francioli et al., 2021, Nowrotek et al., 2019, Martinez et al., 2017). This is much more accurate than previously described methods and allows an estimation of abundance of sequences and the clustering of similar sequences together into operational taxonomic units (OTU) (Shendure and Ji, 2008). This method however has some shortcomings when compared to more recent third generation sequencing methods, which can reveal sequences of larger fragments of the genome, leading to more accurate identifications to the species and even strain level (Johnson et al., 2019). Modern long read sequencing technology has enabled the assembly of metagenomes directly from environmental samples without culturing the organism first (Wilkins et al., 2019). Despite these recent advances, Bilen et al. (2018) reported that 66 % of updates in the human microbiome in the timeframe of 2015 - 2018, were achieved using culturing methods and describes culturing as an essential part of microbiome analysis.

Whole genome sequencing (WGS) data is useful in determining the relationship between bacteria within a sample and between different samples with a better resolution than 16S rRNA metagenomic sequencing (Devanga Ragupathi et al., 2018). The high resolution of WGS based single-nucleotide polymorphism (SNP) calling, compared to single or

multi gene sequencing, enables the detailed tracking of bacterial origins in disease outbreaks but could also be used for the tracing the origin of microbial spoilage in milk or other food (Carroll et al., 2019). WGS data can assist in predicting an isolate ability to produce toxins, biofilms or spores, for which an identification to the species level is often insufficient (Huang et al., 2021, Miller et al., 2018).

Metagenomic and WGS sequencing techniques are promising tools for the food industry as they enable advancements in the tracking of pathogens, investigation of contamination events, the prediction of virulence or spoilage abilities in bacteria as well as the assessment of a products microbiota during food production (Grutzke et al., 2019, Jagadeesan et al., 2019, Allard et al., 2018, Goodwin et al., 2016).

However, all sequencing based research studying the diversity of bacterial spores, can be insufficient, if the DNA extraction method used, is not suited for bacterial spores, which can happen due to their tough spore coat (Martin-Laurent et al., 2001). When planning to extract DNA from environmental samples, further errors can be introduced by the sample matrix. In the case of milk, there are two well-documented problems for sequencing. The first is the presence of lipids in the milk, which can inhibit sequencing (Terry et al., 2002). The second is the presence of casein protein in milk, which inhibits PCR and sequencing, if present at approximately 1 % total concentration (the average milk sample has a concentration of around 2.8 %) (Fox and McSweeney, 1998, Rossen et al., 1992). The extraction of DNA from cow feed may also result in difficulties for downstream applications. Cow feeds in New Zealand are plant based and plant acidic polysaccharides like pectin or xylan are known inhibitors of PCR and so are some plant lipids including maize oil (Pandey et al., 1996, Rossen et al., 1992).

However, multiple commercial kits are available which have shown satisfactory results for DNA extraction from bacterial spores in environmental samples (Dixon et al., 2017, Molsa et al., 2016, Desneux and Pourcher, 2014, Rusenova et al., 2013). A consensus on which kit or method is most suited has not been reached based on current literature.

2.5 Potential sources of spore-forming bacteria in raw milk

2.5.1 Transfer of bacterial spores into milk from the cow skin

Milk within the udder of a healthy cow is assumed to be almost sterile but becomes contaminated during milk harvesting and milk processing (Miller et al., 2015b, Gill et al., 2006, Tolle, 1980). The skin of the udder can function as a vector for microorganisms to be transferred into the milk during the milk harvesting process (Figure 2-1) (Gill et al., 2006). The udder skin microbiome not only changes depending on the season, but varies depending on the farm (Verdier-Metz et al., 2012). This supports the hypothesis that farm management practices influence the udder skin microbiome and therefore the bacteria present in milk.

The spore-forming bacteria are a concern in raw milk as they have the potential to survive dairy manufacturing and contaminate final product (Quigley et al., 2013). Spores on a cow's udder are believed to originate from contact with soil or cow faeces (Giffel, 1997, Christiansson et al., 1999). Soil and faecal contact of the udder is difficult to avoid. However, well trained milking shed staff and cleaning procedures of the udder skin can reduce the level of bacterial spores in the milk (Evanowski et al., 2020). Magnusson et al. (2006) investigated different cleaning techniques and found that cleaning with a wet synthetic towel and drying with paper towels as most effective to reduce bacterial spore contamination in the milk. It does, however, add additional time, labour and material costs to the milking process. This is why cleaning of the udder before milking is uncommon in New Zealand and has been phased out in the 1970's/80's and is now only used when teats are visibly dirty (DairyNZ, 2014). Additional to this, liquid residues of the cleaning fluids on the udder can help to transfer bacteria into the milk (Galton and Merrill, 1988).

The role of the cow udder skin microbiome in the quality of milk requires investigation to determine the sources of heat resistant spore-forming bacteria, since they are difficult to

remove from milk during processing, making them likely to end up in final product (McHugh et al., 2017). Since the source of bacterial spores on udder skin, are mostly soil and faeces and as cattle feed is the main source of spores in cow faeces, the bacterial spores in cattle feed should be investigated (Magnusson et al., 2007, Vissers et al., 2007a).

Vacheyrou et al. (2011) showed, that bacteria on the udder skin are also found in the barns and the milking environment and that those bacteria on the udder are found in raw milk. This was confirmed by Monsallier et al. (2012) who showed that the types of bacteria on the cow's udder vary when different housing or feeds are used. This suggests that the farm environment and management can affect the microbiome of the milk and highlights the cow's udder skin as a vector responsible for the transfer of bacteria (Figure 2-1).

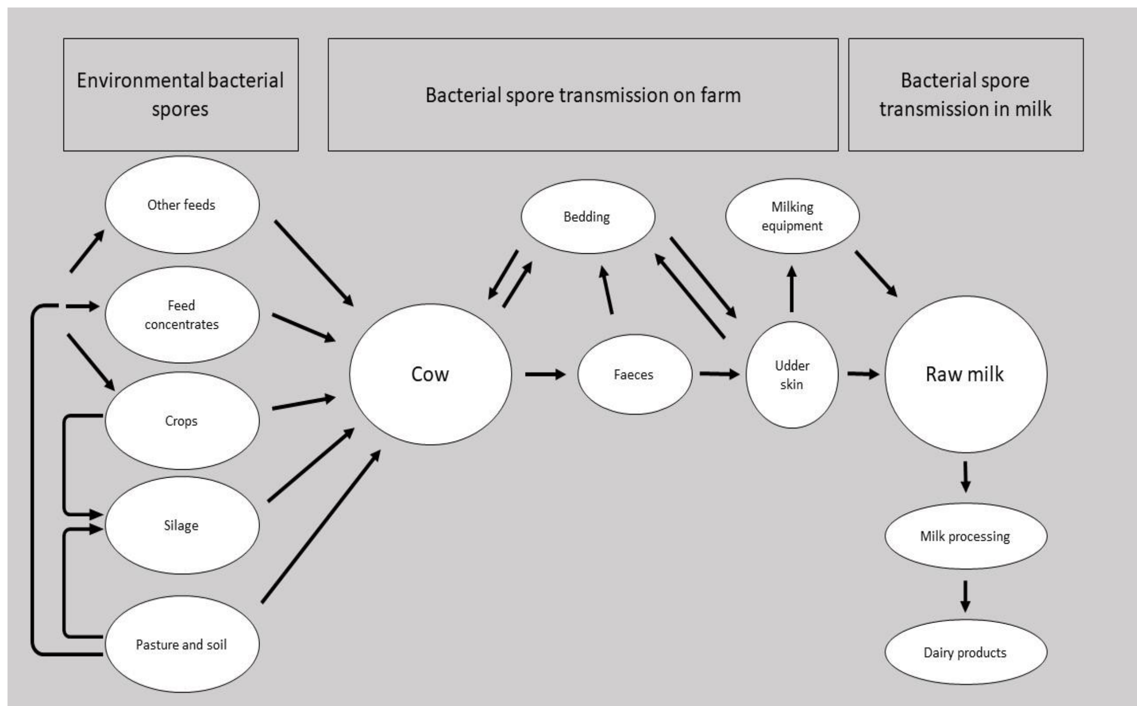

Figure 2-1 Transmission pathways of bacterial spores from the environment into milk and milk processing.

2.5.2 Transfer of bacterial spores through milking equipment, milking procedure, and milk transport

Milking shed equipment can be a key factor of bacterial spore transmission and can harbour spore-forming microorganisms, mostly *Bacillus* species (Scheldeman et al., 2005). *Bacillus* spores can appear after improper handling and cleaning of the equipment and are a problem once they form a biofilm within it (Marchand et al., 2012).

A study by Miller et al. (2015a) showed that pre-milking stripping, can increase mesophilic spores in raw milk. Although the exact reason for that is unknown, it is unlikely due to human – cow udder contact since pre-milking udder massages, seemed to decrease thermophilic spores in the raw milk. In New Zealand neither of these procedures are used, however post-milking udder disinfectant is usually applied, and it was shown to reduce the thermophilic spores in milk (Miller et al., 2015a).

Even after the milk is collected from the farm and transported to undergo processing, further contamination can occur if milk transport tankers are not adequately cleaned. For example, biofilms can form on the interior surfaces of the tanker and carry *Bacillus* spores, which are able to produce heat stable milk damaging enzymes (Teh et al., 2011). The milk microbiome undergoes further changes once it is transferred from tankers into the processing facility (Kable et al., 2016).

2.5.3 Cow feed as a source for bacterial spore contamination in milk

It was reported through tracking changes in *B. cereus* spore counts, that a primary source of spores in milk could be cattle feed through udder contamination with faeces (Vissers et al., 2007a). This concept was also supported by artificial feeding experiments with *B. cereus* spores, resulting in increased *B. cereus* spore counts in faeces and milk, which were confirmed to have originated from the feeding by random amplified polymorphic DNA PCR fingerprinting (Magnusson et al., 2007).

The diets of dairy cattle herds in New Zealand have changed in the last 25 years, from farmers using mostly pasture to an increased feeding with crops, as well as imported and harvested supplementary feed (MPI, 2017). The microbiota of some of the new feeds is unknown and this may influence the milk microbiome in new ways. Information that is available on the microbiota in cow feed is mostly based on traditional microbiological culture dependent methods, which may not provide complete information on the microbiome of cow feed. For some feeds like PKE, tuber feed or feed concentrates, few studies have been conducted investigating the number and diversity of bacterial spores present.

2.5.3.1 Feed concentrates

Scheldeman et al. (2005) reported a correlation between the presence of highly heat resistant spores in raw milk and feed concentrates, which is energy dense, usually dried feed made to address nutritional deficits in the cows (Purcell et al., 2016). Heat resistant spores of *Aneurinibacillus thermoaerophilus*, *Brevibacillus borstelensis*, *Bacillus* spp. and *Paenibacillus* sp. in the raw milk are likely to originate from a feed concentrate (Scheldeman et al., 2005).

Vaerewijck et al. (2001) reported high concentrations of *Bacillus* spores, including species like *B. subtilis, B. pumilus, B. clausii, B. sporothermodurans, B. cereus* or *B. licheniformis,* in concentrate cattle feed. These species are known to produce proteolytic or lipolytic enzymes, or in case of *B. cereus,* also heat stable toxins (De Jonghe et al., 2010). *Bacillus sporothermodurans* spores are due to high heat resistance, a spoilage concern for UHT milk (Scheldeman et al., 2006, Scheldeman et al., 2005). The spores of *B. thermoamylovorans* spores are very heat resistant (106 °C for 30 min) and have been reported in milk and in cattle feed (Eijlander et al., 2019, Scheldeman et al., 2005). *Bacillus thermoamylovorans* has not yet been found in feed concentrates, but is present

in palm wine (Flint et al., 2017). The wine is made from the same source as PKE, suggesting that *B. thermoamylovorans* in raw milk could originate from PKE.

2.5.3.2 Silage

Silage is commonly made from maize and pasture through fermentation using lactic acid bacteria and its proper fermentation is important for its nutritional value, stability and cow health (Pholsen et al., 2016, Kalač, 2011). Once fermented, the microbial composition of silage is different to the bacterial composition of its raw ingredient (e.g. grass or maize etc) and therefore the bacteria that are transferred into milk reflect this change (Doyle et al., 2017). Overall spore counts in silage are reported between 2.7 – 5.5 log_{10}/g depending on the type of silage, sampling site and time of opening if the silage stack (Martin et al., 2019, Driehuis, 2013). Lower spore counts were found in stacks which have been recently opened and a sample taken from the core while moulded parts were avoided (Driehuis, 2013). The mesophilic and thermophilic spores in silage can experience changes across seasons with combined counts of 3.6 log_{10}/g in winter compared with 6.3 log_{10}/g in summer, an increase which could also increase the spore count in milk (Buehner et al., 2014).

In silage over 36 different bacterial spore species have previously been reported (Borreani et al., 2019, Zucali et al., 2015, Driehuis, 2013, Liu et al., 2013). Silage is sometimes considered the primary origin of bacterial spores in milk and has been associated with the transfer of a variety of *Bacillus* spp., *Clostridia* spp., or *Paenibacillus* spp. into raw milk (Driehuis, 2013). In a study of *Clostridium* species found in raw milk, 83 % were also found in pasture or maize silages (Julien et al., 2008). Silage is also associated with the spreading of pathogens like *Clostridium botulinum,* which has been reported to grow in silage, if growth conditions are suitable for this bacterium during the fermentation process (Lynn et al., 1998, Donald et al., 1995, Notermans et al., 1981). However, *C. botulinum* presence is usually only associated with silage if it has been

contaminated with animal carcases e.g. bird carcases (Driehuis, 2013). Silage is also associated with *B. cereus* spores, which are of concern for milk quality because of the spoilage enzymes produced and psychrotrophic growth in chilled raw milk (Porcellato et al., 2021). An increase in *B. cereus* in silage has been correlated with an increase in abundance in cow faeces, the udder skin and ultimately an increased abundance in the raw milk tanks, showing that silage can be transitionally involved in *B. cereus* contamination of raw milk (Vissers et al., 2007a, Giffel, 1997).

Dark and moulded areas can regularly appear in a silage stack, due to aerobic deterioration after opening the stack or insufficient sealing. In these areas, aerobic bacteria including aerobic bacterial spores, start proliferating, and if these areas are not properly discarded prior to feeding, they can lead to increased bacterial spore counts in the milk (Borreani et al., 2019, Liu et al., 2013). In maize silage, areas of aerobic deterioration can include high concentrations of butyric acid-producing bacterial spores, leading to spoilage of cheese (Vissers et al., 2007b, van den Berg et al., 2004). However, in recent years the use of ever improving inoculants in silage production has improved the aerobic stability of silage and thereby potentially reduced the amount of bacterial spores in silage (Nkosi et al., 2011, Rammer et al., 1994).

2.5.3.3 Hay and straw

Scheldeman et al. (2005) found hay and straw to have similar mesophilic thermo-resistant bacterial spore counts compared to silage. However, mesophilic and thermophilic highly heat resistant bacterial spores, in hay and straw showed slightly higher numbers than silage. Silage showed more psychrotrophic highly thermo-resistant bacterial spores than hay or straw. This can be linked to the time of harvest and harvest conditions. Silage is harvested during late spring early summer when temperatures are low and is generally exposed to sunlight for only one or two days, whereas hay is harvested in mid to late summer, and exposed to high temperatures and sunlight for

around three days and has a moisture content below 20 % compared to >60 % for silage (Holmes et al., 2002). Straw is even drier, as it is harvested dry and then further dried down to around 10 % moisture (Holmes et al., 2002).

Most hay and straw bacterial spores were identified as *B. licheniformis B. pallidus*, *Brevibacillus agri* and other *Brevibacillus* spp. (Scheldeman et al., 2005). Spores of *B. cereus* were found in 25 % of hay samples but not in straw samples (Magnusson et al., 2007).

2.5.3.4 Pasture

For pasture, there is some evidence for a correlation between the bacteria on pasture and the bacteria found in milk (Doyle et al., 2017). For spore-forming bacteria, a correlation in the increases of *B. cereus* spores on pasture and *B. cereus* spores in milk was found and indicates that bacterial spores from pasture may contribute to the spores in milk (Slaghuis et al., 1997). Psychrotrophic spores were found to be quite abundant within pasture, comprising up to 23 % of the total spore count (McKinnon and Pettipher, 1983). Reports on the number of bacterial spores in pasture in New Zealand are rare and old but Stout (1960) reported between 10^3 to 10^4 CFU/g of aerobic mesophilic *Bacillus* spores. However, there are no studies within the last decade and results on the spores growing at different temperatures or anaerobic conditions are still missing, despite pasture being one of the most used feeds in cattle worldwide.

2.5.3.5 Other feeds

For many crops fed to cows, the root microbiome has been studied with the purpose to improve yields, but the parts of crops that are usually fed to the cow are not as well investigated (Philippot et al., 2013). This makes it difficult to predict which bacterial spores those crops can transfer to the milk. We know that their surface is likely contaminated with bacterial spores from the soil and faeces, which can be used as

fertiliser (Maciorowski et al., 2007). For tuber feed *Beta vulgaris* ssp., it was reported, that roots contain very low levels of spore-forming bacteria genera, mostly *Bacillus* (0.2 %- 0 %) or *Paenibacillus* (0.2 % - 0 %), unless they were planted in potting soil (6.7 % and 8.3 % respectively) (Zachow et al., 2014). This could be due to the high level of faecal matter in potting soil, since faeces based fertilisers were shown to increase spore counts in grass (Rammer et al., 1994).

Distillers' grain (hydrated distillers' grain) contains mostly lactic acid bacteria as well as some moulds, yeast and some not further identified aerobic and anaerobic spore-forming bacteria (Olstorpe et al., 2010, Serjak et al., 1953). However, the far more common feed concentrate in New Zealand is dried distiller's grain. After the drying process, it is likely that a lot of the lactic acid bacteria or moulds will not survive. The bacterial spores, however, are likely to withstand the heat. So far, the microbial community composition of dried distiller's grain remains unknown.

2.6 Conclusion

Spore forming bacteria can impact milk and dairy product quality due to their ability to produce biofilms, spoilage enzymes and spores. Their spore's resistance to environmental factors like heat, makes them difficult to remove from products and production sites and the best way to control them is to keep them from entering. A wide range of factors like season, milking procedure or used feed types can influence the spores present in milk. This leads to a variety of bacterial spore species which can be found in milk. However, information on the bacterial spores in raw milk and dairy cattle feed currently used in New Zealand is lacking. On New Zealand farms increasing amounts of supplementary feeds are being used and the impact of this trend on the microbiota of milk is currently unknown. The industry could benefit from additional knowledge on which feeds carry the greatest risk for bacterial spore contamination of milk.

The recent developments in sequencing technology, allow us to explore the microbial content of our environment in more detail than in previous decades, which could also be beneficial to exploring milk and cattle feed. So far limited evidence is provided that bacterial spores in milk could originate from feed. However, the diversity of spore species and their transfer from feed to milk remains to be elucidated.

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Chapter 3: Investigation methods and description of the spore-forming bacteria in dairy cattle milk and feed

3.1 Abstract

Dairy cattle in New Zealand are fed a predominantly pasture-based diet. However, since the early 2000s' forage crops and supplementary feeds have been increasingly used. These feeds may have different microbial compositions compared with pasture and may differently affect the bacterial spore diversity present in raw milk. This study examined the bacterial spore populations of cattle feeds and corresponding raw milk in New Zealand. The abundance and diversity of psychrotrophic, mesophilic and thermophilic bacterial spores under aerobic and anaerobic conditions were assessed. Tuber feed and PKE had the greatest counts across most testing conditions. Raw milk samples had the lowest bacterial spores counts present. Isolated spore-forming bacteria were identified using a matrix-assisted laser desorption/ionization time of flight mass spectrometer biotyper (MALDI-TOF). Results showed that almost all (~ 96 %) of the spore-forming bacteria species identified in milk, could also be identified in the feed fed to cattle that produced the milk. Selected samples were also screened using a metagenomic analysis to identify spore forming bacteria species, however, this method was successful for feed samples but not for milk. In addition, different dairy cattle feeds contained different bacteria spore species. Indicating that spores in feeds may influence the spore diversity present in the corresponding raw milk.

3.2 Introduction

Milk within the udder of a healthy cow has few bacteria in it, but it is a rich medium for bacterial growth due to its large array of fats, proteins and carbohydrates (Jenness, 1988, Tolle, 1980). Bacterial contamination of milk can lead to product spoilage or consumer health risks if no effort is made to limit microbial growth post-harvest (Ledenbach and

Marshall, 2009). Of concern are bacterial spores, which can impact milk quality, even after pasteurisation as they can survive heat treatment (Quigley et al., 2013, Ledenbach and Marshall, 2009). These spores can form biofilms within production plants which can act as a reservoir of spoilage bacteria, potentially leading to the need of extensive cleaning measures (Gopal et al., 2015).

Milk can acquire bacteria whilst leaving the teat cistern, from bacteria present on the skin (Verdier-Metz et al., 2012). The spore forming bacteria on the skin of the udder, are believed to originate from contact with soil, feed, bedding and/or cow faeces (Christiansson et al., 1999, Giffel, 1997). Studies have also indicated that there are significant correlations between spores in silage, cow faeces, the udder skin and raw milk (Doyle et al., 2017, Vissers et al., 2007). In another study, a significant increase in *B. cereus* spores in faeces and raw milk was observed when high levels of *B. cereus* spores (10^8 or 10^{10} spores per cow per day) were deliberately fed to cows (Magnusson et al., 2007).

However, the bacterial spore diversity and abundance of dairy cattle feeds are not well studied. Even though dairy cattle in New Zealand are predominantly pasture-fed, the addition of non-pasture feed has more than quadrupled between 1991 (4.2 %of the diet) and 2015 (18 % of the diet), with most of that increase from supplementary feeds (e.g. PKE, tuber feed and Maize silage) from 1.2 % to 8 % over the same period (MPI, 2017).

Most industry standard test for microorganisms in products is based of culture based methods which combined with MALDI-TOF or gene based identification methods can detect the presence of pathogenic or spoilage related microorganisms (MPI, 2016, Ross, 2011). However high throughput sequencing techniques can be, less labour intensive, faster and able to detect a higher diversity of species than a culture-based approach (Grutzke et al., 2019, Zhang et al., 2019). Allowing for more effective food safety assurance or assessments of spoilage bacteria abundance, without the need for incubation (Ryu et al., 2021, Allard et al., 2018). A common method is the sequencing a

marker gene, frequently used is the V3-V4 region of the 16S rRNA gene to identify the bacteria (Francioli et al., 2021).

To better understand the spore abundance and diversity, present in New Zealand dairy cattle feeds, and in the corresponding raw milk, were investigated in this study using metagenomics complementary to culture-based methods.

3.3 Methods

3.3.1 Sample collection

Samples from 27 farms were taken in four time periods over 12 months in different regions of New Zealand (Detailed description: Table A-1). The time periods were: summer (late October 2017 - February 2018), autumn (March – April 2018), winter (May - June 2018) and spring (September – early October 2018). In the initial collection in summer 2017, 21 out of the total of 41 milk samples were collected and 24 out of the total of 39 pasture samples were collected due to logistical constraints (Table 3-1). The rest were sampled over the following seasons.

Table 3-1 Samples tested for bacterial spore counts (by sample type and season).

Samples tested for spore count	Total samples	Samples collected by season (Summer/ Autumn/ Winter/ Spring)
Milk	41	21/9/6/5
Pasture	39	24/5/5/5
Chicory and plantain	13	7/3/0/3
Pasture silage	11	3/0/7/1
Maize silage	14	5/6/3/0
Tuber feed	11	5/3/2/1
Concentrate	17	9/2/3/3
PKE	14	5/4/4/1

A milk sample, and a corresponding sample of all feed types used within the three weeks prior to the collection of the milk sample, were collected from each farm, and samples were grouped in one of seven sample types for analysis: chicory and plantain (n=13) [*Cichorium intybus* n=8 and *Plantago lanceolata* n=5]; concentrate (n=17) [pelletised concentrate (n=8), dried distiller grain (DDG) (Maize; *Zea mays* or Barley; *Hordeum vulgare* n=4), DDG + PKE (*Elaeis guineensis*) mix (n=2), DDG + tapioca (*Manihot esculenta*) mix (n=1), DDG + tapioca + canola (*Brassica napus*) mix (n=1), PKE + Barley mix (n=1)]; pasture (n=39) [mix of ryegrass (*Lolium perenne)* and white clover (*Trifolium repens*)]; pasture silage (n=11); PKE (n=14); maize silage (n=14); tuber feed (n=11) [fodder beet (*Beta vulgaris)* n=6 and turnips (*Brassica rapa)* n=5].

To collect feed samples, commercial plastic bags (OfficeMax, USA) were used, which contained < 50 CFU/bag residue of microorganisms, making them adequate for sample collection according to the American Public Health Association (APHA) standards (Wehr and Frank, 2004) (Appendix A, Figure A-1). Approximately 200 g wet weight of sample were collected for each feed. Samples from grazed feeds (pasture, chicory and plantain), were either hand sampled by tearing off using the sampling bag or cutting with sterile scissors two cm above the ground, simulating the manner in which a cow would consume pasture. Silage samples were taken from the centre area of the stack face approximately 10 cm deep into the face. Tuber feeds were bagged whole, whilst concentrates and PKE samples were bagged from the storage location (silo or bunker). At the same time as the feed collection, two 50 ml milk samples were aseptically taken from the bulk tank vat, in sterile 50 ml centrifugation tubes (BD Biosciences, USA). Samples were then chilled to 4 °C and transported to the lab, where samples were frozen at -20 °C until testing.

3.3.2 Sample preparation

Chicory and plantain, pasture and pasture silage samples were prepared by blending a 10 g sample with 100 ml sterilised (0.22 µm membrane filtered and autoclaved water

(milliQ water)). Tuber feed samples were prepared by cutting five g of the top of the bulb, five g of the bottom of the bulb and five g of the leaves with a sterile scalpel and blending in a stainless-steel blender with 150 ml sterile milliQ water. The blenders were cleaned between samples using soap and water first, followed by sterilisation, using a modified version of a protocol from Kemp and Smith (2005). The blenders were run with 300 ml of 10 % (v/v) sodium hypochlorite solution (42g/l) (Pental, Australia) for 10 seconds and then incubated for 10 min at room temperature. The blenders were rinsed three times with 200 ml sterilised milliQ water and the blender was either dry autoclaved or left at 105 °C overnight to deactivate any remaining sanitiser residue.

Of the remaining feed types (PKE, concentrate and maize silage), a five g sample was dissolved in 25 ml sterile milliQ water and mixed by vortex for at least 15 seconds. Milk samples were tested as received once defrosted.

3.3.3 Heat treatment for spore selection

A heat treatment for 12 min at 80 °C was used to inactivate vegetative cells (Kent et al., 2016). Eight ml of the prepared liquid sample was transferred to a 15 ml sterile centrifugation tube, briefly centrifuged, and submerged in a pre-heated 80 °C water bath. A control tube containing water and a thermometer measuring the temperature of the liquid inside the tube was included in the water bath. Once the control tube reached 80 °C, the treatment was timed for 12 min. After the heat treatment, the samples were cooled in a 4 °C water bath for at least 15 min.

3.3.4 Enumeration of spore populations in milk and feed samples

For microbial counts, one ml of sample was plated directly onto plates and a serial 10-fold dilution series of the sample was prepared using nine ml sterile peptone water and one ml of heat-treated sample. Of the diluted sample, 100 µl were spread on MPCA

(Oxoid ltd., England) supplemented with five g/l of agar (Merck, Germany) for aerobic incubation, and DRCA (differentially reinforced *Clostridium* broth (Thermo Fisher Scientific, USA), with 15 g/l Agar (Merck, Germany)) for anaerobic incubation. For each sample, three sets of both MPCA (for aerobic incubation) and three sets DRCA (for anaerobic incubation) were prepared for incubation at 10 °C, 37 °C, and 55 °C. Resulting colonies were counted after 48 h (37 °C and 55 °C), or 120 h (10 °C).

3.3.5 Enrichment of bacterial spores in milk and feed samples

An enrichment procedure for spores from milk and feed samples was used to recover low quantities of spores as described in previously (Martinez et al., 2017, Kent et al., 2016). For samples collected in summer, one ml of the heat-treated sample of cattle feed solution or milk, was added to nine ml of sterilised milk and enriched at 37 °C or 55 °C for six h or at 10 °C for two days. Then 500 µl of the enriched sample were spread onto MPCA plates in triplicate. All plates were incubated at 37 °C or 55°C for five days, or 10 °C for 14 days under aerobic conditions. After incubation the spores were washed off the plates using nine ml of buffered peptone water and the resulting spore solution was heat treated at 80 °C for 12 min. From this enriched solution, the bacterial spores were cultured on MPCA aerobically and on DRCA anaerobically at 10 °C, 37 °C and 55 °C, then single colonies were selected for identification.

Samples from all other seasons (autumn, winter, spring) were enriched by adding one ml of heat-treated sample to nine ml of sterilised milk and enrichment at 37 °C or 55 °C for 24 h or 10 °C for five days, followed by a microbial count. This method was chosen as tests of five samples, produced high numbers of spores, and showed no significant difference in the bacterial spore species, when compared with the summer enrichment method (tested with a PERMANOVA, Appendix A, Table A-3 and Table A-4). This method was faster allowing processing of more samples. Culturing of the resulting bacteria colonies was carried out as described for the summer method.

3.3.6 Identification of cultured bacterial spores in milk and feed samples

For all sample types, at least 10 samples were analysed for their spore-forming bacteria species and a total 116 samples were analysed (milk n=31; pasture n=21; chicory and plantain n=10; pasture silage n=10; maize silage n=10; tuber feed n=11; PKE n=11; concentrates n=13).

Colonies were selected from plates after initial and enrichment testing, under aerobic and anaerobic conditions, at all three temperatures based on differences in morphology. All spore-forming bacterial species from a sample across all different testing conditions, were assumed to represent the detectable bacterial spores in that sample. The colonies were sub-cultured on Trypticase soy agar (TSA) (BD diagnostics systems, Germany) and incubated at the temperature under which they were originally isolated. Single colonies were prepared for MALDI-TOF (Bruker, USA) identification using the full extraction method described by Lindsay et al. (2014). In brief: Biological material from a colony was transferred into a mixture of 300µl deionised water and 900µl ethanol and frozen till analysis. Ethanol was removed by centrifugation at 9659 g for 2 min and the pellet was resuspended in 30 µl of 70% formic acid followed by mixing with 30 µl of acetonitrile (Merck, Germany). After centrifugation (9659 g for 2 min), 1 µl of supernatant was dried on the MALDI target and covered by 1 µl of cyano-4-hydroxycinnamic acid matrix (Bruker, USA). In total, 1741 isolates were identified at the species level.

3.3.7 Data analysis of culture results

Bacterial spore counts in cattle feed and milk were log_{10} transformed and analysed using ANOVA mixed effect models and Tukey`s tests in Minitab 18 (Minitab LLC, USA). The significance of oxygen availability and region on spore-forming bacterial counts were analysed using a mixed effects ANOVA model using the fixed variables of oxygen

availability, sample type, season, and temperature and region as a random variable with an interaction term of sample type and temperature. To determine the significance of sample type and temperature a mixed effect ANOVA model was run for aerobic and anaerobic counts separately with the fixed effects of sample type, season and temperature with region fitted as a random effect as well as an interaction term of sample type and temperature.

To analyse the influence of seasons on each sample type, mixed effect ANOVA models and Tukey`s tests were created in Minitab 18 for each sample type separately with the fixed effects of season, temperature and oxygen availability and region as random variables. Results were excluded when less than three samples of a sample type were tested in a season.

The average species found per sample type (total number of species found across sample type / number of samples of sample type) and the Shannon diversity index (*H*`) were calculated for each sample type. The later was calculated using the formula $H` = -\sum_{i=1}^{S} p_i \ln p_i$ where *S* is the number of species identifications across all samples of a sample type (species richness) and *pi* is the proportion of *S* made up of the *i*-th species. Evenness (*E*) was determined as $E=(H`/H_{max})*100$ where H_{max} = ln (*S*) (Shannon, 1948).

Bacterial spore species from different sample types were analysed for differences using a permutational multivariate analysis of variance (PERMANOVA) using sample type and season as variables in the PAST software (version 3.26) (Hammer et al., 2001). The PERMANOVA was based on Bray-Curtis distances and P-values were adjusted by the Holm-Bonferroni correction.

3.3.8 Sample selection and sample preparation for metagenomic analysis

For a metabarcoding-16S rRNA marker gene analysis, DNA was extracted from two milk, a PKE and a tuber feed sample. For each sample type, the analysis was carried out as

previously described (see section 3.3.3 Heat-treatment for spore selection)-step following the summer enrichment method (on plates) described in (see section 3.3.5 Enriched testing of milk and feed samples), for each of the three temperatures 10°C, 37 °C and 55 °C creating four extracts per sample and a total of 16 extracts for analysis.

3.3.9 DNA extraction and sequencing

The Nucleo Spin Soil DNA extraction kit (Macherey Nagel, Germany) was used according to the manufacturer's instructions. The DNA quality and concentration were measured using a Microvolume Spectrometer (Berthold Technologies, Germany) and the abundance of 16S rRNA gene sequences was confirmed in a PCR using universal 16S primers (27F & 515R).

Extracted metagenomic DNA samples were sequenced by the NZGL sequencing service (now: Massey Genome Service, New Zealand) where libraries were prepared following the 16S rRNA Metagenomic amplicon library preparation protocol for the V3 and V4 region with dual index single step PCR (Illumina, USA). Reads were generated using a MiSeq system (250 bp) (Illumina, USA).

3.3.10 Metagenomic data analysis

Raw sequences were mapped against the PhiX genome using Bowtie2 (Langmead et al., 2009). Any hitting PhiX sequences were removed from the results and fastq files were reconstructed using the SamToFastq.jar program from the Picard suite (Wysoker et al., 2013). Adapters were removed using the "fastq-mcf" program from the ea-utils suite of tools (Aronesty, 2013).

The Qiime2 pipeline under default settings was used for further analysis (Bolyen et al., 2019). The following plugins were used in the processing: BIOM (McDonald et al., 2012), Pandas (McKinney, 2010), DADA2 (Callahan et al., 2016), Scikit-learn (Pedregosa et al.,

2011) and q2-feature-classifier (Bokulich et al., 2018). To identify sequences, the SILVA rRNA gene database was used (http://www.arb-silva.de/, accessed 17.10.2019, Quast et al. (2013)). The output files were filtered to only include species level identifications to be comparable to the MALDI-TOF results and only include genera, which have known spore-forming members.

The spore forming bacteria species reads, detected from the same samples after heat-treatment and enrichments, were combined into one observation to represent the average bacterial spore population. To adjust for higher read counts after enrichment, the number of reads of each species after enrichment was adjusted by $N^{0.5}$ before combining with the reads after heat treatment (Jonsson et al., 2016).

3.4 Results and discussion

3.4.1 Quantification of bacterial spores in cattle feed and corresponding raw milk

The mean bacterial spore counts in cattle feed or milk samples for each of the three temperatures investigated under aerobic and anaerobic conditions are presented in Table 3-2 and Table 3-3, respectively. Sample types were found to cluster into three to six groups depending on the incubation temperature and oxygen availability. Across all incubation conditions, the greatest counts of spores per sample type were found in tuber feed and PKE while raw milk samples had the lowest counts of spores (Table 3-2 and Table 3-3).

Table 3-2 Mean and standard deviation (Stdev) of aerobic bacterial spore counts in raw milk and different dairy cattle feeds at 10 °C, 37 °C or 55 °C.

	10 °C		37 °C		55 °C	
Sample type	Mean*	Std	Mean*	Std	Mean*	Std
Milk	0.7^{d}	0.5	1.3^{e}	0.6	0.8^{e}	0.4
Chicory and plantain	1.9^{bc}	0.7	2.5^{cd}	0.8	1.9^{d}	0.4
Maize silage	1.5^{c}	0.3	2.5^{d}	1.1	2.3^{cd}	1.0
Concentrate *feeds*	1.4^{c}	0.3	3.1^{bcd}	1.1	3.2^{bc}	1.2
Pasture	2.3^{b}	0.9	3.1^{cd}	0.9	2.3^{d}	0.7
Grass silage	2.0^{bc}	1.2	3.8^{abc}	1.5	3.2^{bcd}	1.3
PKE	1.7^{bc}	0.9	4.1^{ab}	0.9	4.1^{a}	0.9
Tuber feed	3.7^{a}	0.6	4.4^{a}	0.6	3.4^{ab}	0.6

* in log_{10} CFU / g or ml, calculated using mixed effect ANOVA models, means with different letters are significantly different ($P<0.05$)

Table 3-3 Mean and standard deviation (Stdev) of anaerobic bacterial spore count in raw milk and different dairy cattle feeds at 10 °C, 37 °C or 55 °C.

	10 °C		37 °C		55 °C	
Sample type	Mean*	Std	Mean*	Std	Mean*	Std
Milk	0.6^{d}	0.2	1.2^{c}	0.6	0.9^{d}	0.5
Chicory and plantain	1.5^{abc}	0.0	2.3^{b}	0.7	1.8^{cd}	0.3
Maize silage	1.3^{c}	0.1	2.6^{b}	1.5	2.6^{bc}	1.2
Concentrate *feeds*	1.4^{bc}	0.4	3.1^{ab}	1.1	2.9^{b}	1.3
Pasture	1.8^{a}	0.7	2.9^{b}	0.9	2.3^{bc}	0.8
Grass silage	1.3^{bc}	0.0	3.2^{ab}	1.3	2.8^{bc}	1.4
PKE	1.4^{bc}	0.3	3.9^{a}	1.0	4.1^{a}	0.9
Tuber feed	1.6^{ab}	0.5	4.0^{a}	0.5	2.8^{bc}	0.8

* in log_{10} CFU / g or ml, calculated using mixed effect ANOVA models means with different letters are significantly different ($P<0.05$)

The region where the cattle feed and milk were collected did not show a significant influence on the quantity of bacterial spores detected ($P = 0.104$), indicating that the geographical differences within New Zealand do not influence the quantities of bacterial spores in New Zealand's cattle feed or milk (Table A-2).

The availability of oxygen had a significant ($P < 0.001$) effect on the quantity of spores recovered (Table A-2). The anaerobic spore counts were on average ~ 10 % lower than the aerobic spore counts (2.4 vs. 2.6 $\log_{10}$ average spores / g or ml) (Table 3-2 and Table 3-3). The reason for this difference not being more prominent, could be due to approximately 60 % of species were identified as facultative anaerobes, meaning that these bacteria would grow on both aerobic and anaerobic plates.

The incubation temperature at which the quantity of bacterial spores was tested had a significant effect on the number of spore-forming bacteria recovered ($P < 0.001$) (Table A-2). Spore counts were significantly different ($P < 0.001$) between all three temperatures in aerobic and anaerobic counts, indicating that the three temperatures selected different species.

The added interaction term between incubation temperature and sample type in the mixed effect ANOVA model showed a significant impact ($P < 0.001$) on the spore counts detected (Table A-2). This indicates that the number of psychrotrophic, mesophilic or thermophilic spore-forming bacteria varied with sample types. The origin of these differences could be due to the environments in which the feeds are produced, stored or potentially consumed. Feeds grazed *in situ* such as pasture, chicory and plantain and tuber feed, grouped among samples with greater counts of psychrotrophic spore-forming bacteria compared to their thermophilic spore-forming bacterial counts, while for feeds that go through drying processes during production, like PKE and concentrate feeds had greater thermophile counts.

For raw milk samples, the average level of mesophilic spore-forming bacteria isolated was 10 CFU / ml which is similar to reports in previous studies of between 10 – 100 CFU / ml (Coorevits et al., 2008, McGuiggan et al., 2002). Up to 10^3 CFU / ml mesophilic bacterial spores have been reported before when tested at 30 °C instead of 37 °C (Scheldeman et al., 2005). The quantity of thermophilic bacterial spores was lower than mesophilic spores which have been reported in previous research of spores in New

Zealand's raw milk (Scott et al., 2007). Psychrotrophic spores in the milk were around 4 CFU / ml which aligns with the reported values in literature which found that 77 % of milk samples contain between 1 – 20 CFU / ml of psychrotrophic spores (Matta and Punj, 1999).

For pasture samples, the average number of mesophilic spores was around 5 $x10^3$ CFU /g, similar to the 10^3 CFU /g reported by Stout (1960). No significant difference was found between the bacterial spore counts in pasture and chicory & plantain. Since bacteria colonise a plant in grater quantity epiphytically (on the surface of the plant) than endophytically (in the interior of the plant), this similarity could be explained by them growing in the same environment and having similar amounts of spores attach to them (Turner et al., 2013).

In maize silage and pasture silage, aerobic mesophilic bacterial spores were around 10^3 CFU / g and 10^4 CFU / g respectively, which is within the range reported in literature of 10^3 – 10^4 CFU / g and 10^4 - 10^5 CFU / g for maize silage and pasture silage, respectively (Driehuis, 2013). No other differences were found between the silage samples spore counts ($P > 0.05$). The origin of the difference could lie within in the bacterial spore population of the ensilaged raw material.

For concentrate feeds, previous studies in the Netherlands reported mesophilic bacterial spore counts of 10^5 – 10^6 CFU / g (Scheldeman et al., 2005), which is greater than the 10^3 CFU / g detected in this study. However, the study of Scheldeman et al. (2005) did not report the exact types of concentrates studied and could have included PKE as a feed concentrate, resulting in higher numbers.

The highest bacterial counts in this study, were recorded in tuber feeds. To the authors knowledge, there are no reports of bacterial spore counts in tuber feeds but the high counts detected in this study are assumed to originate from its close proximity to soil which can contain up to 10^9 spores per gram of dry soil (Brandes-Ammann et al., 2011).

The season had a significant effect on the number of bacterial spores found (P < 0.001). These seasonal variations were most prominent in pasture where counts in summer were significantly lower than those in autumn (P < 0.001) and spring (P < 0.001), but not significantly different to winter samples (P = 0.767) (Table 3-4).

Table 3-4 Mean[a] [b] ± standard deviation bacterial spore counts of cattle feed and corresponding raw milk, across the seasons.

Sample type	Summer	Autumn	Winter	Spring
Milk	0.7 ±0.4 (B)	0.8 ±0.4 (A/B)	1.1 ±0.5 (A)	1.1 ±0.6 (A)
Pasture	2.2 ±0.6 (C)	3 ±0.9 (A)	2.4 ±0.6 (B/C)	2.8 ±1.1 (A/B)
Chicory and plantain	1.8 ±0.4 (A)	2.2 ±0.6 (A)	NT	1.9 ±0.2 (A)
Pasture silage	2.1 ±1 (A)	NT	2.9±1 (A)	4.1 (-)
Maize silage	1.9 ±0.8 (A)	2.0 ±0.6 (A)	2.6 ±1.2 (A)	NT
Tuber feed	3.2 ±0.3 (A)	3.3 ±0.6 (A)	3.6 ±0.4 (-)	3.0 (-)
Concentrate	2.3 ±0.7 (A)	1.4 ±0.2 (-)	3.4 ±0.6 (B)	3 ±0.3 (A B)
PKE	3.2 ±1.2 (A)	3.1 ±0.6 (A)	3.4 ±0.6 (A)	2.9 (-)

[a] in $\log_{10}$ CFU / g or ml, calculated in mixed effect ANOVA models
[b] Means with different letters denote significant differences within feed between seasons (P<0.05) based of Tukey tests, which was only calculated if 3 or more samples of one sample type were tested in a season
"-" = grouping is missing due to low sample size
"NT" = no sample was tested

The seasonal variation in bacterial spore counts on pasture is likely explained by seasonal weather changes. The greatest counts were observed in samples collected during autumn and spring (3 and 2.8 average bacterial spores in $\log_{10}$ CFU/g respectively), indicating that a combination of warmer temperatures with adequate water supply could increase the quantity of bacterial spores in pasture.

A seasonal effect was seen in milk and concentrates, where summer samples were lower than winter (P < 0.01) for both and in the case of milk, these were lower than spring samples (P < 0.001). No seasonal variation was observed in the other feed types (Table 3-4). The seasonal variation in spore counts of bovine milk have previously been

reported for the Northern hemisphere, but contradict each other, either showing increases in summer (Buehner et al., 2014, Vissers et al., 2007) or decreases in summer (Kable et al., 2016) compared with the rest of the year. The results presented in this study are similar to Kable et al. (2016) who found low bacterial spore counts in summer and proposed that the season with the highest bacterial spore counts in milk depends on the country's climate, feeding practices and the grazing period. Most of the samples were collected in 2018, which was the second warmest year ever recorded in New Zealand (NIWA, 2019). The average yearly temperature of 13.4 °C in New Zealand that year, was similar to the 14.2 °C (California) in Kable et al. (2016), and more distant from the temperatures of 10.1 °C (Netherlands) in Vissers et al. (2007) and 7.1 °C (South Dakota) in Buehner et al. (2014) (NIWA, 2019, NOAA, 2019, KNMI, 2016). In this study, pasture and milk, both showed significantly higher bacterial spore counts in spring compared with summer ($P \leq 0.001$), indicating the possible transfer of spores from the feed into the milk (Table 3-4).

3.4.2 Identification of bacterial spore species in cattle feed and corresponding milk by MALDI-TOF analysis

Across all sample types, 80 spore-forming bacterial species were found across all sample types (Figure 3-1) (full dataset in Appendix A Table A-5). Of those 80 species, 36 were only detected in one or two sample types. However, a few species were detected across almost all sample types. All eight sample types contained *Bacillus cereus*, *B. licheniformis*, *B. subtilis* and *B. thermoamylovorans*, with *B. licheniformis* also being found in the most samples overall (found in 104 / 116 samples). Seven of the eight sample types contained *B. megaterium*, *B. pumilus*, *B. smithii*, *B. wiedmannii*, *B. weihenstephanensis, Clostridium sporogenes* and *Lysinibacillus sphaericus.* Of these 11 bacterial spore species, *B. cereus*, *B. licheniformis*, *B. smithii, B. subtilis*, *B. thermoamylovorans, B. pumilus* and *Clostridium sporogenes* were previously described in milk and cattle feed, (Masiello et al., 2014, Coorevits et al., 2008, Julien et al., 2008,

Scheldeman et al., 2005, Vaerewijck et al., 2001). *Bacillus weihenstephanensis, B. wiedmannii, B. megaterium, L. sphaericus* have only been previously reported in raw milk (Miller et al., 2015, Masiello et al., 2014, Coorevits et al., 2008). To the authors' knowledge this is the first report of these species in cattle feed.

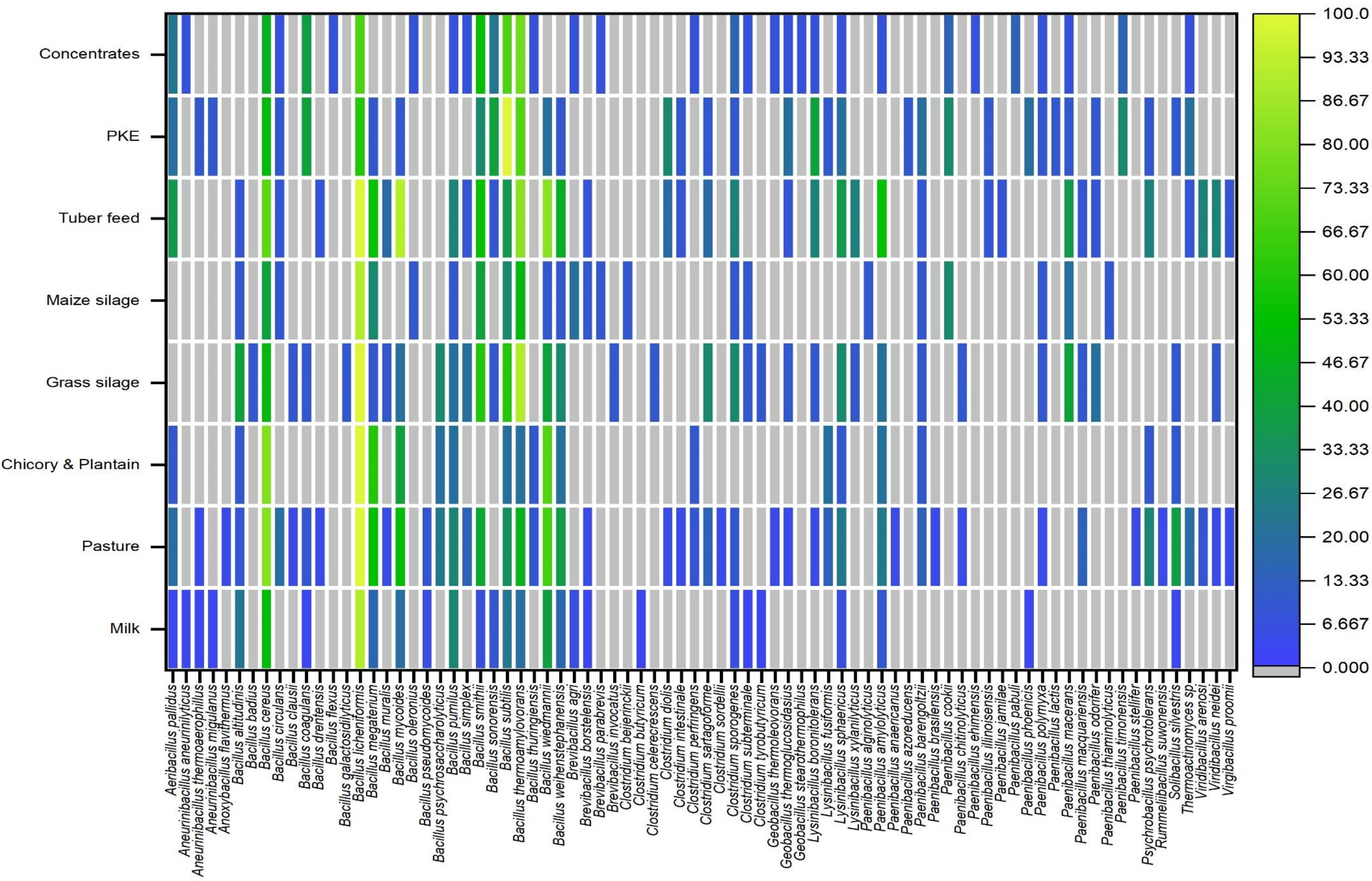

Figure 3-1 Heat map of spore-forming bacteria identified in cow feed and milk using MALD-TOF. Values are the percentage of samples that contained a species for each sample type. Grey boxes indicate the absense of the species, while abundance is shown on a color scale from blue (low) to yellow (high), as indicated on the right.

The diversity within sample types differed but the evenness of the sample types were between 83 – 92 %, indicating that the diversity within those samples was relatively even (Table 3-5). The greatest diversity of bacterial spore species was found in tuber feed, pasture, pasture silage and PKE, all of which showed a Shannon diversity index (*H`*) of 3.3 or more and the average sample contained eight or more different bacterial spore species. The lowest diversity was detected in milk, maize silage, chicory & plantain, with an *H`* of 2.8, 2.8 and 2.7 respectively and less than six species on average per sample. Concentrates were intermediate with an *H`* of 3.1 and around six species on average per sample. This roughly aligns to that PKE and tuber feed also contained greater quantities of spores compared to chicory & plantain or milk (Table 3-2 and Table 3-3).

Table 3-5 Bacterial spore species diversity in raw milk and cow feed indicated in Shannon diversity (H'), the maximum H' (H'-max), evenness and the average number of spore-forming species identified within each sample type.

	Milk	Pasture	Chicory and plantain	Pasture silage	Maize silage	Tuber feed	PKE	Concen trate
H` Shannon diversity	2.8	3.5	2.7	3.3	2.8	3.3	3.3	3.1
H'-max	3.4	3.9	3.0	3.6	3.2	3.6	3.6	3.5
Evenness	83	88	89	91	88	91	92	88
Avg. species per sample	4.1	9.7	5.7	9.3	5	11.6	8.4	6.2

Of the 80 bacterial spore species found, 51 were exclusively found in feed samples, 27 were found in feed and corresponding milk samples and one species (*Clostridium butyricum*) was found only in one milk sample. *Clostridium butyricum* has previously been recovered from milk, silage, soil and animal intestine (Cassir et al., 2016, Driehuis, 2013, Cremonesi et al., 2012).

The 23 % (18 / 79 species) of spore-forming bacterial species, which were identified most often in cattle feed samples (70 % (489 / 697) of all species identifications in feed), were all but two also found in milk. In milk these 16 species made up 57 % (16 / 28

species) of the bacterial spores found in milk and accounted for 83 % (106 / 128 identifications) of all species identifications across milk samples. Showing that the most common bacterial spore species in cattle feed are also the most common bacterial spore species in milk. This indicates a possible link between cattle feed as a source of milk contamination.

The composition of spore-forming bacterial species in milk or cattle feed in the literature is inconsistent, possibly due to different detection and selection methods used (Kent et al., 2016). However, culture-dependent studies indicate that *Bacillus* and, in some cases, *Paenibacillus* are the dominant bacterial spores in milk, with *B. circulans*, *B. licheniformis*, *B. pumilus*, *B. subtilis, B. cereus* and *B. weihenstephanensis* commonly reported (Masiello et al., 2014, Coorevits et al., 2008). The results presented in this study also identified *Bacillus* species (85 % of identified species) as the most common in milk, followed by *Clostridium* (4 %) and *Paenibacillus* (3 %). The six most commonly identified bacterial spore species in milk in this study, were *B. licheniformis* (contained in 90 % of milk samples), *B. cereus* (52 %), *B. pumilus* (29 %) and *B. subtilis* (23 %), as well as, *B. wiedmannii* (39 %) and *B. altitudinis* (23 %). *B. weihenstephanensis* (16 %) was less commonly found in milk samples. *B. circulans* was not identified within the milk.

Pasture silage and maize silage are the most investigated cattle feeds reported in the literature and are suspect sources of bacterial spores in milk. Silages have been identified as a potential source of *Clostridia* spp., *Bacillus* spp., or *Paenibacillus* spp.in raw milk (Driehuis, 2013, Julien et al., 2008). Driehuis (2013) summarized the results of 13 publications, which identified 26 aerobic and anaerobic spore-forming species in pasture maize and mixed silages. Since then, five other studies have identified an additional 16 spore-forming bacteria species in silage (Yin et al., 2021, Borreani et al., 2019, Driehuis et al., 2016, Zucali et al., 2015, Liu et al., 2013). Of the 42 species reported, 17 were also found in the present study and a further 30 species were found in silage for the first time in this study (Table 3-6).

Table 3-6 Summary of the bacterial spore species found in silage previously reported in literature, and present in this study. The total number of species in each category is included (n).

Novelty	Genus	Species
Previously detected in literature but not in this study (n=25)	*Bacillus*	*B. firmus; B. lentus; B. spiralis; B. sphaericus; B. sporothermodurans*
	Clostridium	*C. acetobutyricum; C. aerotolerans; C. aminovalericum; C. amygdalinum; C. baratii; C. bifermentans; C. disporicum; C. felsineum; C. guangxiense; C. jejuense; C. neuense; C. saccharolyticum; C. xylanolyticum*
	Other genera	*Brevibacillus choshinensis; L. fusiformis; Paenibacillus pabuli; P. peoriae; P. polymyxa; P. validus; P. thermophilus*
Previously detected, and also in this study (n=17)	*Bacillus*	*B. cereus; B. circulans; B. clausii; B. coagulans; B. licheniformis; B. oleronius; B. pumilus; B. sonorensis; B. subtilis*
	Clostridium	*C. beijerinckii; C. celerecrescens; C. sporogenes; C. tyrobutyricum*
	Other genera	*Br. borstelensis; P. barengoltzii; P. macerans; P. polymyxa*
Newly detected in this study (n=31)	*Bacillus*	*B. altitudinis; B. badius; B. galactosidilyticus; B. megaterium; B. muralis; B. mycoides; B. psychrosaccharolyticus; B. simplex; B. smithii; B. thermoamylovorans; B. wiedmannii; B. weihenstephanensis*
	Clostridium	*C. sartagoforme; C. subterminale*
	Other genera	*Br. agri; Br. parabrevis; Br. invocatus; Ly. boronitolerans; Ly. sphaericus; Ly. xylanilyticus; P. alginolyticus; P. amylolyticu; P. cookii; P. ehimensis; P. macquariensis; P. odorifer; P. thiaminolyticus; Geobacillus thermoglucosidasius; Solibacillus silvestris; Viridibacillus neidei*

In previous studies, 20 spore-forming bacterial species were identified in concentrate feeds (Table 3-7). The most common were *Aeribacillus pallidus*, *B. cereus*, *B. licheniformis, B. pumilus and B. subtilis* (Scheldeman et al., 2005, Vaerewijck et al., 2001). These were also found in concentrates analysed in the present study, as well as 24 species which were novel to concentrate feeds (Table 3-7). Eight species previously described in concentrates were not found in this study (Scheldeman et al., 2005, Vaerewijck et al., 2001).

Table 3-7 Summary of the bacterial spore species found in concentrates previously reported in literature, and present in this study. The total number of species in each category is included (n).

Novelty	Genus	Species
Previously detected in literature but not in this study (n=8)	*Bacillus*	*B. amyloliquefaciens; B. clausii; B. sporothermodurans*
	Other genera	*Aneurinibacillus thermoaerophilus; Br. borstelensis; P. illinoisensis; P. thiaminolyticus; Sporosarcina psychrophile*
Previously detected, and also in this study (n=12)	*Bacillus*	*B. cereus; B. circulans; B. flexu; B. licheniformis; B. oleronius; B. pumilus; B. smithii; B. sporothermodurans; B. subtilis*
	Other genera	*Br. agri; Ly. sphaericus; Aeribacillus pallidus*
Newly detected in this study (n=24)	*Bacillus*	*B. coagulans; B. simplex; B. smithii; B. sonorensis; B. thermoamylovorans; B. thuringiensis*
	Clostridium	*C. perfringens; C. sporogenes; C. subterminale*
	Other genera	*An. aneurinilyticus; Br.parabrevis; G. thermoleovorans; G. thermoglucosidasiu; G. stearothermophilus; Ly. boronitolerans; L. sphaericus; P. amylolyticus; P. cookii; P. ehimensis; P. pabuli; P. polymyxa; P. macerans; P. timonensis; Thermoactinomyces* sp.

While research data is available on the bacterial community in soil and the rhizosphere, the identity and abundance of bacterial spores present on the pasture leaves are not as well reported. The few studies on the bacterial spore species in pasture leaves have found *B. cereus, B. circulans, B. megaterium, B. mycoides* and *B. thuringiensis* (Damgaard et al., 1998, Giffel, 1997, Stout, 1960). All these species were found in the present study, as well as 45 species which were not previously described as present in pasture.

Tuber feed bulbs had not been research extensively for their spore-forming bacteria, prior to this study. Previous work focusing on the rhizosphere showed tuber feeds can contain *B. ginsengihumi*, *Brevibacillus reuszeri P. koleovorans*, *P. sediminis* and *Paenisporosarcina indica* (Zachow et al., 2014). This study examined pieces of the bulb and leaves to emulate how a cow consumes tuber feed. This resulted in 31 species which

were not previously described in tuber feed, potentially due to different parts of the plant being tested.

To the author's knowledge, no reports about the bacterial spore species in PKE or chicory and plantain were published until this study.

To understand whether there is a similarity between the spore-forming bacteria found in cattle feed and the corresponding milk samples, a PERMANOVA analysis was used (Table 3-8). Results showed that the spore species found in PKE and concentrate feeds were similar, but significantly different to all other sample types. In addition, even though PKE and tuber feeds contained the most diverse and largest number of bacterial spores (Table 3-2, Table 3-3 and Table 3-5), their spore species were significantly different from each other (Table 3-8). Similar bacterial species were present in pasture, chicory and plantain, and tuber feeds. It was hypothesised that these feed groups would have overlapping bacteria species due to growing in, or in proximity to, the soil. Pasture silage and maize silage contained similar populations, which is likely due to similarities in their production process. From the PERMANOVA analysis, the spore-forming bacteria species in milk were not different from those found in chicory and plantain or in maize silage (Table 3-8).These three sample types represent the sample types with the lowest bacterial spore counts and diversities (Table 3-2, Table 3-3 and Table 3-5). The few bacterial spore species found within these samples are the most common bacterial spores, giving these sample types similar bacterial spores (Table 3-5 and Table 3-8).

Table 3-8 PERMANOVA comparison of bacterial spore species present in cattle feed and milk.*

Sample type	Milk	Pasture	Pasture silage	Maize silage	Chicory and plantain	Concen trate	PKE	Tuber feed
Milk		<0.0001	<0.0001	0.0293	0.5481	<0.0001	<0.0001	<0.0001
Pasture	<0.0001		0.0031	0.0004	0.0661	<0.0001	<0.0001	0.2636
Pasture silage	<0.0001	0.0031		0.0539	0.0006	0.0319	0.003	0.0096
Maize silage	0.0293	0.0004	0.0539		0.002	0.0679	<0.0001	<0.0001
Chicory and plantain	0.5481	0.0661	0.0006	0.002		<0.0001	<0.0001	0.0002
Concentrate	<0.0001	<0.0001	0.0319	0.0679	<0.0001		0.3091	<0.0001
PKE	<0.0001	<0.0001	0.003	0.0001	<0.0001	0.3091		0.0002
Tuber feed	<0.0001	0.2636	0.0096	0.0001	0.0002	<0.0001	0.0002	

* calculated from Bray-Curtis distances and P-values were Holm-Bonferroni corrected, significant values ($P<0.005$) are indicated in grey

The identification of the same bacterial spore species in both cattle feed and milk, indicates that the bacterial spores within milk could originate from cattle feeds. Observed differences in the bacterial spore species of different cattle feeds could be utilised in strategies to reduce bacterial spore counts in milk.

The enrichment procedure for bacterial spores using milk as a growth medium, indicated that of the 80 bacterial spore species from milk and cattle feed, 56 were able to grow in milk under the conditions used in this study and showing the potential for these spore species to influence milk quality (Table 3-9). Further research is required to determine if all bacterial spore isolates found in this study pose a quality risk for milk products.

Table 3-9 List of the bacterial spore species divided into species found after enrichment in milk and species not detected after enrichment in milk.

Growth in milk	Genus	Species
Detected after enrichment in milk (n=56)	*Bacillus*	*B. altitudinis; B. cereu; B. circulans; B. coagulans; B. licheniformis; B. megaterium; B. muralis; B. mycoides; B. pseudomycoides; B. pumilus; B. simplex; B. smithii; B. sonorensis; B. subtilis; B. thermoamylovoran; B. thuringiensis; B. wiedmannii; B. weihenstephanensis*
	Clostridium	*C. beijerinckii; C. butyricum; C. dioli; C. intestinal; C. perfringens; C. sartagoforme; C. sporogenes; C. subterminale; G. thermoleovorans; G. thermoglucosidasius; Ly. boronitolerans; Ly. fusiformis; Ly. sphaericus; Ly. xylanilyticus; P. amylolyticus; P. barengoltzii; P. brasilensis; P. cookii; P. chitinolyticus; P. jamilae; P. pabuli; P. phoenicis; P. polymyxa; P. macerans; P. macquariensis; P. odorifer; P. thiaminolyticus; P. timonensis; P. stellifer*
	Other genera	*An. pallidus; An. aneurinilyticus; An. thermoaerophillus; An. migulanus; Br. agri; Br. Borstelensis; Br. Parabrevis; V. arenosi; V. neidei*
Not detected after enrichment in milk (n=24)	*Bacillus*	*B. badius; B. clausii; B. drentensis; B. flexus; B. galactosidilyticus; B. oleronius; B. psychrosaccharolyticus*
	Clostridium	*C. celerecrescens; C. sordellii; C. tyrobutyricum*
	Other genera	*Anoxybacillus flavithermus; Br. invocatus; G. stearothermophilus; P. alginolyticus; P. anaericanus; P. azoreducens; P. ehimensis; P. illinoisensis; P. lactis; Psychrobacillus psychrotolerans; Rummeliibacillus suwonensis; Solibacillus silvestris; Thermoactinomyces* sp*; V. proomii*

3.4.3 Analysis of bacterial spores in cow feed and milk using metagenomics

Culture-dependent experiments indicate that milk had the lowest numbers of bacterial spores while tuber feed and PKE had the greatest. These samples were selected for a 16S rRNA metagenomic analysis to compare a culture-independent approach to detect bacterial spores with bacterial spores obtained through culture.

Of the two milk samples analysed, one returned a bacterial spore identified down to the species level, *P. ginsengihumi*, and the other one failed to return reads from bacterial spore species (Table A-6). This indicates that this method of testing was not well suited

to identify bacterial spores down to the species level in milk. A likely reason for that is the low levels of bacterial spores within the milk. A likely reason for that is the low levels of bacterial spores within the milk, despite efforts to enrich the bacteria. For future approaches, a step to remove DNA which is not from bacterial spores, using a heat treatment followed by a propidium monoazide treatment or a different DNA removal method would be recommended (Stinson et al., 2019). For a better yield of spores from milk, longer incubation steps than those used in this study could be considered but that could lead to reduced detectable diversity and alter the quantity ratios between the more abundant and less abundant species naturally present.

In PKE, 34 bacterial spore species were detected and 18 in tuber feed. The most common bacterial spore species were from the genus of *Bacillus* and *Paenibacillus* (Figure 3-2 and Figure 3-3). Both PKE and tuber feed contained *P. ginsengihumi* which was detected in the milk sample.

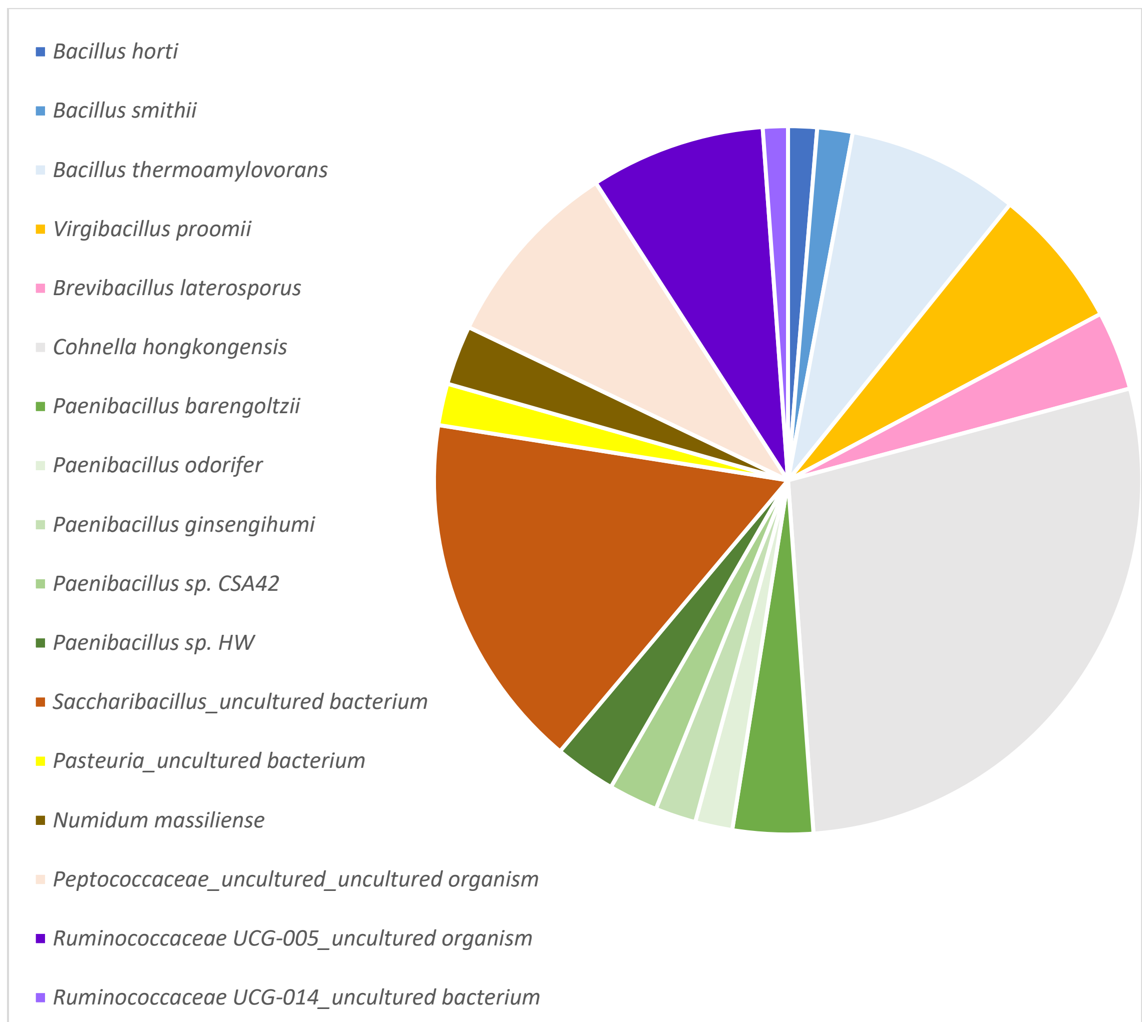

Figure 3-2 Bacterial spore reads per species, in percent of total reads, found in tuber feed. Species are ordered according to their taxonomic relationship.

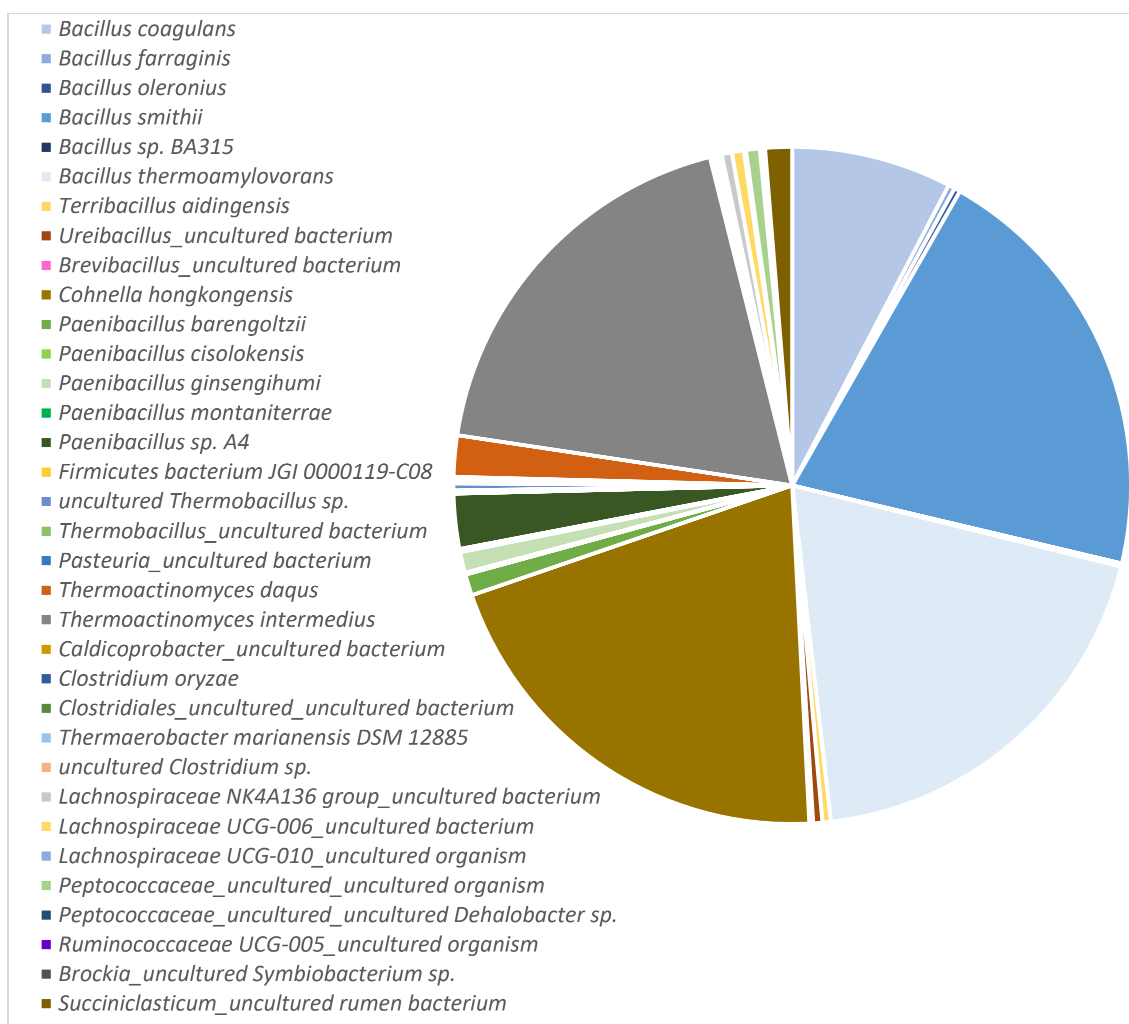

Figure 3-3 Bacterial spore reads per species, in percent of total reads, found in PKE. Species are ordered according to their taxonomic relationship.

Using metagenomics, some reads were identified as uncultured bacteria; 16/34 identified species in the PKE sample and 5/18 identified species in the tuber feed sample. These species are unlikely to be picked up with culture-based methods and indicating that around 47 % and 28 % of spores in PKE and tuber feed respectively, wouldn't be detected. However, these results represent two feed samples, further research is needed to deem how many bacterial isolates within cattle feed are truly unculturable.

For cattle feed, the diversity of bacterial spore species found, was higher using the metagenomics methods than using the culture dependent methods, while it was lower in milk (Table 3-10).

Table 3-10 Average number of spore species found per sample in PKE, tuber feed and milk samples, by culture dependent MALDI-TOF or culture independent metagenomic-16S rRNA analysis, as well as how many species were found in both.

Sample type	Metagenomic 16S rRNA analysis	MALDI-TOF analysis average species per sample type	Same species in both
PKE	34	8	4
Tuber feed	18	12	4
Milk	0.5	4	0

Metagenomics using 16s rRNA sequencing methodology may be more useful when used in conjunction with culture-based methods, to provide a more complete picture of the bacterial spore species within cattle feeds.

Comparing the identification by MALDI-TOF and 16S rRNA metagenomics sequencing, across tuber feed and PKE samples, only around 22 % (6/21) of the identified genera and 6 % (5/82) of species were the same, which is similar to previously observed differences of 15 % between the methods (Bilen et al., 2018). A reason for the differences is that the 16S rRNA sequencing can detect unculturable bacterial spore species, reducing the chance of detecting all the same species as the culture-based method. The differences in species identified, could have originated from the accuracy of the identification methods themselves. The SILVA 16S rRNA gene database trained identifier for the 16S rRNA gene identification has a bias, influenced by which strains are available in it, just like the Brunker database used by the MALDI-TOF, which may lead to differences in species identified.

A limitation of this investigation is the sample size with only two milk, one PKE and one tuber feed samples undergoing 16S rRNA metagenomic analysis. However further 16S rRNA sequencing would be required to gain insight into bacterial species across multiple samples to make a claim on the unculturable spores in cattle feed or milk.

3.5 Conclusion

This study identified the bacterial spore populations in dairy cattle feeds, some of which had not previously been investigated, and found significant differences in the bacterial spore populations between feed types. It showed that most bacterial spore species identified in raw milk were also present in the feeds fed to the dairy cows producing that milk. The data supports the hypothesis that bacterial spores may transfer from the feed into the milk. It further produced evidence for what species of bacterial spores can grow within milk. Metagenomic analysis can be used to assist the exploration of bacterial spore species in cattle feed but needs improved methodologies for the detection of spore DNA in milk. The knowledge obtained throughout this study, is intended to aid in the control of bacterial spore contamination in dairy production.

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Chapter 4: Genomic comparison of bacterial spores from dairy cattle feed and milk

4.1 Abstract

Cattle feed has been purported as a potential source of spore-forming bacteria in milk, which can lead to product spoilage. To identify links between bacterial spore isolates from milk and cattle feed, this study sequenced the genomes of 109 spore-forming bacteria from 9 different species isolated from dairy cattle milk and feed. No isolates from feed and milk were found to directly match (<4-7 SNPs depending on the species), based on the limits set for this study. Closely related genomes (<60 SNPs) from milk and feed were however found for *B. licheniformis* and *B. pumilus* isolates. Multiple isolates were identified that matched or were closely related to other isolates from the same feed or milk source, which occurred more frequently in milk isolates than feed isolates. This indicates that the diversity of genomes is potentially greater in feed than in milk.

This study presents evidence for a transfer chain of bacterial spores from cattle feed to milk, however a more intensive study is required to confirm.

4.2 Introduction

Bacterial spores once present in milk, are difficult to remove from products as they can survive cleaning and control methods, commonly used in dairy production and can lead to product spoilage (Doyle et al., 2015, Gopal et al., 2015, Ledenbach and Marshall, 2009). The ideal approach to limit the amount of bacterial spores in dairy products, is to prevent the spores from entering into milk, for which knowledge of their origin is advantageous (McHugh et al., 2017). A hypothesis for the origin of bacterial spores in milk is that the spores originating in cattle feed pass through the digestive tract and contaminate the skin of the udder from where they transfer into the milk (Buehner et al., 2014, Vissers et al., 2007b, Gill et al., 2006). Studies examining this transfer usually

identify spore species in a potential source and raw milk using species specific culturing methods or single gene-based approaches like 16S rRNA sequencing (Martin et al., 2019, Gupta and Brightwell, 2017, Vissers et al., 2007a, Scheldeman et al., 2005). Conclusions are then drawn based on correlations of the abundance or absence of species in both the source and milk. These methodologies only allow identification to the genus or species level, however, there are often multiple strains within a species. In the case of *B. cereus* there are approximately 160 strains listed on the National Center for Biotechnology Information (NCBI) database (www.ncbi.nlm.nih.gov/taxonomy/?term=1396[uid] accessed 18.11.2020). Methods based on WGS can compare isolates beyond the species level allowing detailed tracking of bacteria (Schurch et al., 2018). Increasingly used as a measure of divergence to distinguish between bacterial strains are SNP analyses, due to their high resolution and evolutionary stability (Van Ert et al., 2007, Keim et al., 2004, Pearson et al., 2004).

A threshold of SNP differences between isolates which are considered clonal or related must be defined before analysing bacterial genomes, to avoid falsely connecting more distantly related isolates (Jia et al., 2019, Salipante et al., 2015). Within this threshold analysis there is a “clonal” or “match” threshold (isolates very recently separated) and a “clustering” threshold (isolates recently diverged - they are closely related but not directly descended) and the remainder defined as “unrelated” or “distantly related”. This is often done for clinical isolates to determine if outbreaks are related or determining the specific source of pathogenic bacteria (Carroll et al., 2019, Walker et al., 2018). Some studies determine their own thresholds while others rely on literature. Pightling et al. (2018) reviewed 17 studies that tracked bacteria based of SNPs and recommends under 20 SNPs for matching isolates, however, they encourage all available information on the genomes be taken into account when determining a threshold. This study took both current data and the literature into account to determine a threshold for matching genomes at 4-7 SNPs and genomes which cluster together at 60 SNPs. The aim of this study was to use WGS analysis to provide evidence to test the hypothesis that cattle

feed is a source of bacterial spores in milk and to examine the genotypic diversity of bacterial spore species in cattle feed and milk.

4.3 Methods

4.3.1 Bacterial isolates

Bacterial isolates were isolated from cattle feed and milk and identified by MALDI-TOF as described in chapter 3. The sample origin and MALDI-TOF identifications are listed in Table 4-1 (detailed version in Table B-1). A total of 109 isolates, 65 from cattle feed and 44 from milk, were selected for sequencing. The isolates belonged to 10 different species, of which nine were *Bacillus* species commonly found in milk and cattle feed, with a focus on *B. licheniformis* as it was the most common bacterial species across cattle feed and raw milk and one (*Paenibacillus phoenicis*) which was rarely found (Chapter 3; Figure 3-1). Isolates were chosen from different farms and seasons to assess differences in genomes over different geographic and temporal conditions. The isolates g117, g197 and g213 were sequenced twice as controls leading to a total of 112 genomes used in this study.

Table 4-1 Origin and species of isolates[a].

Species	Total number of isolates	From milk	From feed	Number of farms isolates originated from	Number of seasons isolates originated from	Growth temperature
B. cereus	18	5	13	4	3	37°C
B. licheniformis	55	24	31	6	4	37°C
B. mycoides	5	2	3	1	2	30°C
B. pumilus	8	5	3	1	2	37°C
B. subtilis	6	4	2	2	3	37°C
B. thermoamylovorans	8	1	7	1	2	55°C
B. weihenstephanensis	5	1	4	1	2	30°C
B. wiedmannii	2	1	1	1	1	30°C
P. phoenicis	2	1	1	1	1	37°C

[a] Detailed description can be found in appendix B (Appendix B Table B-1)

4.3.2 DNA extraction, library preparation and sequencing

To sequence the genomes of a126, a131, a321, a157, a189, a191, a338, g117, g197, g213, t93 and t417, the isolates were taken from cryo-cultures (-80°C), streaked onto TSA agar and grown over night at 37°C. The genomic DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Germany), as per the manufacturer's specifications except for the use of a lysozyme lysis buffer instead of the manufacturer´s ATL buffer.

Libraries were prepared from genomic DNA using the Nextera XT library preparation kit (Illumina, USA) as per the manufacturer's instructions except for increasing the number of cycles in the amplification step to 15 to increase yield. Two of the samples, t93 and a131 failed to amplify using the Nextera XT library preparation kit so they were prepared using the Illumina DNA prep kit (Illumina, USA). The library(s) were QC checked using the PerkinElmer GX Touch HT Instrument using the HT DNA NGS 3K Reagent Kit (PerkinElmer, UK) and by qPCR following the Illumina's Sequencing Library qPCR Quantification Guide. Sequencing was performed with Illumina HiSeq X sequencing (Illumina, USA) at the Fonterra Research and Development Centre (New Zealand).

All other isolates and a repeat culture of g117, g197 and g213 isolates, were taken from cryo-cultures and streaked onto TSA agar and grown over night at their respective growth temperature (Listed in appendix B, Table B-1). Isolates were then transferred onto TSA and grown for up to 48 h until sufficient sized colonies for picking were available. DNA was extracted using the Quick-DNA Fungal/Bacterial Miniprep kit (Zymo Research, USA) as per the manufacturer's instructions. Libraries were prepared using the Illumina DNA prep kit (Illumina, USA) and sequencing was carried out on a MiSeq (Illumina, USA).

4.3.3 Data analysis

Sequencing reads were assessed for their quality using FastQC (version 0.11.9), trimmed using Trimmomatic (version 0.39) and de-novo assembled using SPAdes (version 3.14.0) (Bolger et al., 2014, Bankevich et al., 2012, Andrews, 2010). The assembly statistics were gathered using Quast (version 5.0.2) (Gurevich et al., 2013).

Variant calling between the genomes of the same species based of their MALDI-TOF identification was carried out using Snippy (version 4.6.0), Gubbins (version 2.3.1) and SNP-dists (version 0.7.0) (Seemann, 2018, Croucher et al., 2015, Seemann, 2015). When using Snippy the “snippy-multi” command and the “snippy” command were used, with the multi option used if more than two isolates were to be compared or default state for two isolates. Gubbins was run with default settings, with the exception of filter percentage which was set to greater than 20 % of the genome (average length of unaligned genome to the reference was five percent). The reference genomes for the analyses, were chosen randomly from the available milk isolates for each bacterial species. If more than one isolate was filtered out during variant calling, additional analyses were run using the other milk isolates as references. After that step, the milk isolate was chosen with the fewest other isolates filtered out of the analysis and filtered out isolates were aligned to each other. Isolates that did not align to any other isolate, were declared an independent cluster. The isolates SNP differences were then visualised in heat maps using GraphPad Prism version 9 (GraphPad Software, USA).

Due to reports discussing if *B. weihenstephanensis* and *B. mycosis* are indeed sperate species, an additional analysis looking for clustering between isolates within those species was included (Liu et al., 2018).

4.4 Results and discussion

4.4.1 Estimation of SNPs thresholds for matching isolates, highly related isolates and distantly related isolates

Single nucleotide polymorphism thresholds to identify isolates as matching are set to determine transmission routs of bacterial infections (Walker et al., 2018, Mair-Jenkins et al., 2017, Taylor et al., 2015). A recommended threshold from literature is less than 20 SNPs, with consideration of any additional relevant data in the study, when a bootstrap analysis shows a monophyletic relation in more than 90 % of iterations (Pightling et al., 2018). To gather additional data on matching isolates in this dataset, the three *B. licheniformis* isolates g117, g197 and g213 were sequenced twice to provide an internal control between the two sequencing labs. The genomes of the isolates once assembled were on average 4,276,346 ±59753 base pairs (bp) long (Table B-2). The genome assemblies of g117, g197 and g213 from each sequencing run were compared to each other. This comparison found, three, four and one SNPs respectively between the genomes of different sequencing runs. This indicates that an average of 2.7 ± 1.5 SNPs could be introduced by sequencing error or mutation (during growth of the isolates for DNA extraction) per 4,276,346 bp or an error/mutation rate of 6.24E-07 per base. This leads to the conclusion, that an isolate which has transferred from cow feed to milk, should not display more SNPs that that. With this data, a threshold value of SNPs (X) for calling matching isolates was calculated by using the formula:

$$X = (r_{em} * A_{len}) + (2\sigma_{em} * A_{len}).$$

Were A_{len} is the average alignment length in bp r_{em} is the error/mutation rate per bp and σ_{em} is the standard deviation of r_{em}. The resulting threshold values the investigated species are presented in Table 4-2. Between four and seven SNPs were expected to call the genome of two isolates a match for this present study. In the literature the threshold of SNPs to call related isolates differs between authors and species and can vary from zero to ≤ 37 SNPs as the review of 27 studies showed (Schurch et al., 2018). For *Bacillus*

isolates, studies have used thresholds of four, eight, 12 and 20 SNPs, which aligns with the thresholds used in this study (Frentzel et al., 2020, Carroll et al., 2019, Glasset et al., 2018).

The low number of SNPs found between the two sequencing runs of g117, g197 and g213 shows that the two methods used, were similar enough to compare the genomes from the different runs.

Table 4-2 Average length of genomes and calculated SNP threshold to call isolates a match, per species.

Species	*B. thermoamylovorans*	*B. pumilus*	*B. subtilis*	*B. licheniformis*	*B. cereus*	*B. mycoides*	*B. weihenstephanensis*
Avg. length of genome alignment in bp	3,076,651	3,392,766	3,943,386	3,974,407	4,897,311	4,911,821	5,276,010
Max SNPs expected if isolates are matching	4	5	5	5	7	7	7

After reviewing the 1280 genome comparisons measured (Figure 4-2 - Figure 4-10), a trend was observed where most isolates (99 % of total isolates) were either <60 SNPs (7 %) or > 300 SNPs apart (92 %) (Figure 4-1). Following this observation of the distribution of SNP counts between isolates, a threshold of <60 SNPs was chosen to define closely related but not matching isolates which form a cluster together. It is similar to the reported values of 10-115 SNP's for closely related but not matching *Bacillus* isolates (Stevens et al., 2019, Okinaka et al., 2011). The distance between different

clusters of *Bacillus* isolates has previously been reported as between 226 - 390 SNPs (Stevens et al., 2019, Girault et al., 2014). A genome difference of 60 SNPs also aligns roughly with being ten times the SNP distance to call a match. Isolates of the same species with more than 60 SNPs between them will be referred to as distantly related.

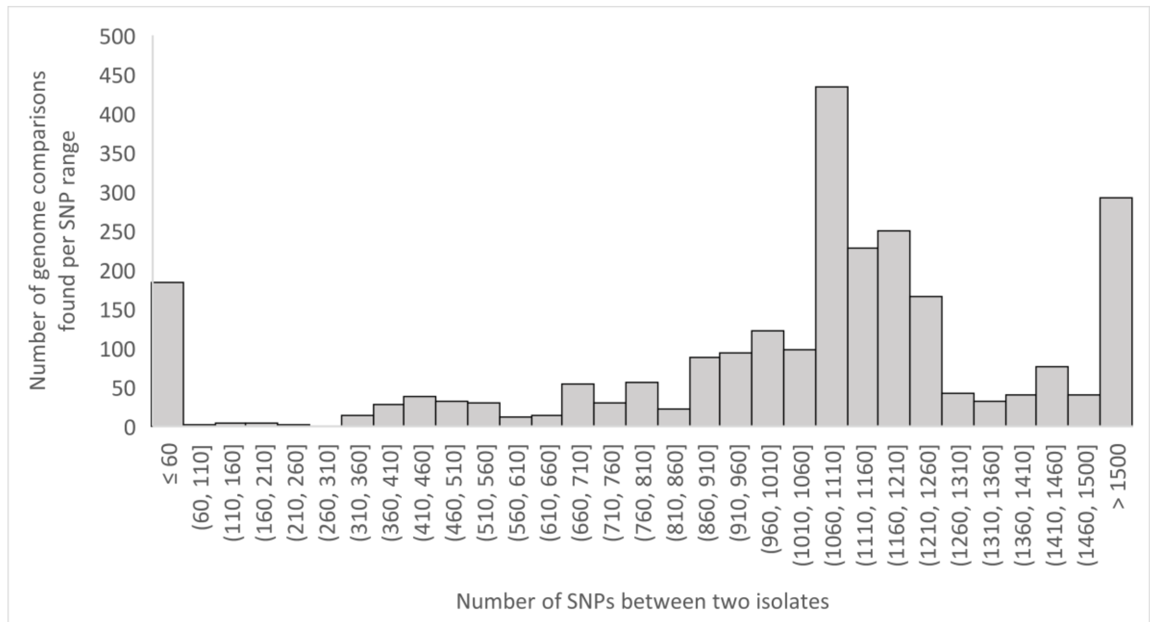

Figure 4-1 Histogram of the number of genome comparisons which found SNPs in the range between 60 and 1500 SNPs in steps of 50 SNPs.

4.4.2 Genome comparison of bacterial spore isolates from dairy cattle milk and feed

The exact numbers of SNPs found between the isolates of each species, is listed in Figure 4-2 - Figure 4-10 with the exception of the isolates from *B. wiedmannii* and *P. phoenicis* (t213, t489, t417 and t93), as their isolates failed to align more than 80 % of their genomes. Their isolates were all defined as separate strains and therefore excluded from further analysis. Among the remaining 105 isolates genomes, 22 matching isolates (SNP difference ≤ threshold) were found (Summary of matches can be found in Table 4-3 and a summary of clusters identified in Table 4-4). In all cases matches were between isolates from the same sample type, farm and season. Of the matching isolates,

12 were milk isolates which matched another milk isolate and 10 were pasture isolates which matched another pasture isolate.

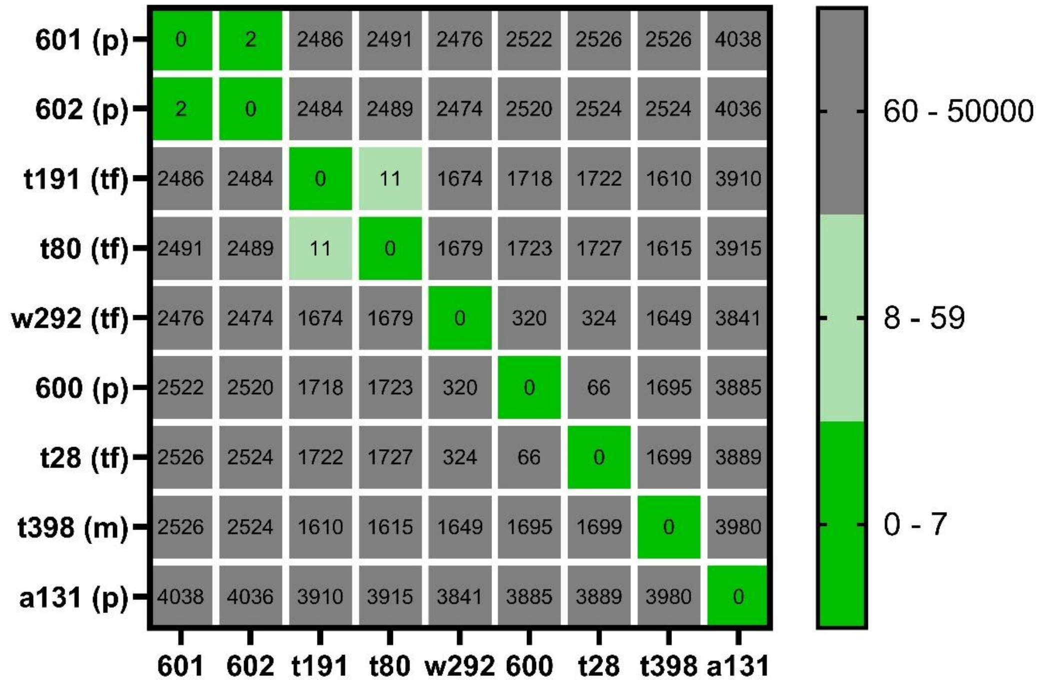

Figure 4-2 Observed SNPs between isolates from the first set of B. cereus isolates, which aligned to the reference isolate t398. Dark green fields indicate matching isolates, light green highly similar isolates, grey distantly related isolates and white fields with crosses indicate that an isolate did not align to another isolate. Isolate originated from milk (m), pasture (p) or tuber feed (tf), which is indicated in brackets following the isolate names on the left.

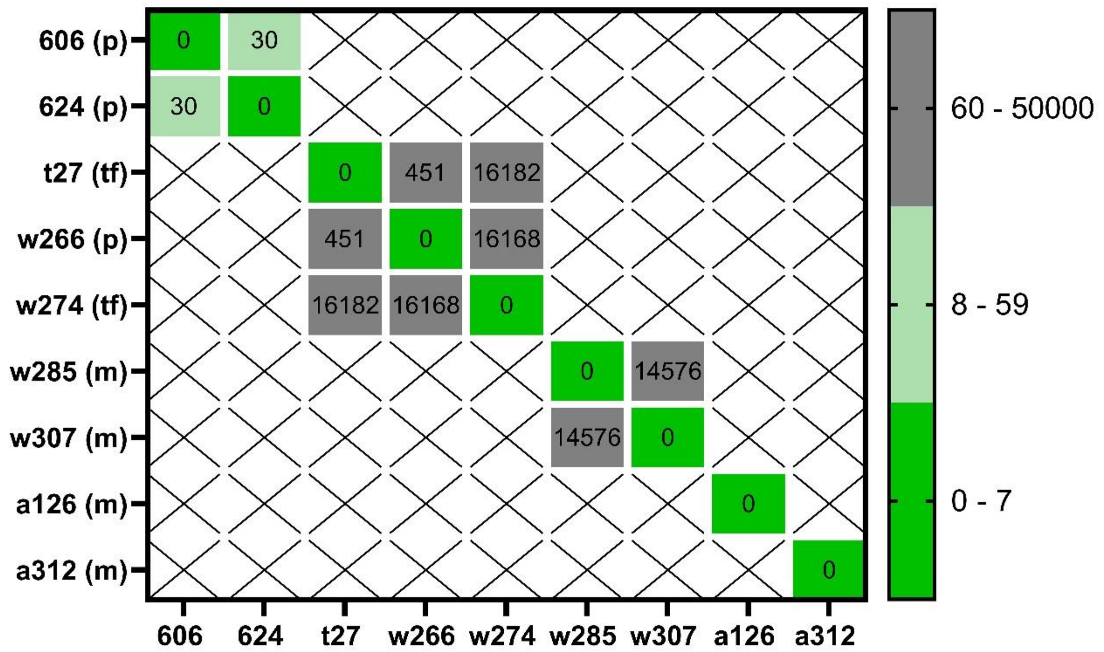

Figure 4-3 Observed SNPs between B. cereus isolates which did not align with the reference in the first set of B. cereus isolates but instead to 606, t27 or w285. Dark green fields indicate matching isolates, light green highly similar isolates, grey distantly related isolates and white fields with crosses indicate that an isolate did not align to another isolate. If an isolate originated from milk (m), pasture (p) or tuber feed (tf) is indicated behind the isolate names on the left..

60 - 50000

6 - 59

0 - 5

Figure 4-4 **Observed SNPs between isolates from the first set of *B. licheniformis* isolates, which aligned to the reference isolate 349. Dark green fields indicate matching isolates, light green highly similar isolates, grey distantly related isolates and white fields with crosses indicate that an isolate did not align to another isolate. If an isolate originated from milk (m), maize silage (ms), pasture (p), PKE (pke) or tuber feed (tf) is indicated behind the isolate names on the left.**

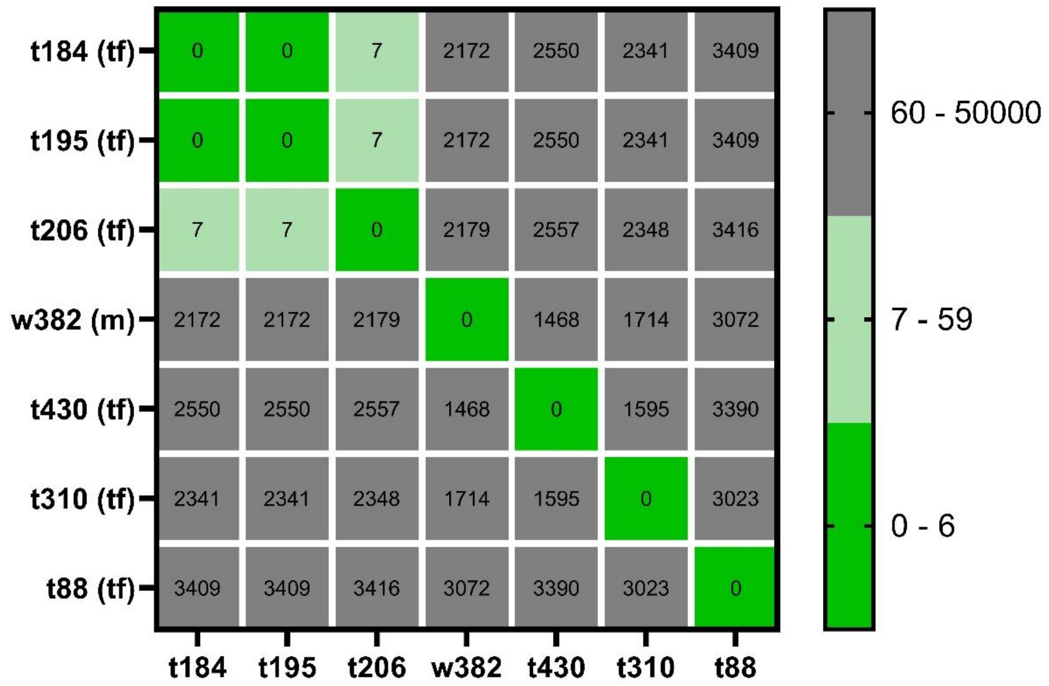

Figure 4-5 Observed SNPs between B. licheniformis isolates which did not align with the reference in the first set of B. cereus isolates but instead to the reference isolate w382. Dark green fields indicate matching isolates, light green highly similar isolates, grey distantly related isolates and white fields with crosses indicate that an isolate did not align to another isolate. If an isolate originated from milk (m) or tuber feed (tf) is indicated behind the isolate names on the left.

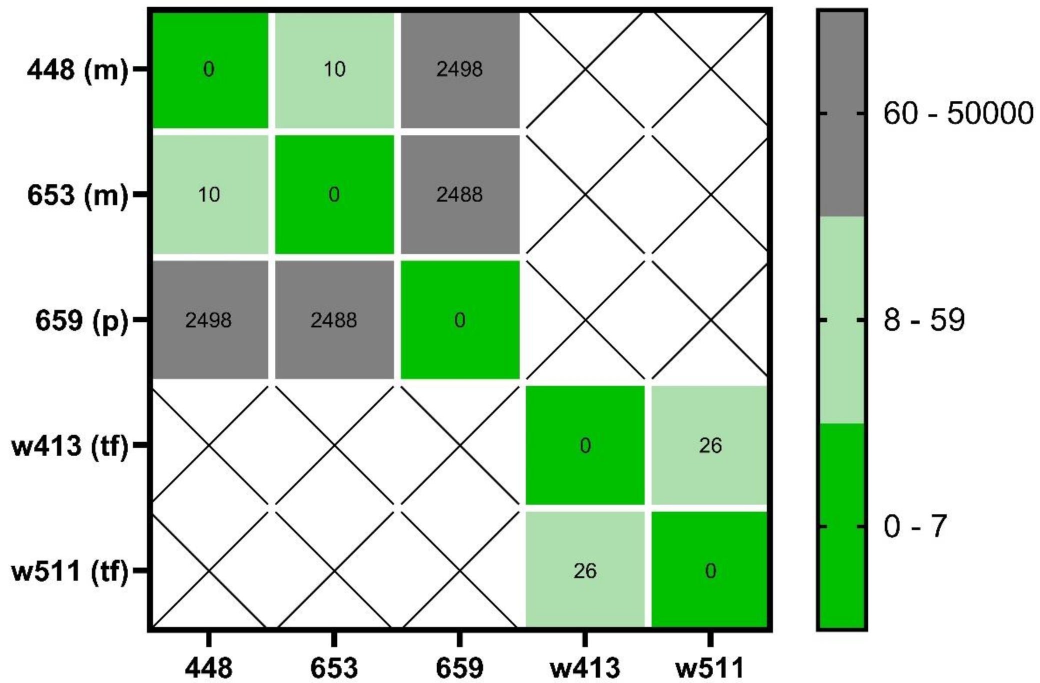

Figure 4-6 Observed SNPs between B. mycoides isolates which aligned to the reference isolates 448 or w413. Dark green fields indicate matching isolates, light green highly similar isolates, grey distantly related isolates and white fields with crosses indicate that an isolate did not align to another isolate. If an isolate originated from milk (m), pasture (p) or tuber feed (tf) is indicated behind the isolate names on the left.

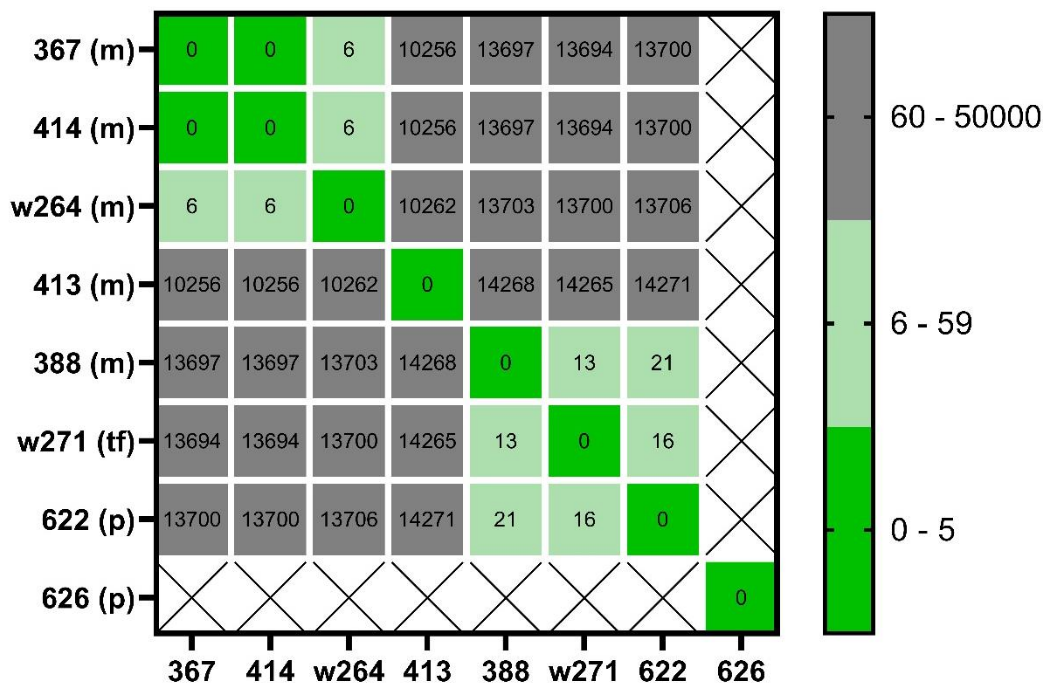

Figure 4-7 Observed SNPs between B. pumilus isolates aligned to the reference isolate 388. Dark green fields indicate matching isolates, light green highly similar isolates, grey distantly related isolates and white fields with crosses indicate that an isolate did not align to another isolate. If an isolate originated from milk (m), pasture (p) or tuber feed (tf) is indicated behind the isolate names on the left.

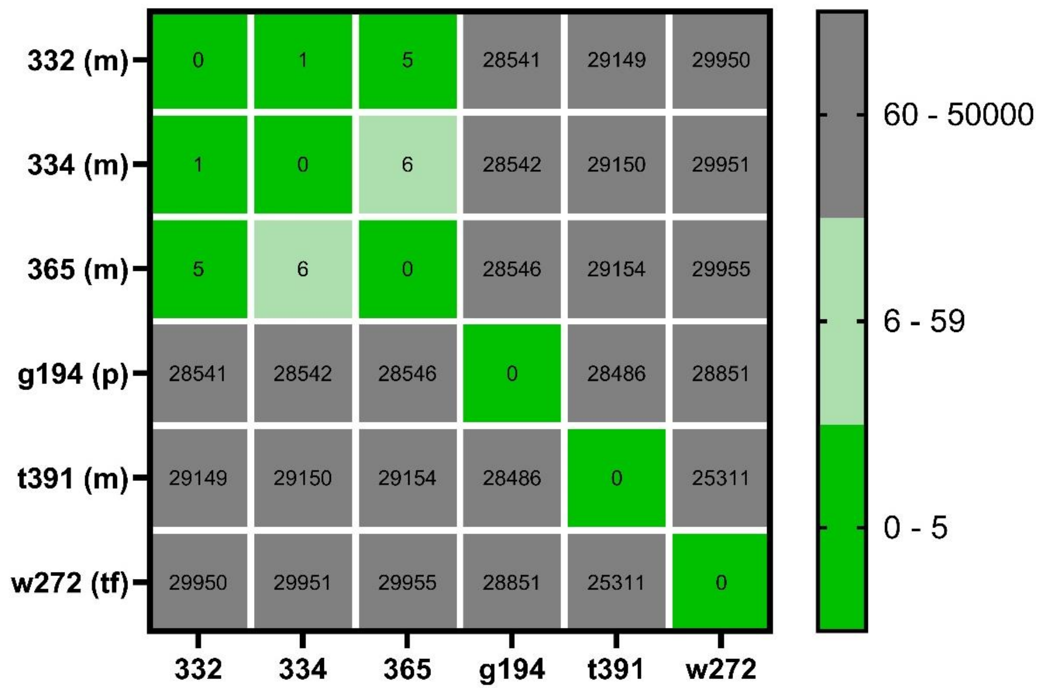

Figure 4-8 Observed SNPs between B. subtilis isolates aligned to the reference isolate 334. Dark green fields indicate matching isolates, light green highly similar isolates, grey distantly related isolates and white fields with crosses indicate that an isolate did not align to another isolate. If an isolate originated from milk (m), pasture (p) or tuber feed (tf) is indicated behind the isolate names on the left.

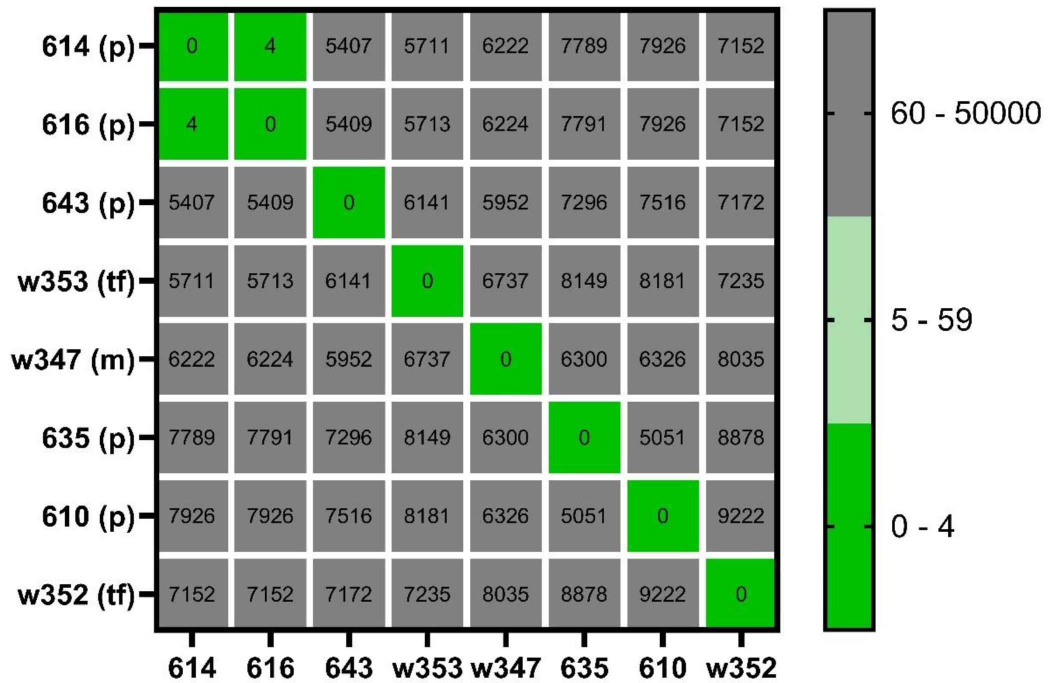

Figure 4-9 Observed SNPs between B. thermoamylovorans isolates aligned to the reference isolate w347. Dark green fields indicate matching isolates, light green highly similar isolates, grey distantly related isolates and white fields with crosses indicate that an isolate did not align to another isolate. If an isolate originated from milk (m), pasture (p) or tuber feed (tf) is indicated behind the isolate names on the left.

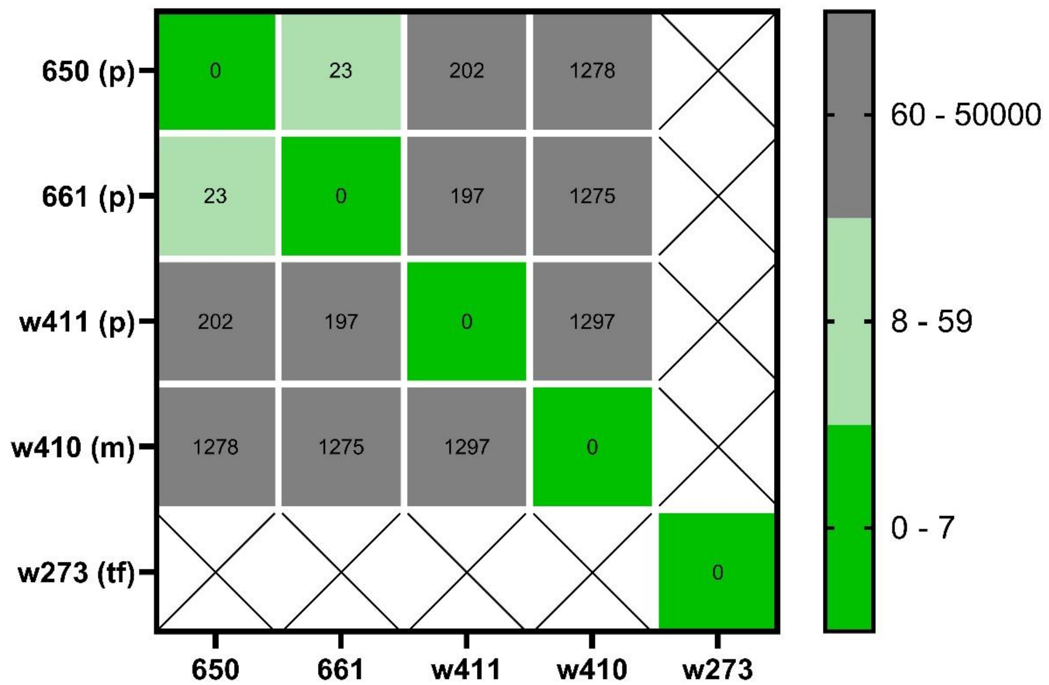

Figure 4-10 Observed SNPs between B. weihenstephanensis isolates aligned to the reference isolate w410. Dark green fields indicate matching isolates, light green highly similar isolates, grey distantly related isolates and white fields with crosses indicate that an isolate did not align to another isolate. If an isolate originated from milk (m), pasture (p) or tuber feed (tf) is indicated behind the isolate names on the left.

Table 4-3 Origin and SNP differences of isolates whose genomes matched.

	Isolate 1					Isolate 2			
Species	Season	Farm	Sample	ID	SNPs	Season	Farm	Sample	ID
B. cereus	Summer	26	Pasture	601	0	Summer	26	Pasture	602
B. licheniformis	Summer	26	Milk	366	0	Summer	26	Milk	428
B. licheniformis	Summer	26	Pasture	640	0	Summer	26	Pasture	609
B. licheniformis	Spring	7	Pasture	g103	1	Spring	7	Pasture	g117
B. licheniformis	Spring	7	Pasture	g134	0	Spring	7	Pasture	g260
B. licheniformis	Autumn	36	Milk	t396	0	Autumn	36	Milk	t415
B. licheniformis	Autumn	36	Milk	t406	1	Autumn	36	Milk	t501
B. pumilus	Summer	26	Milk	367	0	Summer	26	Milk	414
B. subtilis	Summer	26	Milk	332	1	Summer	26	Milk	334
B. subtilis	Summer	26	Milk	332	5	Summer	26	Milk	365
B. thermoamylovorans	Summer	26	Pasture	614	4	Summer	26	Pasture	616

Among the total of 44 milk isolates, 40 had at least one other milk isolate in their respective species, while across all 65 feed isolates, 63 had a second feed isolate among the other isolates of their species they could have matched. Matches between milk isolates were observed in 30 % (12 / 40) of isolates and thereby more common than in cattle feed samples where 16 % (10 / 63) matches were found. This was also observed when looking at isolates which were closely related ($\leq$60 SNPs), a summary of which can be found in Table 4-4. Among the milk isolates 61 % (27 / 44) joined a cluster with other closely related isolates compared to 45 % (29 / 65) of feed isolates, indicating that feed samples potentially have greater genome variation than the milk isolates.

Table 4-4 Summary of clusters of closely related isolates found per species across sample typs, seasons and farms.

Species	*P. phoenicis*	*B. wiedmannii*	*B. weihenstephanensis*	*B. mycoides*	*B. subtilis*	*B. thermoamylovorans*	*B. pumilus*	*B. mycoides*	*B. cereus*	*B. licheniformis*
Total isolates	2	2	5	5	6	8	8	5	18	55
Clusters total	2	2	4	4	4	7	4	4	15	27
Clusters across sample types	0	0	0	0	0	0	1[b]	0	0	7[a]
Clusters between feed and milk	0	0	0	0	0	0	1	0	0	5
Clusters between different feeds	0	0	0	0	0	0	0	0	1	3
Clusters across seasons	0	0	0	0	0	0	2	0	0	8
Clusters across farms	0	0	0	0	0	0	0	0	0	9

[a] Number of clusters and sample types cluster occurred in: 3x milk, tuber feed; 1x milk, pasture; 1x milk, pasture, PKE; 1x maize silage, pasture; 1x pasture, tuber feed

[b] Sample types in cluster: milk, pasture, tuber feed

This trend of lower diversity in milk samples compared to feed, was also seen when comparing how many isolates from a sample type did not cluster together with any other isolate, where 36 % (15 / 42), 44 % (12 / 27) and 56 % (18 / 32) of isolates from milk, pasture and tuber feed respectively, were distantly related (>60 SNPs) to all other isolates. These results indicate a higher genomic diversity in tuber feed isolates followed by pasture then milk. This is supported by previous findings which indicated that tuber feeds contain a higher number of spores and higher diversity of bacterial spore species compared with pasture, which itself contained a higher quantity and diversity of bacterial spore species than milk (Chapter 3). These differences in population size could partially contribute to differences in genome diversity, as bacterial isolates grown to greater numbers go through more generations, providing a greater chance to obtain different mutations, due to different selective pressures in the different environments (Bosshard et al., 2017, Foster, 2007). A potential reason for the lower genomic diversity in milk is that only a small portion of the spores a cow is exposed to from cattle feed, transfer into milk. The causes of this could be due to, death in the digestive tract or good in shed hygiene practises to reduce transfer into the milk from the udder skin (Magnusson et al., 2007, Magnusson et al., 2006). There may be some selective pressures exerted upon bacteria, selecting certain isolates to make it into milk at greater rates. This could be linked to findings of bacterial spores species found in milk, possessing adaptations to milk processing environments (Ostrov et al., 2019, Burgess et al., 2017). The greater bacterial diversity observed in tuber feed could be explained by the rich diversity of microbiota in soil, which can contain up to 10^9 spores per gram of dry soil (Orem et al., 2019, Brandes-Ammann et al., 2011, Logan and De Vos, 2011). No assessment can be made about the genetic diversity of isolates from maize silage or PKE since less than 3 samples were tested.

The matching isolates found, originated for the same farm and sample type in all cases (Table 4-3). No isolates from cattle feed and milk were found to match in this study. Of clusters of highly similar isolates (< 60 SNPs) 9 % (6 / 69 clusters) contained milk and

feed isolates clustered together (Table 4-4). This indicates that bacterial spores in feed and milk can be genetically similar, indicating a shared linage between these bacteria. However, under the requirements for matching isolates within this study, no conclusive evidence was found for a link. Most isolates in this study (80 %) did not match with any other isolate, indicating that the diversity of genomes was larger than the number of samples tested could observe. Bacterial cells of the same origin will accumulate SNPs over time, so that a population of bacterial cells which originated from the same cell, can be represented by multiple genomes which are denoted by different numbers of SNPs between them (Ford et al., 2011). Obtaining a statistically significant sample size when there is no previous data available on the genome diversity of bacterial spores in cattle feed and milk, makes the selection process challenging. The only species in which clusters of highly similar isolates between milk and feed isolates were identified, were *B. pumilus* and *B. licheniformis*, which had 25 % (1/4) and 19 % (5/27) of their clusters respectively containing both feed and milk isolates. This indicates that in cattle feed these species potentially have lower genomic diversity than other species. In comparison *B. thermoamylovorans* and *B. cereus*, which had at least the same number of isolates tested as *B. pumilus* (8, 18 and 8 isolates sequenced respectively) showed no clustering across milk and cattle feed samples.

As well as no matches between feed and milk isolates, there were no matches found between isolates which originated from different feeds, seasons or farms. The absence of such matches indicates that the matches that were found are unlikely to be the result of laboratory contamination. Additionally, thresholds chosen to designate matching isolates in this study, are narrow enough to adequately select for recent transfer events. Some similarities between isolates of different origin can be found when looking at the clustering of highly similar genomes (< 60 SNPs distance). Among species with isolates from different feed types (all species except for *B. wiedmannii* and *P. phoenicis*), 65 clusters formed, of which four (6 %) contained isolates from different feeds. These clusters between different feed types were found in *B. licheniformis* and *B. pumilus*

isolates (Table 4-4). Only *B. cereus*, *B. licheniformis* and *B. subtilis* contained samples from different farms (Table 4-1). The isolates of these three species formed 42 clusters of which 9 (21 %) contained isolates from different farms (Table 4-4). This clustering between farms occurred only in *B. licheniformis*. This similarity of isolates across distances could be due to the ability of *B. pumilus* and *B. licheniformis* to spread airborne between farms or sample types (Bayle et al., 2021). However, *B. cereus*, *B. mycoides* and *B. subtilis* have also been found to be capable of airborne spread, but did not build clusters across different sample types or farms in this study (Bayle et al., 2021, Fang et al., 2016, Roque et al., 2016). This adds further evidence towards a higher diversity of genomes across *B. cereus*, *B. mycoides* and *B. subtilis* compared with the others. These findings align with an early report of suspected high genetic diversity between *B. mycoides* members and more recently reported findings that isolates identified as *B. mycoides* can have less compact clusters compared with other *Bacillus cereus* group members (Carroll et al., 2020, von Wintzingerode et al., 1997). The diversity of *B. subtilis* genomes is reported to be larger than related species, partially due to its ability to acquire xenologous copies of genes, which in part confers its ability to survive in a diverse set of ecological niches (Brito et al., 2018, Earl et al., 2007). However, the assumptions on the diversity of genomes between and within different species should be regarded with caution due to uneven sample sizes, until further research is conducted.

The clustering of highly similar genomes indicated, that among the species with isolates from different seasons (all species except for *B. wiedmannii* and *P. phoenicis*), 65 clusters formed, of which 10 contained isolates from different seasons (Table 4-4). This was observed in *B. licheniformis* (30 %; 8 / 27) and *B. pumilus* (50 %; 2 / 4) species clusters. This adds more evidence to the conclusion of a lower genome diversity within these species drawn from their clustering across different farms, seasons and feed types.

This lower diversity could be due to these species having closely related species which make their species definition narrower. As genomic technologies become more commonly used for the definition of a species the reported genomic diversity may reduce

as new species are defined from subpopulations of an existing species. This recently occurred for *B. licheniformis*, splitting into *B. licheniformis* and *B. paralicheniformis* (Dunlap et al., 2015). *Bacillus pumilus* while very similar to *B. licheniformis* is genetically closer to *B. safensis*, *B. stratosphericus*, *B. altitudinis* and *B. aerophilus*, such that 16S rRNA sequencing cannot identify differences, this narrow definition of the species might lead to a lower diversity of genomes within the species (Liu et al., 2013). Another reason could be *B. licheniformis* having a greater genome stability over time. A comparison of over 100 genomes from *B. licheniformis* and *B. paralicheniformis* from around the world, spanning over 130 years apart, showed that all but one still only clustered into two clades, one *B. licheniformis* and one *B. paralicheniformis*, indicating a certain level of consistency within the genomes across regions and time (Agerso et al., 2019).

Among the five *B. mycoides* and five *B. weihenstephanensis* isolates, one *B. mycoides* and two *B. weihenstephanensis* isolates did indeed cluster together (Appendix B Table B-3). Indicating that these two species belong to the same species (Carroll et al., 2020, Liu et al., 2018). It may also indicate that MALDI-TOF identification has some limitations when it comes to closely related species like the *B. cereus* group members and highlights the importance of multipronged approaches to identification like the incorporation of genome sequencing, if exact identifications are required (Ha et al., 2019).

To the authors knowledge this is the first study to investigate the potential transfer of bacterial spores from cattle feed into milk using whole genome sequences. It is also the first instigation of bacterial spore genome diversity in New Zealand cattle feed and milk. For definite proof of a transfer of bacterial spores from cattle feed into milk, matching isolates would need to be found, which are rarer than closely related isolates, concluding that more feed isolates would need to be sequenced. This study has laid a foundation which can be used as an indication of expected genome diversities in the farm environment, to better estimate the number of genomes needed to find a definitive answer if bacterial spores from cattle feed can migrate into milk. Based on the data acquired, future studies looking for a connection between bacterial spores in feed and

milk should focus on one farm, timepoints close together and isolates from *B. licheniformis* and *B. pumilus* to maximise the chances of discovering matching isolates.

4.5 Conclusion

Comparing the genomes of 109 bacterial spore isolates from cattle feed and milk indicated that the diversity of genomes was greater in cattle feed than milk. Within the parameters set by the study, no matching genomes were found between milk and cattle feed but highly similar genomes were found, supporting the hypothesis that that the bacterial spores from milk analysed in this study could have originated from cattle feed. A more intensive study on a single farm would add support to this hypothesis by increasing the chance of detecting genome matches from cattle feed and milk.

Some evidence was found that the diversity of genomes in cattle feed and milk is lower across *B. licheniformis* and *B. pumilus* than *B. cereus, B. mycoides, B. subtilis*, *B. thermoamylovorans* and *B. weihenstephanensis*.

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Chapter 5: Potential of spore-forming bacterial isolates to effect milk quality characteristics.

5.1 Abstract

Spore-forming bacteria in milk, can affect milk quality during processing differently, depending on their genetic makeup. In this study, a set of 39 spore-forming bacteria belonging to 7 different species, isolated from raw milk, were screened for biofilm formation on polystyrene. Twenty isolates formed biofilms, and therefore have the potential to cause problems in dairy manufacturing plants. These isolates were further analysed for their abilities to form biofilms on stainless steel in milk, grow in milk at different temperatures, secrete milk degrading enzymes and produce spores that survive heat treatments. Results showed an almost universal ability to grow in milk and secrete protease and lipase enzymes. All 20 isolates tested showed some ability to form biofilms in milk and produce heat resistant spores, however significant differences between some isolates were observed. The genetic background for the observed differences was studied resulting in the discovery of a set of 23 genes which could in the future be used to assess the spoilage potential of a spore-forming isolates, as well as indications for additional gene functions relating to biofilm formation and enzyme secretion. Overall, two *Bacillus licheniformis* isolates, 349 and w382, were identified as having the greatest potential to affect milk quality during processing.

5.2 Introduction

Bacterial spores can spoil milk and milk products as they survive the heat treatments in milk processing used to control microorganisms (Kmiha et al., 2017, Ranieri and Boor, 2009). However, not every bacterium which is able to sporulate, poses the same spoilage potential to food production (Porcellato et al., 2021). A spore-forming isolate, which is able to proliferate in milk and form biofilms in the processing plant, increases the potential

of a bacterium to cause spoilage (Huang et al., 2021, Tirloni et al., 2017, De Jonghe et al., 2010).

Biofilms provide the bacterial cells with a protective layer which allows their survival in harsh conditions, e.g. food processing environments (Sadiq et al., 2017). Within processing equipment biofilms have the potential to survive cleaning and permanently damage metal on which they form (Gupta and Anand, 2018, Bremer et al., 2006). They can shed bacterial cells and spores into product, and secrete proteolytic or lipolytic enzymes affecting product quality (Gopal et al., 2015). The presence of high numbers of bacteria is often enough to reduce the value of product (FSANZ, 2016). Bacteria in biofilms are able to withstand higher temperatures than planktonic bacteria showing the importance to avoid them even in processing areas with high temperatures (Simmonds et al., 2003). The formation of a biofilm is advantageous to milk contaminating bacteria, and bacteria with the genetic ability to form strong biofilms is associated with the presence of that isolate in milk (Ostrov et al., 2019). Mesophilic strains have a greater proteolytic ability compared with thermophilic strains of the same species when compared at their respective optimal growth temperature, therefore this study focuses on isolates which are capable of forming biofilms and grow under mesophilic conditions (Lucking et al., 2013).

In the well-researched spore-forming bacterium *Bacillus subtilis*, 72 genes are involved in biofilm formation and 479 involved in spore formation (Subtiwiki.com accessed 6.4.2021 (Zhu and Stulke, 2018)). For most of these genes, it is unknown if there are alleles that increase or decrease the potential of an isolate to affect the quality of milk (Zhu and Stulke, 2018). It is also unknown which alleles can be expected to be found in raw milk isolates. Additional knowledge of the genetic potential for milk spoilage by an isolate could help assess the impact of specific isolates on product quality (Huang et al., 2021).

In this study spore-forming isolates from raw milk were analysed for their ability to form biofilms, grow in milk, produce spoilage enzymes, survive heat treatments and investigate gene variants which could potentially lead to milk spoilage.

5.3 Methods

5.3.1 Bacterial isolates

A total of 39 bacterial spore isolates, (isolate in Chapter 3), were selected for this study (Table 5-1). Their genomes were sequenced and compared (Chapter 4; Figure 4-2 - Figure 4-10, Table B-2). Isolates were revived and freshly grown from -80°C cultures on TSA (BD diagnostics systems, Germany) prior to testing.

Table 5-1 Identity and growth temperature of the tested isolates as well as indication if they are genetically closely related.

Species identification from MALDI-TOF	ID	Growth temperature	Closely related to other isolates based of WGS comparison
B. cereus	t398	37°C	-
B. cereus	w285	37°C	-
B. cereus	w307	37°C	-
B. mycoides	448	37°C	A
B. mycoides	653	30°C	A
B. weihenstephanensis	w410	30°C	-
B. wiedmannii	t489	37°C	-
B. pumilus	367	30°C	B
B. pumilus	388	37°C	-
B. pumilus	413	37°C	-
B. pumilus	414	37°C	B
B. pumilus	w264	37°C	B
B. subtilis	332	37°C	C
B. subtilis	334	37°C	C
B. subtilis	365	37°C	C
B. subtilis	t391	37°C	-
B. licheniformis	282	37°C	D
B. licheniformis	304	37°C	-

Species identification from MALDI-TOF	ID	Growth temperature	Closely related to other isolates based of WGS comparison
B. licheniformis	326	37°C	E
B. licheniformis	349	37°C	-
B. licheniformis	358	37°C	F
B. licheniformis	366	37°C	E
B. licheniformis	389	37°C	-
B. licheniformis	428	37°C	E
B. licheniformis	g213	37°C	G
B. licheniformis	t396	37°C	H
B. licheniformis	t406	37°C	F
B. licheniformis	t414	37°C	E
B. licheniformis	t415	37°C	H
B. licheniformis	t423	37°C	G
B. licheniformis	t501	37°C	F
B. licheniformis	t505	37°C	G
B. licheniformis	t507	37°C	G
B. licheniformis	w240	37°C	-
B. licheniformis	w262	37°C	-
B. licheniformis	w326	37°C	D
B. licheniformis	w337	37°C	F
B. licheniformis	w382	37°C	-
B. licheniformis	w389	37°C	G

[a] Same letter (A-H) indicates that isolates genomes clustered together (< 60 SNP's) while (-) indicates no genome in the list was closely related to that isolate. The comparison is detailed in Chapter 4.

5.3.2 Biofilm formation on polystyrene surfaces

A biofilm assay was preformed according to the method detailed in O'Toole (2011). An overnight culture of all isolates was grown in Tryptic soy broth (TSB) (BD diagnostics systems, Germany) at 37 °C (16 h). From the overnight culture, 100 µl of inoculate was diluted in 9.9 ml sterile TSB, from which 100 µl (containing ~5 x10^4 CFU/ml) was transferred to a 96 flat bottom well polystyrene microtiter plate in five replicates, and in biological duplicates. Plates were incubated statically at 37 °C for 24 h and the optical density (OD) of each well was measured at 620 nm. The culture medium was then removed, and the plates were washed twice with autoclaved water. The wells were air

dried at 37 °C for 20 min. Each well was stained using 150 µl of 0.1 % (w\v) crystal violet (CV) solution for 15 min at room temperature. Plates were then rinsed four times with water by submerging. Plates were dried upside down at 37 °C for at least 2 h and 150 µl of 99 % ethanol was added to each well and left for 15 min at room temperature. From each well, 125 µl of the solubilized CV were added to a new 96 well plate and the absorbance of the liquid was quantified in a plate reader (PerkinElmer, USA) at 550 nm.

For data analysis, the OD of stained attached bacteria were averaged (AB) and divided by the average OD of stained control wells (NC), containing medium only to calculate a biofilm formation index (BFI) (BFI = AB / NC) (Naves et al., 2008, Soto et al., 2006). The BFI of isolates was sorted into 4 categories: negative <2, weak 2–3.99, moderate 4–5.99 and strong ≥6 (Naves et al., 2008).

5.3.3 Biofilm formation on stainless steel and the detection of attached cells by culture and scanning electron microscopy

Before use, stainless steel coupons (1 cm * 1 cm) were soaked in acetone (98 %) overnight, followed by 3 rinses in deionised water. The coupons were then suspended in a saturated detergent solution and sonicated for 30 min followed by 3 more rinses in deionised water followed by autoclaving to sterilise.

Each isolate that formed a biofilm on polystyrene in the earlier experiment was grown in 9 ml TSB at 37 °C for 16 h. From this culture, 20 µl were added to 2 ml UHT milk (resulting in ~1 x10^4 CFU/ml) in a 24 well microtiter plate with each well containing a sterile stainless-steel coupon. These plates were incubated for 24 h at 37 °C and 150 rpm. After incubation, a microbial count of the UHT milk was executed, and the cells attached to the coupons were counted. To enumerate the attached cells, each coupon was removed from the growth medium using sterile tweezers and washed by pipetting onto one edge of the coupon with 1 ml of sterile peptone water three times, the coupon was then rotated by 90 ° to the next edge and the wash step was repeated. The coupon was transferred

into a sterile 25 ml glass tube, filled with 5 ml of 0.5 % buffered peptone water and 5 g of 3-6 mm glass beads and mixed by vortex for 1 min at 60 rpm to release biofilm cells attached to the coupon into the liquid. The number of cells released from the biofilm this way was measured with a microbial plate count.

Biofilm microbial counts on stainless steel in milk, were log_{10} transformed and a one-way ANOVA and Tukey's test was performed using Minitab 18 (Minitab LLC, USA).

The abundance of an actual biofilm on the coupons was confirmed for selected isolates (326, 388, t505, w337 and w382) using electron scanning microscopy (SEM) in the Manawatu Microscopy and Imaging Centre (Massey University, New Zealand). Samples were prepared and washed the same way as described above, followed by fixation in a 0.1 M phosphate buffer containing 3 % (w/v) glutaraldehyde and 2 % (w/v) formaldehyde, at room temperature for 24 h. Samples were then washed for 10 min in phosphate buffer three times, followed by ethanol dehydration using a graded ethanol series before critical point drying. Samples were mounted on aluminium stubs and sputter-coated with 100 nm of gold (Bal-Tec SVD050, USA) and viewed using a Quanta 200 scanning electron microscope (FEI Co., USA) at an accelerating voltage of 20 kV.

5.3.4 Heat resistance of spores

Isolates were grown on TSA plates for 8 days at 37 °C, after which spores were washed off using 9 ml sterile peptone water (Eijlander et al., 2019). Spores were then centrifugated at 5,000 g for 10 min and washed 3 times by resuspending in 9 ml sterilised milliQ water as described in Shields et al. (2012). The samples were kept on ice between washings and finally resuspended in 6 ml milliQ water and frozen at -20 °C as aliquots of 1 ml. Spores were thawed at 10 °C when required for analysis.

To assess an initial spore count, 50 µl of the spore solution was heat treated at 80°C for 12 min and diluted using serial 10-fold dilutions in sterile peptone water followed by

plating on TSA using the drop plate method (Naghili et al., 2013). Another 10 µl sample was observed under a light microscope to confirm the abundance of spores and ensure they did not form any large cell aggregates.

The thermal treatment of the spores was carried out using a PCR thermocycler (Proflex, Thermo Fisher Scientific, USA) (Kim et al., 2021). Fifty µl of the spore solution was transferred into PCR tubes. The spore solution was then subjected to a temperature of 90 °C. After the spore solution reached 90 °C (20 sec) three PCR tubes were taken out after 5, 10, 30 and 60 min, respectively and transferred onto ice for at least 10 min. The experiment was carried out as biological duplicates. To quantify the surviving spores, 10 µl of the heat-treated spore solution, was pipetted into 9.99 ml of sterile peptone water to achieve a 10^{-3} dilution which was further diluted to reach the desired dilution factor. The diluted spore solutions and remaining spores from the PCR tube, were then plated out using the drop plate method. The plates were then incubated overnight at 37°C and the colonies were counted the next day.

Decimal reduction times (D_{90}-values) were calculated for each measurement, using the slope of a linear trendline based on the log_{10} transformed microbial count data. The D_{90}-values were compared in a one-way ANOVA and Tukey's test using Minitab 18 (Minitab LLC, USA).

5.3.5 Enzyme secretion detection

Calcium caseinate agar (Sigma-Aldrich, USA) and tributyrin agar (Sigma-Aldrich, USA) were prepared according to the manufacturer's recommendations. Isolates were inoculated as a dot in the middle of the agar and incubated at 37 °C for at least 48 h during which plates were observed at regular intervals for the creation of a halo, indicating protease or lipase production respectively (Zhang et al., 2020).

5.3.6 Growth in milk at psychrophilic and mesophilic temperatures

Overnight cultures of isolates were grown in 9 ml TSB at 37 °C for 16 h. One ml of cells was harvested using 10.000 g for 5 min and washed three times with sterile milliQ water to remove free dehydrogenases. From the washed cell suspension, 90 µl were added to 8.9 ml of UHT milk together with 10 µl of sterile 1 % Triphenyl tetrazolium chloride (TTC). The milk was then incubated at 7.5, 10, 15, or 37 °C for 36 days or until the colour change reached its maximum. Changes in colour were observed using a Lovibond 1000 (Tintometer GmbH, Germany) with its 3/40A and 3/40B chloride module (Tintometer GmbH, Germany) every 24 h at 37 °C and every 3 days at lower temperatures, until a 2.5 reading was reached on 3/40B (Jo et al., 2021, Sahoo and Anjaneyulu, 1997).

5.3.7 Genomic data analysis

The genomes of isolates which had clustered together in chapter 4 (Figure 4-2 - Figure 4-10) but performed differently to each other in the analyses were annotated using Prokka (version 1.14.5) (Seemann, 2014). Genomes were then compared using Snippy (version 4.6.0), Gubbins (version 2.3.1) (Croucher et al., 2015, Seemann, 2015). Genes with non-synonymous mutations were screened for their respective functions using online databases (UniProt, Subtiwiki) and literature (UniProt, 2019, Zhu and Stulke, 2018).

5.4 Results and discussion

5.4.1 Biofilm formation

5.4.1.1 Biofilm formation on polystyrene

Of the 39 isolates tested, 20 showed some capacity to form a biofilm on polystyrene (BFI $\geq$2) (Table 5-2). Those 20 isolates were one *B. cereus*, two *B. pumilus*, one *B. subtilis*

and 16 *B. licheniformis* isolates. Among all *B. licheniformis* isolates, 70 % (16/23) of isolates formed a biofilm, while other species showed a lower prevalence for biofilm formation with 40 % (2/5), 33 % (1/3) and 25 % (1/4) for *B. pumilus, B. cereus* and *B. subtilis* respectively. The *B. weihenstephanensis* (0/1), *B. wiedmannii* (0/1) and *B. mycoides* (0/2) isolates did not form a detectable biofilm.

The 20 biofilm positive isolates were selected to be used for further experiments investigating their biofilm formation on stainless steel, enzyme production, growth in milk and their spores heat stability.

Table 5-2 Biofilm formation index of selected isolates and their species identification. Cells highlighted in grey are positive for biofilm formation on polystyrene.

Species	ID	BFI of isolates on polystyrene[a]	BFI strength category[b]
B. licheniformis	w382	43.0	+++
B. licheniformis	304	18.6	+++
B. licheniformis	g213	18.4	+++
B. licheniformis	326	10.6	+++
B. licheniformis	t414	7.3	+++
B. licheniformis	349	6.0	+++
B. licheniformis	t501	5.6	++
B. licheniformis	t507	5.2	++
B. licheniformis	w337	4.9	++
B. licheniformis	t406	4.6	++
B. cereus	t398	4.2	++
B. licheniformis	358	4.0	+
B. licheniformis	t423	3.9	+
B. licheniformis	w389	3.9	+
B. licheniformis	t415	3.2	+
B. pumilus	388	2.8	+
B. pumilus	367	2.2	+
B. licheniformis	t505	2.2	+
B. subtilis	334	2.1	+

Species	ID	BFI of isolates on polystyrene[a]	BFI strength category[b]
B. licheniformis	t396	2.0	+
B. licheniformis	w240	1.9	-
B. pumilus	w264	1.8	-
B. pumilus	414	1.5	-
B. licheniformis	282	1.5	-
B. licheniformis	366	1.5	-
B. pumilus	413	1.4	-
B. licheniformis	428	1.4	-
B. cereus	w307	1.3	-
B. licheniformis	389	1.3	-
B. mycoides	448	1.2	-
B. mycoides	653	1.2	-
B. weihenstephanensis	w410	1.1	-
B. licheniformis	w326	1.1	-
B. cereus	w285	1.0	-
B. subtilis	332	1.0	-
B. subtilis	t391	1.0	-
B. licheniformis	w262	1.0	-
B. wiedmannii	t489	0.9	-
B. subtilis	365	0.8	-

[a] Calculated from the isolates average OD (550 nm) of stained attached bacteria divided by the OD of stained control wells

[b] negative <2 (-), weak 2–3.99 (+), moderate 4–5.99 (++) and strong ≥6 (+++); all positive isolates are marked in grey

The strongest biofilms on polystyrene were all formed by *B. licheniformis* isolates. *Bacillus licheniformis* also formed some of the weakest biofilms in this experiment and some of its isolates, did not form a biofilm at all. Differences in the ability to form a biofilm and the strength of biofilm formation within one species or between isolates of different species was reported, previously within different *Bacillus* species and was associated with genetic differences between isolates (Ostrov et al., 2019, Celandroni et al., 2016). The diversity of biofilm production ability in *B. licheniformis* potentially indicates a wide variety of abundant biofilm building genotypes. As shown in chapter 4, the 23 *B.*

licheniformis isolates belong to 11 different genetic clusters (Figure 4-4 and Figure 4-5 as well as Table 5-1). The cluster designated D in Table 5-1, did not form biofilm while those designated F, G and H did. Differences in the ability of *B. licheniformis* to form biofilms has previously been reported for dairy industry isolates (Md Zain et al., 2017). In the *B. licheniformis* cluster designated E, two isolates (326 and t414) formed a strong biofilm (BFI > 5) on polystyrene while two (355 and 428) did not form biofilm. When analysed for genetic differences, non-synonymous mutations of 22 known genes and 10 hypothetical proteins, were found in both isolates 326 and t414 compared with isolates 355 and 428 (Appendix C, Table C-1). A gene database search identified 3 genes among the 22, whose mutation might be responsible for the observed differences. The genes *cheB*, *speA* and *yknZ* have previously been identified as being involved in biofilm formation, this is likely part of the reason for observed differences (Huang et al., 2019, Xu et al., 2016, Burrell et al., 2010). Many of the remaining 19 genes are not yet well defined and could be involved as well, however, the identified genes are a good base from which to further investigate genetic factors in the regulation of biofilm formation in *B. licheniformis*.

This genome-based comparison, as well as all others done in this study, contain some uncertainty due to the hypothetical proteins in the genome potentially being real genes or due to potentially missed mutations located outside the alignment coverage, during the genome comparison (da Costa et al., 2018). Parts of a genome not covered in the alignment can either stem from genes which are only present in one of the genomes or gaps in the sequencing of one of the genomes. These two scenarios cannot be distinguished from each other in the comparison unless fully circular genomes are being used. Since all genomes used in the study are scaffold assemblies which are potentially missing portions of the full genome, this uncertainty exists in the methodology. However, since all genomes compared were already known to be similar, parts of the compared genomes which did not join the alignment were few, at between 0.2 – 2 %, with one outliner at 4 % of the genome's length not joining the alignment. For the majority of the

reference genome length (at least 96 %), alignment coverage is available, leaving potential for error due to these undiscovered areas small and allowing an assessment of the differences until full circular genomes are available. Confidence that identified gene differences are the cause of observed phenotypic differences could be obtained by transcriptional analysis or synthetic rescue experiments (Alberts et al., 2002).

In isolates from *B. cereus*, *B. mycoides*, *B. pumilus* and *B. subtilis* differences in biofilm building ability on polystyrene were less commonly observed, but the number of tested isolates, of these species were also overall lower. Of four *B. subtilis* isolates, only the isolate 334, formed a biofilm on polystyrene. That isolate was genetically closely related to two of the isolates which did not form a biofilm, showing only 4 SNPs difference from isolate 332. Interestingly, between 334 and 332, only one missense mutation was identified, which was located in the *gluP* gene, coding for the rhomboid protease GluP (Appendix C, Table C-2). That same mutation in the same nucleotide was found between 334 and all other *B. subtilis* isolates. The intramembrane proteases GluP, was reported in *B. subtilis*, to have a function in glucose uptake and cell division (Mesak et al., 2004). However, in *Mycobacterium smegmatis*, rhomboid proteases have been shown to influence the formation of biofilms (Kateete et al., 2012). This indicates that *gluP* might have a function in biofilm formation in *B. subtilis* too, although further research is advised.

Of the five *B. pumilus* isolates, 367 and 388 formed a biofilm on polystyrene. Of the other three that did not, two (414 and w264) were closely related to 367 (Table 5-1). An analysis of the genetic differences between those three, did not yield definitive results (Appendix C, Table C-3 and Table C-4). Between 367 and w264, non-synonymous mutations were found in *alsT*, *aspA* and *sasA* and three hypothetical proteins. Out of these, *aspA,* an aspartate ammonia-lyase who's mutation has previously shown to be connected to changes in growth rate and biofilm production, this may be a factor causing the observed differences (Wang et al., 2020). Based on the observations made, isolates 367, 414 and w264, despite being similar genetically, they differed phenotypically,

potentially due to different gene mutations, which only in case of 367 lead to improved biofilm formation.

A similar analysis of gene differences was not possible for the three tested *B. cereus* isolates since their genomes were too different to align. The isolates of *B. mycoides*, *B. weihenstephanensis* and *B. wiedmannii* all failed to form a biofilm on polystyrene and further genomic analysis was not carried out.

5.4.1.2 Biofilm formation on stainless steel in milk

Isolates which formed a biofilm on polystyrene, were further tested for their ability to form a biofilm on stainless steel in milk. All isolates managed to grow in milk reaching an approximate cell density of 10^8 CFU/ml (Table 5-3). The planktonic cell counts grouped the isolates into two groups with the *B. cereus* isolate t398 showing a significantly lower count than the *B. licheniformis* isolates 326, 304 and t414 and the *B. pumilus* isolate 388. All other isolates did not differ significantly from other isolates.

Further differences were found in the number of cells which formed a biofilm on the stainless-steel surface, where the isolates clustered into three groups (Table 5-3). Isolate w337 showed the highest average number of cells attached, which was significantly higher ($P < 0.05$) than the cells attached by isolates t415 and t507. The lowest average counts were found in t507 which had significantly ($P < 0.05$) lower numbers of cells attached than the isolates 349, t505 and w382 in their cultures cell density.

Table 5-3 Number of cells which formed a biofilm on stainless steel in milk, as well as the number of planktonic cells in the milk, each with standard deviation and significant grouping[a].

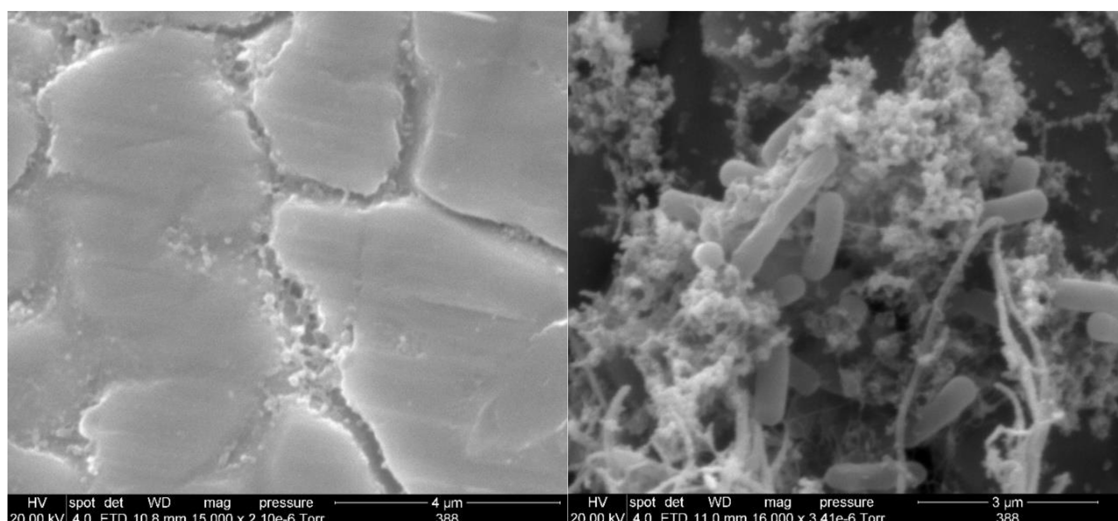
Species	ID	Biofilm cell count avg. in log_{10} CFU/cm^2	Biofilm stdev.	Biofilm grouping[a]	Planktonic cell count avg. in log_{10} CFU/ml	Planktonic stdev.	Planktonic grouping[a]

B. licheniformis	w337	6.1	0.8	A	8.3	0.4	AB
B. licheniformis	349	5.9	0.8	AB	8.4	0.1	AB
B. licheniformis	t505	5.7	1.6	AB	8.2	0.2	AB
B. licheniformis	w382	5.6	1.2	AB	8.3	0.1	AB
B. pumilus	388	5.4	1.1	ABC	8.8	0.1	A
B. subtilis	334	5.3	0.7	ABC	8.6	0.2	AB
B. licheniformis	326	5.2	1.2	ABC	8.9	0.1	A
B. licheniformis	w389	4.9	1.2	ABC	8.5	0.4	AB
B. licheniformis	t406	4.9	1.2	ABC	8.4	0.1	AB
B. licheniformis	t501	4.9	1.6	ABC	8.3	0.1	AB
B. pumilus	367	4.7	0.6	ABC	8.7	0.4	AB
B. licheniformis	g213	4.7	0.7	ABC	8.3	0.6	AB
B. licheniformis	t396	4.7	1.0	ABC	8.1	0.4	AB
B. licheniformis	358	4.7	1.2	ABC	8.2	0.4	AB
B. licheniformis	t414	4.6	1.5	ABC	8.8	0.2	A
B. licheniformis	t423	4.4	0.7	ABC	8.5	0.2	AB
B. licheniformis	304	4.3	1.4	ABC	8.9	0.4	A
B. cereus	t398	4.3	0.6	ABC	7.8	0.2	B
B. licheniformis	t415	3.8	0.8	BC	8.2	0.3	AB
B. licheniformis	t507	3.3	0.4	C	8.1	0.3	AB

[a] Isolates in different groups = $P < 0.05$ as determined in a Tukey test

To confirm that attached cells formed a biofilm on the stainless-steel coupons, SEM images of w337, t505, w382, 388, 326 and a cell-free control were prepared. All tested isolates showed evidence of biofilm formation on the surface (exemplified by isolate 388 in Figure 5-1).

Figure 5-1 Scanning electron microscope image of a stainless-steel coupon after 48h in milk without (left) or with (right) bacterial spore isolate 388 biofilms.



The planktonic counts indicate that all selected isolates will grow in milk at 37 °C. However, of the 20 isolates tested, four isolates showed significantly greater planktonic counts than a *B. cereus* isolate. Since the growth time was 24 h, it could be assumed that all isolates likely reached their stationary phase of growth since the exponential phase of different *Bacillus* species can be observed within 8 h at 37 °C (De Jonghe et al., 2010). This indicates that differences between the *B. cereus* isolate and the four other isolates are due to differences in stationary phase cell density, rather than the growth rate. A spore-forming isolate, able to grow to a high cell density, could increase the total spore count of a product, which is a common quality assessment of milk powder, and thereby threaten product quality (McHugh et al., 2017).

The biofilm formation in milk on stainless-steel showed multiple isolates attaching with different efficiency. The varying isolates were all *B. licheniformis*, indicating that genetic variation between isolates can have a stronger effect than species identity when predicting cell attachment to stainless steel in milk and confirms the results found on polystyrene, which showed the same. It further shows that the species identification is not solely sufficient to assess the potential of an isolate to cause problems in the milk processing environment. The varying ability to produce biofilms as previously been

reported in *Bacillus* isolates (Huang et al., 2021, Kwon et al., 2017). The isolate t505 produced significantly higher numbers of attached biofilm cells on stainless steel in milk compared to the isolate t507, but genetically they clustered together (Table 5-1). This greater biofilm production was also visible of polystyrene where t507 grew a moderate biofilm (BFI = 4 – 5.99) and t505 a weak biofilm (BFI = 2 – 3.99). Between those two isolates, 34 non-synonymous mutations in known genes and 23 in hypothetical proteins were identified. Interestingly, the remaining three isolates from the same genetic cluster (g213, t423 and w389) showed no significant differences to any other tested isolates in the number of biofilm cells. This indicates that there is potentially one mutation which made t507 attach in greater numbers but also a mutation which made t505 attach in lower numbers. For both isolates the closest related isolate was t423 (34 SNP's to both t505 or t507). The isolate t423 did not show any significant differences to other isolates in its ability to form biofilms, which enabled the approach to exclude the mutations which were also found in t423 towards t507 and t505 to narrow the field of genes which had an effect on biofilm formation. This indicated 15 known and 13 hypothetical genes with differences which may have an effect on biofilm production (Appendix C, Table C-5). Of the 15 known genes, two genes *acsA* and *rapI* have been previously reported to be involved in the regulation of biofilm formation and could be the cause of observed differences (Christensen et al., 2021, Hathroubi et al., 2020).

Knowledge of these isolates ability to form such structures will help to assess their potential to cause milk quality issues within a production site to make informed cleaning decisions. Knowledge about milk damaging genotypes further assist in developing accurate detection methods for those isolates and development of processes to avoid such isolates during production.

5.4.2 Growth in milk and production of spoilage enzymes

The 20 isolates which formed a biofilm on polystyrene, were also tested for their ability to grow in milk at different temperatures using a TTC assay, and to secrete lipolytic or proteolytic enzymes (Table 5-4). When grown on 37 °C, all isolates grew to the maximum colour change after 48 h. At 15 °C, growth was observed in isolates t398 and 388 after four days. At 7.5 °C or 10 °C growth was not observed within the maximum incubation time of 36 days. After their maximum incubation time, the cultures at 7.5 °C and 10 °C, were shifted to 37 °C which lead to growth in all cases. To confirm, microbial counts were conducted after incubation at 10°C for 36 d. All cultures were found to contain living cells with between 100-3100 CFU/ml.

The lipase secretion was observed in all isolates and the secretion of protease in all isolates except g213 and t398 (Table 5-4).

Table 5-4 Bacterial spore isolates, their species and ability to grow at different temperatures or secrete milk spoilage enzymes at 37°C.

Species	ID	Growth at 7 °C	Growth at 10 °C	Growth at 15 °C	Lipase	Protease
B. pumilus	367	-	-	-	+	+
B. pumilus	388	-	-	+	+	+
B. subtilis	334	-	-	-	+	+
B. cereus	t398	-	-	+	+	-
B. licheniformis	t396	-	-	-	+	+
B. licheniformis	t406	-	-	-	+	+
B. licheniformis	t414	-	-	-	+	+
B. licheniformis	t415	-	-	-	+	+
B. licheniformis	t423	-	-	-	+	+
B. licheniformis	t501	-	-	-	+	+
B. licheniformis	t505	-	-	-	+	+
B. licheniformis	t507	-	-	-	+	+
B. licheniformis	304	-	-	-	+	+
B. licheniformis	326	-	-	-	+	+
B. licheniformis	349	-	-	-	+	+

Species	ID	Growth at 7 °C	Growth at 10 °C	Growth at 15 °C	Lipase	Protease
B. licheniformis	358	-	-	-	+	+
B. licheniformis	w337	-	-	-	+	+
B. licheniformis	w382	-	-	-	+	+
B. licheniformis	w389	-	-	-	+	+
B. licheniformis	g213	-	-	-	+	-

(–) indicates a negative outcome and (+) a positive

At 15 °C *B. cereus* isolate t398 reached maximal colour change after 9 days whilst *B. pumilus* isolate 388 required 14 days to reach the maximum. Previous work reports *B. cereus* growth in milk at psychrotrophic temperatures as low as 4 °C (Porcellato et al., 2021, Tirloni et al., 2017, Borge et al., 2001). Growth at 15 °C for some *B. pumilus* isolates, has been reported previously (From et al., 2007).

The TTC assay proved useful, for the fast and easy observation of bacterial growth within a milk substrate at different temperatures. This method has been previously described for the investigation of bacterial growth in food (Yamaguchi et al., 2003).

All isolates grew in milk at 37 °C and temperatures around that value can be found in milk processing environments, showing that, if they form a biofilm within the right area, all isolates tested are a potential risk to milk product quality (Scott et al., 2007, Bylund, 2003). All isolates survived in milk at 7 °C for up to 36 days, which indicates that once in the product, the isolates could continue to impact products shelf life. The species used in this study, have previously been reported to be able to grow in milk (Porcellato et al., 2021, Dat et al., 2012, De Jonghe et al., 2010). Despite that and the fact that these isolates were originally found in milk, an assessment of their actual ability to grow in milk was needed since bacterial spores can lay dormant in many environments without proliferation and their milk damaging potential would be lower, if they were unable to proliferate in milk (Ulrich et al., 2018).

All 20 isolates tested were able to secrete proteolytic enzymes and 18 of the 20 isolates produced lipolytic enzymes. This aligns with previous research on *Bacillus* species that reported that most isolates produced proteases while fewer produced lipases, indicating that protease production is more prevalent than lipase (Mehta et al., 2019). It was further reported that while *B. cereus* produced protease they did not produce lipase whilst *B. pumilus* isolates can produce both and *B. licheniformis* produces protease, but some isolates do not produce lipase, as was the case for respective isolates in this study (Mehta et al., 2019). In the present study, only one *B. subtilis* isolate was tested, which produced protease, but not lipase. The ability of *B. subtilis* isolates to produce lipase has been reported in a previous study (De Jonghe et al., 2010). The only *B. licheniformis* isolate which did not produce lipase was g213. Its genome was annotated and compared to its closest related isolates in the study, t423 and t507, which found 17 non-synonymous mutations in the genome of g213 which were present in both t423 and t507 as well as mutations in five hypothetical proteins (Appendix C, Table C-6). A gene responsible for the observed differences could not be identified, however a mutation in the *oppF* gene stands out. This gene is involved in oligopeptide transport and may have an influence on the secretion of lipases but further work is needed to confirm this (Peltoniemi et al., 2002).

5.4.3 Heat stability of bacterial spores

Isolates which formed a biofilm on polystyrene, were also tested for their spore's heat resistance (Table 5-5). The isolates grouped into 9 groups, in which the spores of *B. licheniformis* isolates t501, t507, t415, t423 and t406 showed the highest D_{90}-values. The lowest measured D_{90}-values were observed in the spores of *B. pumilus* isolates, whilst *B. subtilis* spores grouped in the middle of the spectrum of isolates. No D_{90}-value is reported for the *B. cereus* isolate since it did not survive the 90°C heat treatment to produce D_{90}-value.

Table 5-5 Average D_{90}-value at 90°C, standard deviation and significant groups of tested bacterial spore isolates.

ID	Species	Average D_{90}-value (min)	Standard deviation	Grouping[a]
t501	*B. licheniformis*	121.2	20.9	A
t507	*B. licheniformis*	120.5	33.7	A,B
t415	*B. licheniformis*	91.6	124.1	A,B,C,D,E
t423	*B. licheniformis*	87.6	21.1	A,B,C
t406	*B. licheniformis*	73.6	39.7	A,B,C,D,E,F
349	*B. licheniformis*	65.0	20.9	B,C,D,E,F,G
w382	*B. licheniformis*	50.5	4.2	C,D,E,F,G,H
358	*B. licheniformis*	35.8	6.7	C,D,E,F,G,H
304	*B. licheniformis*	33.6	0.7	C,D,E,F,G,H
334	*B. subtilis*	30.8	4.9	E,F,G,H
t396	*B. licheniformis*	29.9	2.6	D,E,F,G,H
w389	*B. licheniformis*	21.7	0.4	F,G,H
326	*B. licheniformis*	18.3	2.7	F,G,H
t505	*B. licheniformis*	18.2	0.2	F,G,H
g213	*B. licheniformis*	17.9	5.1	F,G,H
w337	*B. licheniformis*	16.7	2.4	G,H
t414	*B. licheniformis*	12.2	1.3	G,H
388	*B. pumilus*	6.0	1.2	H
367	*B. pumilus*	5.8	1.3	H
t398	*B. cereus*	0.0	0.0	-

[a] Isolates with different letter denote significant differences $P < 0.05$ as determined in a Tukey test

Differences in heat resistance between isolates of the same species as found here (Table 5-5) was previously reported among others in the *B. subtilis* group and *B. cereus* isolates (Lima et al., 2011, van Asselt and Zwietering, 2006). It is further supported by recent findings that *B. weihenstephanensis* isolates can rapidly adapt to produce spores of higher heat stability after being exposed to heat multiple times, indicating that few changes need to be acquired to alter the heat stability of spores (Kim et al., 2021).

The average D_{90}-value of the *B. licheniformis* isolate spores was 49 min but had a large standard deviation of 40 min, due to the large variation between isolates. In the literature, *B. licheniformis* isolates D_{90}-values vary from 1.57 to 29.9 min but have also been reported to survive at 100°C for 30 min and potentially ultra-high temperature processing at 125 °C for 30 s or 138°C for 4 s (Pereira et al., 2007, Giffel et al., 2002, Vyletelova et al., 2002, Rodriguez et al., 1993). The *B. licheniformis* isolates t501 and t507 were significantly more heat resistant than 13 of the 18 of the other tested isolates. Whilst not closely related to each other, they both had closely related isolates, which were less resistant. Isolate t501 was 34 SNPs different to its closest related isolate w337, and isolate t507 was 49 SNPs different to its closest related isolate w389, however w337 and w389 were significantly less heat resistant than t501 and t507. No mutation was found to be the same between those pairs of isolates (Appendix C, Table C-7 and Table C-8). This indicates that they acquired their higher heat resistance independently through different mutations. Prominently in t501 and t507, mutations in four (*che*Y, *oxd*D, *yfl*N and *yhe*D) and 10 genes (*com*P, era, *feu*B, *ger*AA, *hsl*O, *mnm*G, *pbp*F, *pep*F1, *sle*B and *ywq*J) respectively were found which are thought to be involved in spore formation or heat response and likely responsible for the high heat resistance (Ramirez-Guadiana et al., 2017, Meeske et al., 2016, Nagler et al., 2016, Zheng et al., 2016, Runde et al., 2014, Marchadier et al., 2011, Chao et al., 2006, Comella and Grossman, 2005, Costa et al., 2004, van Ooij et al., 2004, Minkovsky et al., 2002, McPherson et al., 2001, Yamamoto et al., 2000). Overall, multiple *B. licheniformis* isolates achieved D_{90}-values higher than the 60 min maximum exposure time. These isolates could be tested again at greater temperatures or longer times for more accurate estimates.

The D_{90}-value of the *B. subtills* isolate was 29.9 min. Previously observations for *B. subtilis* were 30.4 min or 32.8 min, similar to what was observed in this study, though larger differences could have been expected if more isolates were tested due to the intra species variation (Berendsen et al., 2015, Cho et al., 1999, Rodriguez et al., 1993).

While the *B. cereus* isolate in this study, did not survive 90°C for 5 min, the spores did survive 80°C for 12 min in previous experiments (Chapter 3). Spores of *B. cereus* have previously been reported to survive at 90 °C, with D_{90}-values up to 10.1 min but as low as 1.59 min depending on the isolate and testing conditions (Byrne et al., 2006, Simmonds et al., 2003, Fernández et al., 2001). Since the shortest time tested was 5 min, a 99 % reduction in viable spores could have occurred for the less resistant *B. cereus* isolates. A more detailed D_{90}-value for the *B. cereus* isolate could be reached by sampling at shorter time intervals periods.

Of the isolates able to withstand 90°C, the lowest D_{90}-values were observed for the *B. pumilus* species isolates, with an average D_{90}-value of 6.3 min. Few studies have investigated *B. pumilus* heat resistance, however so far a D_{95}-value of 3.6 min was reported, which is less that the observed value in this study but was measured at a 5 °C higher temperature (Janštová and Lukášová, 2001).

Overall, the results indicate *B. cereus* and *B. pumilus* as the least heat resistant and thereby of lesser concern to milk production. Within a processing environment for milk powder production, milk is exposed to up to 120 °C in the spray dryer, however only the outside of the milk particles are exposed and being counteracted by evaporative cooling, exposing the inside to much lower temperatures around 60 - 70 °C allowing the survival of spore forming and non-spore forming bacteria (Alvarenga et al., 2018, Blazquez et al., 2018, Park et al., 2016, Schuck, 2013). Thereby all isolates tested in this study could be considered as potential contaminants for milk powder. The highest damage potential stems from isolates with the high D_{90}-values since they are likely to survive heat treatments at a greater rate, making it more likely that regulatory specifications are exceeded (McHugh et al., 2017). All species tested have been reported in powders before with *B. licheniformis* being most prevalent (Li et al., 2019, McHugh et al., 2017). It should be considered that the conditions spores are grown under like temperature or medium can influence their heat resistance and there are many potential growth environments for bacteria in milk processing (Bressuire-Isoard et al., 2018, Marchand et

al., 2012). Therefore, to predict the heat resistance of spores, heat resistance under different growth conditions is required to confirm results.

The heat resistance of *Bacillus* spores can increase when the spores are attached to stainless steel (Simmonds et al., 2003). It can be speculated that isolates like 349 or w382 would be of particular concern since they were the only isolates that were the top third of isolates with the greatest biofilm formation on steel in milk, as well as in the top third of the most heat resistant spores.

5.5 **Conclusion**

Spore-forming bacteria from raw milk were tested for their potential to impact milk quality. A broad screening test selected isolates which were able to form biofilms. More detailed testing on the biofilm positive isolates showed, that while most isolates were able to secrete proteases and lipases and were able to grow in milk, differences were found in their ability to form biofilms on stainless steel in milk and produce heat resistant spores. Tested *B. subtilis* and *B. pumilus* isolates formed average biofilms (no significant differences to other isolates) however, *B. pumilus* formed among the least heat resistant spores. Isolates from *B. cereus* formed average biofilms and the least heat resistant spores but were able to grow at 15 °C in milk. *Bacillus licheniformis* isolates formed the most biofilm on stainless steel in milk and the most heat resistant spores, however, some of the *B. licheniformis* isolates were among the weakest biofilm producers and some of the least heat resistant spores in this study, confirming that species identification alone is insufficient to identify isolates with milk damaging potential.

In the process of assessing the milk damaging potential of the isolates, several gene alleles were identified that could lead to the increase in biofilm formation (n=8) and the production of more heat resistant spores (n=14). For example, the genes *gluP* and *oppF* might have so far undescribed gene functions in biofilm formation and lipase secretion respectively however further work is needed to confirm this. Results indicate that

mutations causing the formation of spores with a higher heat resistance could have been acquired in at least two separate occasions. The mutations identified in this study may be used to help assess isolates milk damaging potential without having to test a culture, which could be especially useful in case a genome can be obtained directly from an environmental sample.

5.6 References

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Chapter 6: Summary and general discussion

6.1 Introduction

Spore-forming bacteria are important contaminants in milk and dairy products, due to their ability to withstand measures which limit the growth of non-spore-forming bacteria like pasteurisation, drying or sanitisers (Porcellato et al., 2021, Wedel et al., 2020, Watterson et al., 2014). The milk damaging potential of spore-forming bacteria, can vary depending on the species and strain and they can impact milk safety, quality and shelf life (Mehta et al., 2019, Schmidt et al., 2012). There are limits on certain spore-forming bacteria in dairy products and knowledge of their origin and abilities can be used to design strategies to ensure product quality (FSANZ, 2016).

In a healthy cows udder, milk contains few microorganisms, but can become contaminated during milking from bacteria present on the skin of the udder (Miller et al., 2015b, Gill et al., 2006, Tolle, 1980). Which bacteria contaminate the udder's skin, depends on multiple factors including contamination with faeces, bedding, milking hygiene, season, region and animal feed (Feligini et al., 2015, Miller et al., 2015a, Vacheyrou et al., 2011, Heck et al., 2009, Gill et al., 2006, McKinnon et al., 1990). Despite feed being one of the factors identified as a potential source, for most feeds the quantity and identity of spore-forming bacteria present in them is not well understood (Magnusson et al., 2007, Vissers et al., 2007b). The diet of New Zealand's dairy cows is predominantly pasture-based, however the usage of supplementary feeds has quadrupled in the last 25 years (MPI, 2017). The continued trend of adding new supplementary feeds and increasing the amount of already commonly used once, indicates a study into the spore-forming bacteria in such feeds is required (MPI, 2017).

6.2 Bacterial spore population diversity in cattle feed and raw milk

There is limited knowledge about bacterial spores in most cattle feed except that some spore species found in cattle feed can also be found in the raw milk and other farm sources (Borreani et al., 2019). This work focussed on New Zealand's most used cattle feeds, analysing the quantity and identity of bacterial spores within them. Spore-forming bacteria capable of growing under psychrotrophic, mesophilic or thermophilic temperatures either in the presence or absence of oxygen were enumerated and identified. Significant differences between the number of spore-forming bacteria from different feeds were found and despite silage being the most researched feed, regarding bacterial spore content, the largest number of spores and highest diversity of species were found in tuber feed and PKE that are regarded as new supplementary feeds (MPI, 2017).

All species but one, found in milk were also found in cattle feed used to produce the milk, indicating that spores from feed could get transferred into milk. Significant differences were found between the spore species in different feed types, showing that different feeds carry different potential to impact milk quality based on the numbers and species of spores carried. This may be useful to the milk industry as well as individual farmers to make feeding decisions to avoid high spore counts in raw milk, since avoiding spore-forming bacteria can be easier than removing them from dairy products (Ortuzar et al., 2018).

A metagenomic approach to assess the bacterial spore species in two different cattle feeds was successfully utilised, however similar results were not achieved for milk, likely due to the low number of bacterial spores in New Zealand's raw milk and high background of non-spore-former and cow DNA. The one spore-forming bacteria species found in milk using the genetic analyses was however also found in the two feed samples.

This research identified the number and identity of bacterial spores in cattle feeds, most of which had not previously been reported in these feeds. The data supports the hypothesis that bacterial spores can transfer from cattle feed into milk, however correlation of species abundance between milk and feed, does not necessarily prove causation.

6.3 The connection between bacterial spores in cattle feed and raw milk

Most data on the transfer of bacterial spores from feed to milk is based on the appearance of certain species in both feed and milk or correlations of increased spore levels in feed and milk (Martin et al., 2019, Gupta and Brightwell, 2017, Scheldeman et al., 2005, Vissers et al., 2007a). To provide more definitive proof, 109 bacterial spore genomes from cattle feed and milk were sequenced and compared using SNP differences. Genomes from milk and feed were found to be closely related (<60 SNPs) but did not meet the criteria required to call them identical by decent or matching. The results indicate a higher genome diversity within a species in cattle feed compared to raw milk and some evidence was found that the genome diversity is lower in *B. licheniformis* and *B. pumilus* species compared to *B. cereus*, *B. mycoides*, *B. subtilis* or *B. thermoamylovorans*. Those species genomes also appeared to be more stable over time and geographical distances. This work assessed for the first time the genome diversity within bacterial spore species, in cattle feed and milk. This will lay a foundation for future work, to examine a smaller selection of spore species from few feed source and milk in greater detail, to provide more conclusive evidence of the transfer of bacterial spores from cow feed to milk.

6.4 Bacterial spore's spoilage potential of milk products

A selection of the newly collected and sequenced isolates was examined for their milk damaging potential, to investigate the genotypes associated with milk damage. Isolates were pre-screened for biofilm formation on polystyrene and if able, analysed for their ability to grow in milk at different temperatures, form biofilm on stainless steel in milk, secrete proteases or lipases, as well as the heat resistance of their spores. These experiments intended to assess the isolates fitness to survive or colonise a dairy processing plant. All isolates that passed the pre-screening for biofilm production on polystyrene, were capable of growth in milk and most secreted potentially milk damaging enzymes. Significant differences were found between isolates in the heat resistance of their spores and biofilm formation on stainless steel. These abilities did not group according to species identities, indicating that a species identification alone is not proof to which level an isolate can affect milk quality, which previous work also concluded (Huang et al., 2021, Porcellato et al., 2021, De Jonghe et al., 2010). However, the isolates identified as potentially most milk damaging all belonged to *B. licheniformis*.

Differences in milk damaging potential between closely related isolates were used to investigate the genes responsible for milk damage and identified 22 gene alleles that could be responsible for increased milk damaging potential. This information can be used to screen other isolates for their potential to damage milk, which could be especially useful if combined with the sequencing of genomes directly for environmental samples (Wilkins et al., 2019, Lasken, 2012). In this work, indications of additional gene functions were discovered in *gluP* and *oppF* but further confirmatory experiments are needed.

6.5 Overall conclusions

This work has produced additional knowledge to decrease the number of bacterial spore levels in raw milk as well as insight to detect and avoid the most likely milk damaging bacterial spores in the milk processing environment.

The spore-forming bacteria in commonly used cattle feeds of New Zealand apart from silage, were largely unknown prior to this work especially for newly introduced supplementary feeds. Tuber feeds and PKE were identified as containing the highest number and diversity of bacterial spores. The results from this work could help to provide advice around feed use for farms struggling with elevated bacterial spore counts. This work also showed that most spore-forming bacteria species in cattle feed are also found in the raw milk. It was shown that the genomes of those spores in feed and milk can be closely related, adding more evidence that spores from feed can transfer into the milk. However, a direct match between the genomes of spore-forming bacteria isolated from feed and milk was not found. Groundwork was established for bacterial spore interspecies genome diversity in feed and raw milk, to assist in future study designs investigating the transfer of spores from feed to milk.

The milk damaging potential of 39 spore forming bacteria isolates was assessed, identifying mayor differences, and demonstrating that species identification is insufficient to predict biofilm formation, the heat resistance of spores or enzyme secretion. The work identified multiple gene alleles associated with increased milk damaging abilities.

6.6 Future work

Further work is required to improve the used metagenomic analysis for the investigation of spore-forming bacteria diversity in raw milk. Improving DNA extraction methods and sequencing technology should assist with this and should consider heat treatment and DNA removal steps prior to DNA extraction to remove background DNA for optimal outcomes.

Change alone is eternal so eventually an assessment of newly added feeds will be necessary to assure quality milk production. Besides different types of feed use through time, different countries vary in the types of feed used, thereby the analysis of additional feed types could improve the data for use worldwide.

For more definitive proof of the transfer of spores from cattle feed to milk, a large set of *B. licheniformis* and *B. pumilus* isolates should be tested taken from one farm at the same time of the year to improve the chances of recovery of matching isolates. Chances could be further improved by using selective agar to lower the number of isolates in the selection pool.

The potentially novel gene functions which alter the milk damaging potential of bacterial spores, could be confirmed by mutagenesis. Additional experiments to assess the milk damaging potential of isolates could be, quantifying the protease and lipase secretion and growth curves in milk. Potential differences in those analyses could also be tracked back to genetic differences, adding to the list of milk damaging gene alleles identified in this work. This list could be used in combination with whole genome sequencing from environmental samples to detect especially milk damaging isolates before or soon after they enter the milk processing environment to provide improved strategies to control these bacterial spores.

6.7 References

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Appendix A: Supplementary material Chapter 3

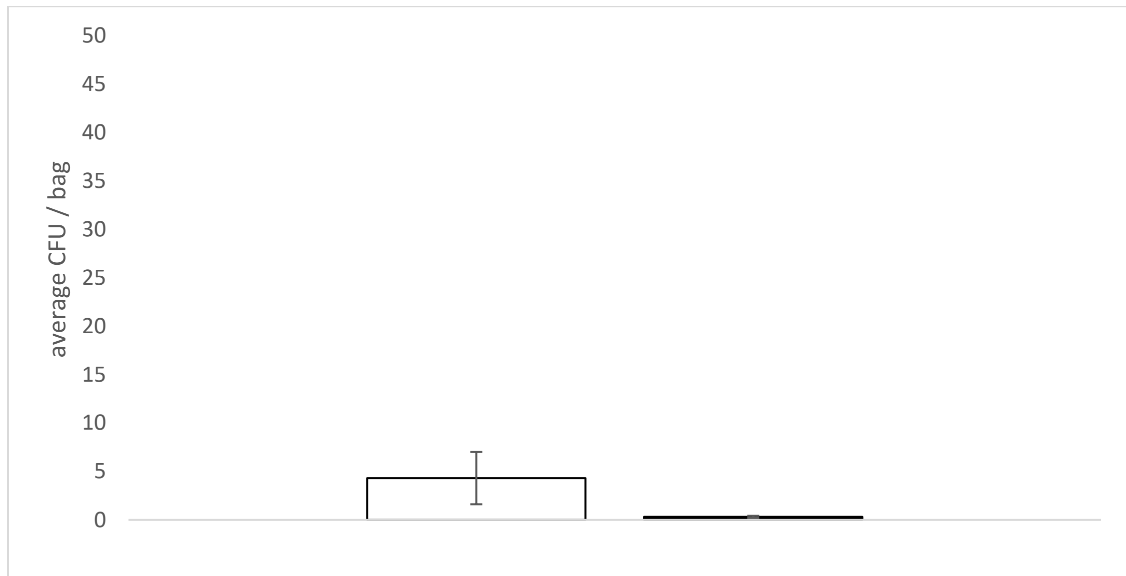

Figure A-1 Average number of bacteria detected in sampling bags tested (n= 20) as per described method in American Public Health Association (APHA) standards chapter 14 in the 18th edition for containers, which determines bags suitable for testing if they contain on average under 50 CFU/bag under aerobic (white) and anaerobic (black) conditions.

Table A-1 Samples analysed for their Bacterial spore count with the region of origin and season of sampling.

Sample type	Bay of plenty	Canterbury	Manawatu-Wanganui	Northland	Southland	Taranaki	Waikato
Milk	Winter 1x	Summer 4x, Autumn 4x	Summer 7x, Autumn 3x, Winter 1x, Spring 2x	Summer 4x	Summer 1x, Winter 1x, Spring 1x	Summer 2x, Spring 1x	Summer 3x, Autumn 2x, Winter 3x, Spring 1x
Pasture	Winter 1x	Summer 4x, Autunm 2x, Winter 1x, Spring 1x	Summer 7x, Autumn 1x, Winter 1x, Spring 2x	Summer 3x	Summer 1x, Spring 1x	Summer 2x, Spring 1x	Summer 7x, Autumn 2x, Winter 2x
Grass silage	Winter 1x		Summer 2x, Winter 1x, Spring 1x		Winter 1x	Summer 1x	Winter 4x
Maize silage		Summer 2x, Autumn 4x	Summer 1x, Autumn 1x, Winter 1x				Summer 2x, Autumn 1x, Winter 2x

PKE		Autumn 2x	Summer 1x, Autumn 1x, Winter 1x		Summer 1x	Summer 1x, Spring 1x	Summer 2x, Autumn 1x, Winter 3x
Chicory & plantain		Autumn 1x, Spring 1x	Summer 3x, Autumn 2x, Spring 2x				Summer 4x
Tuber feed			Summer 3x, Autumn 2x, Winter 1x	Summer 1x	Spring 1x, Winter 1x	Summer 1x	Autumn 1x
Concentrat e	Winter 1x	Spring 1x	Summer 4x, Autumn 2x, Spring 1x,		Spring 1x		Summer 5x, Winter 2x

Table A-2 Output of a mixed effects model: Bacteial spore count versus region, sample, season, temperature and oxygen

Method

Variance estimation	Restricted maximum likelihood
DF for fixed effects	Kenward-Roger

Factor Information

Factor	Type	Levels	Values
Region	Random	7	Bay of plenty, Canterbury, Manawatu-Wanganui, Northland, Southland, Taranaki, Waikato
Sample	Fixed	8	Chicory&Plantain, Concentrates, Tuber feed, Grass silage, Maize silage, Milk, Pasture, PKE
Season	Fixed	4	Autumn, Spring, Summer, Winter
Temperature	Fixed	3	10 °C, 37 °C, 55 °C
Oxygen	Fixed	2	Aerob, Anaerob

Variance Components

Source	Var	% of Total	SE Var	Z-Value	P-Value
Region	0.037462	6.02%	0.029690	1.261783	0.104
Error	0.585169	93.98%	0.027211	21.504514	0.000
Total	0.622632				

-2 Log likelihood = 2295.276302

Tests of Fixed Effects

Term	DF Number	DF Den	F-Value	P-Value
Sample	7.00	929.97	144.03	0.000
Season	3.00	823.61	20.07	0.000
Temperature	2.00	924.89	205.53	0.000
Oxygen	1.00	924.89	29.33	0.000
Sample*Temperature	14.00	924.89	13.68	0.000

Model Summary

S	R-sq	R-sq(adj)
0.764964	65.75%	64.76%

Table A-3 Bacterial species founda after sample enrichment in milk and on a plate (SE) or purely in milk (EE) in samples with high (1x PKE), medium (2x DDG) and low (2x Milk) spore content.

Enrichment method	EE	SE	EE	SE	EE	SE	EE	SE	EE	SE
Sample	Milk	Milk	Milk	Milk	DDG	DDG	DDG	DDG	PKE	PKE
Aneurinibacillus thermoaerophilus	0	0	0	0	0	0	0	1	0	0
B. cereus	1	1	0	0	0	1	0	1	1	1
B. circulans	0	0	0	0	0	0	1	0	0	0
B. coagulans	0	0	0	0	1	1	0	0	1	0
B. lichenoformis	1	1	1	1	1	0	1	1	0	1
B. mycoides	0	0	1	1	0	0	0	0	0	0
B. pumilus	0	0	1	1	0	0	0	0	0	0
B. smithii	0	0	0	0	1	1	1	0	1	0
B. sonorensis	0	0	0	0	0	0	0	0	0	1
B. subtilis	0	0	0	0	1	0	1	1	1	1
B. thermoamylovorans	1	1	0	0	1	0	0	0	1	0
B. wiedmannii	1	1	0	1	0	0	0	0	0	0
Lysinibacillus boronitolerans	1	1	0	0	0	0	0	0	0	0
L. sphaericus	0	1	0	0	0	0	0	0	0	0
Paenibacillus barengoltzii	0	0	0	0	0	0	0	0	1	0
P. chibensis	0	0	1	0	0	0	0	0	0	0

[a] species found indicated by 1 and not found by 0

Table A-4 P-valuesa of a PERMANOVA based of Bray-Curtis distances after Holm-Bonferroni correction, on bacterial spore species populations after sample enrichment in milk and on a plate (SE) or purely in milk (EE).

Enrichment method	EE	SE
EE		0.765
SE	0.765	

[a] significant value (P <0.005)

Table A-5 Percent of samples with positive detection of different bacterial spore species in milk and cattle feed.

Species	Milk	Pasture	Chicory and plantain	Grass silage	Maize silage	Tuber Beet	PKE	Concent rate
Aeribacillus pallidus	3	19	10	0	0	36	20	23
Aneurinibacillus aneurinilyticus	3	0	0	0	0	0	0	8
Aneurinibacillus thermoaerophillus	3	5	0	0	0	0	10	0
Aneurinibacillus migulanus	3	0	0	0	0	0	10	0
Anoxybacillus flavithermus	0	5	0	0	0	0	0	0
B. altitudinis	23	10	10	40	10	9	0	0
B. badius	0	0	0	10	0	0	0	0
B. cereus	52	81	80	50	40	73	50	46
B. circulans	0	19	0	0	10	9	10	8
B. clausii	0	5	0	10	0	0	0	0
B. coagulans	3	10	0	10	0	0	40	38
B. drentensis	0	5	0	0	0	9	0	0
B. flexus	0	0	0	0	0	0	0	8
B. galactosidilyticus	0	0	0	10	0	0	0	0
B. licheniformis	90	100	100	100	90	100	60	69
B. megaterium	16	52	60	10	30	55	10	0
B. muralis	0	5	0	10	0	18	0	0
B. mycoides	19	52	40	20	0	91	10	0
B. oleronius	0	0	0	0	10	0	0	8
B. pseudomycoides	6	10	0	0	0	0	0	0
B. psychrosaccharolyticus	0	24	20	30	0	0	0	0
B. pumilus	29	29	20	20	10	27	0	8

Species	Milk	Pasture	Chicory and plantain	Grass silage	Maize silage	Tuber Beet	PKE	Concent rate
B. simplex	0	14	0	20	0	9	0	8
B. smithii	6	43	0	60	40	55	30	54
B. sonorensis	10	0	0	10	0	9	40	23
B. subtilis	23	33	20	60	30	36	100	69
B. thermoamylovorans	19	48	20	90	50	82	70	77
B. thuringiensis	0	10	10	0	0	0	0	8
B. wiedmannii	39	67	70	40	10	82	20	0
B. weihenstephanensis	16	38	20	30	10	45	10	0
Brevibacillus agri	6	0	0	0	20	0	0	8
Brevibacillus borstelensis	3	5	0	0	10	9	0	0
Brevibacillus parabrevis	0	0	0	0	10	0	0	8
Brevibacillus invocatus	0	0	0	10	0	0	0	0
C. beijerinckii	0	0	0	0	10	0	0	0
C. butyricum	3	0	0	0	0	0	0	0
C. celerecrescens	0	0	0	10	0	0	0	0
C. diolis	0	5	0	0	0	18	30	0
C. intestinale	0	5	0	0	0	9	10	0
C. perfringens	0	10	10	0	0	0	0	8
C. sartagoforme	0	14	0	30	0	18	10	0
C. sordellii	0	5	0	0	0	0	0	0
C. sporogenes	6	10	0	30	10	27	10	15
C. subterminale	3	0	0	10	10	0	0	8
C. tyrobutyricum	3	0	0	10	0	0	0	0
Geobacillus thermoleovorans	0	5	0	0	0	0	0	8
Geobacillus thermoglucosidasius	0	5	0	10	0	9	20	8
Geobacillus stearothermophilus	0	0	0	0	0	0	0	8
L. boronitolerans	0	5	0	10	0	27	40	8
L. fusiformis	0	14	20	0	0	0	10	0
L. sphaericus	6	24	10	30	0	36	20	8
L. xylanilyticus	0	0	0	10	0	27	0	0
Paenibacillus alginolyticus	0	0	0	0	10	0	0	0
P. amylolyticus	10	24	20	20	0	55	0	8
P. anaericanus	0	5	0	0	0	0	0	0
P. azoreducens	0	0	0	0	0	0	10	0
P. barengoltzii	0	14	10	10	10	9	20	0
P. brasilensis	0	5	0	0	0	0	0	0
P. cookii	0	0	0	0	30	0	30	15

Species	Milk	Pasture	Chicory and plantain	Grass silage	Maize silage	Tuber Beet	PKE	Concent rate
P. chitinolyticus	0	5	0	10	0	0	0	0
P. ehimensis	0	0	0	0	0	0	0	8
P. illinoisensis	0	0	0	0	0	9	10	0
P. jamilae	0	0	0	0	0	9	0	0
P. pabuli	0	0	0	0	0	0	0	15
P. phoenicis	3	0	0	0	0	0	20	0
P. polymyxa	0	5	0	10	10	0	10	8
P. lactis	0	0	0	0	0	0	10	0
P. macerans	0	0	0	40	20	36	10	8
P. macquariensis	0	14	0	10	0	9	0	0
P. odorifer	0	0	0	20	0	9	10	0
P. thiaminolyticus	0	0	0	0	10	0	0	0
P. timonensis	0	0	0	0	0	0	30	15
P. stellifer	0	5	0	0	0	0	0	0
Psychrobacillus psychrotolerans	0	29	10	0	0	27	10	0
Rummeliibacillus suwonensis	0	5	0	0	0	0	0	0
Solibacillus silvestris	3	38	10	10	0	0	10	0
Thermoactinomyces sp	0	19	0	0	0	9	20	8
Viridibacillus arenosi	0	10	0	0	0	27	0	0
Viridibacillus neidei	0	5	0	10	0	27	0	0
Virgibacillus proomii	0	5	0	0	0	9	0	0

Table A-6 Sequencing statistics of metagenomic 16S rRNA gene analysis.

Sample	Pre extraction treatment	Input	Filtered	Denoised	Merged	Non-chimeric	Spore-related
Milk1	Heat treatment [a]	440595	17744	17481	12389	11929	0
Milk1	Enrichment 10°C	395708	18207	18205	12971	12971	0
Milk1	Enrichment 37°C	443582	20714	20701	20142	19731	88
Milk1	Enrichment 55°C	472754	30072	30042	21422	18684	0
Milk2	Heat treatment [a]	329973	18753	18737	17311	15191	0
Milk2	Enrichment 10°C	1645	0	0	0	0	0
Milk2	Enrichment 37°C	483277	34926	34855	32974	31552	0

Sample	Pre extraction treatment	Input	Filtered	Denoised	Merged	Non-chimeric	Spore-related
Milk2	Enrichment 55°C	576063	45908	45811	43927	42329	0
PKE	Heat treatment [a]	178666	159347	158701	156833	154871	3959
PKE	Enrichment 10°C	237441	212490	212359	212129	211979	214
PKE	Enrichment 37°C	198776	182198	181647	176109	131117	6059
PKE	Enrichment 55°C	290632	166351	166102	163604	132014	75750
Tuber feed	Heat treatment [a]	141182	87454	86451	83071	81027	192
Tuber feed	Enrichment 10°C	184087	160376	159823	157355	148577	20
Tuber feed	Enrichment 37°C	286778	259255	257878	250296	220944	467
Tuber feed	Enrichment 55°C	259464	69696	69427	68132	59638	545

[a] Heat treatment at 80°C for 12 min

Appendix B: Supplementary material Chapter 4

Table B-1 Detailed origin and species identification of used isolates.

ID	Species	Farm	Sample type	Season	Growth temperature
600	*B. cereus*	26	Pasture	Summer	37°C
601	*B. cereus*	26	Pasture	Summer	37°C
602	*B. cereus*	26	Pasture	Summer	37°C
606	*B. cereus*	26	Pasture	Summer	37°C
624	*B. cereus*	26	Pasture	Summer	37°C
a126	*B. cereus*	3	Milk	Autumn	37°C
a131	*B. cereus*	3	Pasture	Autumn	37°C
a321	*B. cereus*	16	Milk	Autumn	37°C
t191	*B. cereus*	35	Tuber feed	Autumn	37°C
t27	*B. cereus*	35	Tuber feed	Autumn	37°C
t28	*B. cereus*	35	Tuber feed	Autumn	37°C
t398	*B. cereus*	35	Milk	Autumn	37°C
t80	*B. cereus*	35	Tuber feed	Autumn	37°C
w274	*B. cereus*	26	Tuber feed	Winter	37°C
w285	*B. cereus*	26	Milk	Winter	37°C
w292	*B. cereus*	26	Tuber feed	Winter	37°C
w266	*B. cereus*	26	Pasture	Winter	37°C
w307	*B. cereus*	26	Milk	Winter	37°C
282	*B. licheniformis*	26	Milk	Summer	37°C
304	*B. licheniformis*	26	Milk	Summer	37°C
326	*B. licheniformis*	26	Milk	Summer	37°C
349	*B. licheniformis*	26	Milk	Summer	37°C
358	*B. licheniformis*	26	Milk	Summer	37°C
366	*B. licheniformis*	26	Milk	Summer	37°C
389	*B. licheniformis*	26	Milk	Summer	37°C
428	*B. licheniformis*	26	Milk	Summer	37°C
609	*B. licheniformis*	26	Pasture	Summer	37°C
640	*B. licheniformis*	26	Pasture	Summer	37°C
a157	*B. licheniformis*	16	Milk	Autumn	37°C
a189	*B. licheniformis*	16	Pasture	Autumn	37°C

ID	Species	Farm	Sample type	Season	Growth temperature
a191	*B. licheniformis*	16	PKE	Autumn	37°C
a338	*B. licheniformis*	16	Maize silage	Autumn	37°C
g103	*B. licheniformis*	7	Pasture	Spring	37°C
g117	*B. licheniformis*	7	Pasture	Spring	37°C
g131	*B. licheniformis*	7	Pasture	Spring	37°C
g134	*B. licheniformis*	7	Pasture	Spring	37°C
g135	*B. licheniformis*	7	Pasture	Spring	37°C
g197	*B. licheniformis*	7	Tuber feed	Spring	37°C
g213	*B. licheniformis*	7	Milk	Spring	37°C
g220	*B. licheniformis*	7	Tuber feed	Spring	37°C
g254	*B. licheniformis*	7	Tuber feed	Spring	37°C
g260	*B. licheniformis*	7	Pasture	Spring	37°C
g291	*B. licheniformis*	7	Tuber feed	Spring	37°C
t104	*B. licheniformis*	7	Tuber feed	Winter	37°C
t108	*B. licheniformis*	35	Tuber feed	Autumn	37°C
t111	*B. licheniformis*	36	Tuber feed	Autumn	37°C
t113	*B. licheniformis*	35	Tuber feed	Autumn	37°C
t124	*B. licheniformis*	7	Tuber feed	Winter	37°C
t184	*B. licheniformis*	35	Tuber feed	Autumn	37°C
t195	*B. licheniformis*	36	Tuber feed	Autumn	37°C
t206	*B. licheniformis*	35	Tuber feed	Autumn	37°C
t238	*B. licheniformis*	7	Tuber feed	Winter	37°C
t305	*B. licheniformis*	7	Tuber feed	Winter	37°C
t310	*B. licheniformis*	7	Tuber feed	Winter	37°C
t36	*B. licheniformis*	35	Tuber feed	Autumn	37°C
t396	*B. licheniformis*	36	Milk	Autumn	37°C
t406	*B. licheniformis*	36	Milk	Autumn	37°C
t414	*B. licheniformis*	35	Milk	Autumn	37°C
t415	*B. licheniformis*	36	Milk	Autumn	37°C
t423	*B. licheniformis*	36	Milk	Autumn	37°C
t430	*B. licheniformis*	7	Tuber feed	Winter	37°C
t501	*B. licheniformis*	36	Milk	Autumn	37°C
t505	*B. licheniformis*	7	Milk	Winter	37°C
t507	*B. licheniformis*	36	Milk	Autumn	37°C
t88	*B. licheniformis*	36	Tuber feed	Autumn	37°C

ID	Species	Farm	Sample type	Season	Growth temperature
w240	*B. licheniformis*	26	Milk	Winter	37°C
w262	*B. licheniformis*	26	Milk	Winter	37°C
w265	*B. licheniformis*	26	Pasture	Winter	37°C
w275	*B. licheniformis*	26	Tuber feed	Winter	37°C
w326	*B. licheniformis*	26	Milk	Winter	37°C
w337	*B. licheniformis*	26	Milk	Winter	37°C
w382	*B. licheniformis*	26	Milk	Winter	37°C
w389	*B. licheniformis*	30	Milk	Winter	37°C
448	*B. mycoides*	26	Milk	Summer	30°C
653	*B. mycoides*	26	Milk	Summer	30°C
659	*B. mycoides*	26	Pasture	Summer	30°C
w511	*B. mycoides*	26	Tuber feed	Winter	30°C
w413	*B. mycoides*	26	Tuber feed	Winter	30°C
367	*B. pumilus*	26	Milk	Summer	37°C
388	*B. pumilus*	26	Milk	Summer	37°C
413	*B. pumilus*	26	Milk	Summer	37°C
414	*B. pumilus*	26	Milk	Summer	37°C
622	*B. pumilus*	26	Pasture	Summer	37°C
626	*B. pumilus*	26	Pasture	Summer	37°C
w264	*B. pumilus*	26	Milk	Winter	37°C
w271	*B. pumilus*	26	Tuber feed	Winter	37°C
332	*B. subtilis*	26	Milk	Summer	37°C
334	*B. subtilis*	26	Milk	Summer	37°C
365	*B. subtilis*	26	Milk	Summer	37°C
g194	*B. subtilis*	7	Pasture	Spring	37°C
t391	*B. subtilis*	7	Milk	Winter	37°C
w272	*B. subtilis*	26	Tuber feed	Winter	37°C
610	*B. thermoamylovorans*	26	Pasture	Summer	55°C
614	*B. thermoamylovorans*	26	Pasture	Summer	55°C
616	*B. thermoamylovorans*	26	Pasture	Summer	55°C
635	*B. thermoamylovorans*	26	Pasture	Summer	55°C
643	*B. thermoamylovorans*	26	Pasture	Summer	55°C
w347	*B. thermoamylovorans*	26	Milk	Winter	55°C
w352	*B. thermoamylovorans*	26	Tuber feed	Winter	55°C
w353	*B. thermoamylovorans*	26	Tuber feed	Winter	55°C

ID	Species	Farm	Sample type	Season	Growth temperature
650	*B. weihenstephanensis*	26	Pasture	Summer	30°C
661	*B. weihenstephanensis*	26	Pasture	Summer	30°C
w273	*B. weihenstephanensis*	26	Tuber feed	Winter	30°C
w411	*B. weihenstephanensis*	26	Pasture	Winter	30°C
w410	*B. weihenstephanensis*	26	Milk	Winter	30°C
t213	*B. wiedmannii*	37	Tuber feed	Autumn	30°C
t489	*B. wiedmannii*	37	Milk	Autumn	30°C
t417	*P. phoenicis*	36	Milk	Autumn	37°C
t93	*P. phoenicis*	36	PKE	Autumn	37°C

Table B-2 Assembly statistics of the isolate genomes.

Assembly	# contigs (>= 0 bp)	# contigs (>= 1000 bp)	Total length (>= 0 bp)	Total length (>= 1000 bp)	Largest contig	GC (%)	N50	L50	# N's per 100 kbp
282	86	40	4338681	4321174	605006	45.78	216727	6	0
304	35	15	4299447	4293481	1755090	45.79	1086010	2	0
326	42	25	4103636	4098650	664416	46.2	389655	5	0
332	53	14	4068265	4057094	1060199	43.7	1057975	2	0
334	50	15	4120764	4109398	1044703	43.66	679553	3	0
349	42	24	4208225	4201987	726180	46.18	290075	5	0
358	47	17	4263998	4255657	1766238	46.02	802000	2	0
365	74	29	4124109	4108973	700588	43.66	306766	4	0
366	45	31	4549746	4545169	557155	45.35	362569	6	0
367	40	20	3730685	3722685	956997	41.48	611291	3	0
388	35	16	3658186	3651834	940908	41.55	440488	3	0
389	41	25	4314864	4310474	936416	45.85	414728	4	0
413	28	14	3638578	3632933	958286	41.59	931813	2	0
414	42	23	3728552	3721412	862965	41.48	611291	3	0
428	44	29	4549896	4544781	556522	45.34	395658	5	0
448	144	69	5816938	5791459	773411	35.16	196841	9	0
600	312	183	5661751	5624792	270755	35.04	73086	21	0
601	81	41	5971831	5957860	950642	34.98	373745	6	0
602	83	38	5971316	5955861	1121553	34.97	373745	5	0
606	292	106	5688265	5636466	431924	35.39	163270	12	0
609	47	18	4200337	4190264	1467534	46.06	477116	3	0
610	471	156	3769705	3686474	227174	37.39	41637	27	0
614	186	72	3857563	3822844	305101	37.33	137872	10	0
616	178	69	3858320	3823545	309533	37.34	141322	9	0
622	55	20	3749860	3738917	952015	41.49	542362	3	0

Assembly	# contigs (>= 0 bp)	# contigs (>= 1000 bp)	Total length (>= 0 bp)	Total length (>= 1000 bp)	Largest contig	GC (%)	N50	L50	# N's per 100 kbp
624	181	80	5672239	5638369	498667	35.38	223223	8	0
626	46	19	3764632	3753765	1046575	41.28	426903	3	0
635	481	168	3945803	3854647	257474	37.1	40947	26	0
640	48	18	4201311	4191062	1467534	46.06	477116	3	0
643	253	102	3915354	3865621	561782	37.19	78153	14	0
650	216	99	6180136	6135511	700255	35.06	173845	9	0
653	141	71	5827245	5804246	773486	35.15	199254	8	0
659	216	84	6130100	6080331	663331	35.1	184937	9	0
661	212	95	6131707	6091554	663110	35.06	285635	8	0
g103	57	30	4309091	4299468	964561	46.02	467079	4	0
g117	56	36	4305707	4299673	964561	46.02	417180	4	0
g131	48	25	4320553	4313614	1020449	45.67	387065	3	0
g134	42	24	4106881	4100482	664775	46.2	392588	5	0
g153	125	85	4167521	4152198	413486	46.15	117464	9	0
g194	46	15	4204919	4195245	852736	43.44	698130	3	0
g197	36	22	4194978	4191199	673793	46.14	355439	5	0
g213	58	36	4303292	4296218	949384	46.01	274293	4	0
g220	40	24	4177106	4171703	1183107	46.22	441331	3	0
g254	33	14	4173374	4166322	2171534	46.15	2171534	1	0
g260	36	24	4104291	4100446	664775	46.2	392588	5	0
g291	34	15	4287325	4279851	1827322	45.85	1095175	2	0
t27	217	103	5262300	5222361	514321	35.48	156414	11	0
t28	202	86	5680395	5646773	827265	35.04	145108	9	0
t36	51	35	4361207	4354561	604548	45.7	333799	5	0
t80	199	122	5454132	5425742	431540	35.09	103554	13	0
t88	116	42	4530083	4507961	577050	45.79	182751	8	0
t93	552	182	4829895	4732192	130838	51.8	51785	31	0
t104	59	33	4264377	4255728	522416	45.84	224705	6	0
t108	39	15	4262424	4255595	1766479	46.02	801813	2	0
t111	37	13	4338628	4331661	1907782	45.75	732140	2	0
t113	65	28	4392091	4378223	444697	45.67	297374	7	0
t124	45	25	4094923	4088217	1198404	46.28	441353	3	0
t184	118	65	4597212	4580447	339103	45.38	169634	10	0
t191	195	76	5460255	5428494	688814	35.08	204137	8	0
t195	109	43	4602769	4584790	472348	45.39	209534	8	0
t206	252	169	4599791	4573431	223338	45.38	67473	19	0
t213	110	49	5929986	5911219	1342454	35.07	228460	7	0
t238	47	22	4194101	4186699	790466	46.06	477330	4	0
t305	40	15	4365575	4358348	1762238	45.72	801330	2	0
t310	86	37	4366382	4351848	849621	45.84	319500	4	0
t391	48	18	4218488	4208906	1068171	43.39	558306	3	0
t396	58	27	4418194	4405339	864084	45.72	452922	4	0
t398	182	74	5929476	5900764	1307851	34.96	171668	7	0
t406	52	16	4318555	4306806	1767413	45.97	771095	2	0
t414	60	44	4207887	4203238	453161	46.18	178554	7	0
t415	96	47	4484756	4462238	677136	45.75	331995	5	0

Assembly	# contigs (>= 0 bp)	# contigs (>= 1000 bp)	Total length (>= 0 bp)	Total length (>= 1000 bp)	Largest contig	GC (%)	N50	L50	# N's per 100 kbp
t417	492	204	4606027	4519809	131315	52.35	47088	31	0
t423	66	35	4232455	4222063	768833	46.18	487601	4	0
t430	69	21	4311911	4297467	673016	45.87	476606	4	0
t489	100	45	5840729	5823870	1084569	35.02	329706	5	0
t501	54	15	4322293	4308740	1613726	45.98	810126	2	0
t505	91	41	4274952	4256319	964570	46.17	331533	4	0
t507	70	31	4169691	4155767	765596	46.18	401187	4	0
w240	47	27	4122671	4116455	720280	46.21	335424	5	0
w262	2351	739	5013148	4498796	76838	45.25	9881	115	0
w264	45	21	3693661	3683631	956993	41.55	575304	3	0
w265	73	28	4213709	4201798	585170	46.07	296466	5	0
w266	164	87	5231567	5203974	583227	35.51	156468	9	0
w271	40	15	3724430	3716508	1005354	41.65	936679	2	0
w272	40	13	4173426	4164526	1531818	43.49	690670	2	0
w273	192	53	5683970	5651543	792841	35.04	241599	6	0
w274	223	98	5455511	5411752	523607	35.46	145220	12	0
w275	69	28	4377965	4364111	748089	45.73	445969	4	0
w285	156	56	5539344	5511449	909967	35.32	258069	6	0
w292	210	87	5622573	5589930	731403	35.07	209020	7	0
w307	177	108	5894682	5868018	471502	35.2	143849	12	0
w326	49	31	4331259	4324481	604647	45.78	347908	5	0
w337	47	17	4263482	4254032	1765857	46.02	803017	2	0
w347	354	113	3797400	3725544	162564	37.28	68049	20	0
w352	166	79	3868759	3840709	268395	37.48	107210	12	0
w353	140	65	3870112	3847243	456706	37.4	111867	9	0
w382	108	50	4318150	4303617	504825	45.91	214831	7	0
w389	50	30	4120427	4113950	911317	46.22	359349	4	0
w410	207	100	6130486	6093337	768249	35.05	189807	8	0
w411	203	98	6295887	6257977	617548	34.98	285670	8	0
w413	197	81	5641598	5601016	415728	35.36	139070	12	0
w511	191	88	5637005	5601616	403938	35.36	136101	13	0
a126	2188	1035	5465851	4929780	53991	35.7	7068	183	0
a131	196	85	5869856	5845928	491130	35	152735	13	0
a157	59	31	4302529	4293434	560425	45.82	362856	5	0
a189	76	38	4268800	4260158	543770	46.16	254550	6	0
a191	68	32	4183963	4173331	651201	46.2	382324	5	0
a321	1259	665	5907610	5635366	137027	35.31	18052	72	0
a338	33	20	4420769	4417954	1193469	45.25	612390	3	0
G117-1	98	42	4318517	4299767	464881	46.03	355363	6	0
G197-1	62	26	4205668	4195368	673793	46.14	388146	4	0
G213-1	124	32	4329914	4301073	964568	46.01	438661	3	0

Table B-3 SNP's found in alignment[a] between B. weihenstephanensis and B. mycoides isolates which clustered together.

Species	Isolate	650	659	661
B. weihenstephanensis	650	0	43	44
B. mycoides	659	43	0	9
B. weihenstephanensis	661	44	9	0

[a] Values below 7 indicate a match and below 60 indicating isolates belong to the same cluster

Appendix C: Supplementary material Chapter 5

Table C-1 Location and effect of mutations found in B. licheniformis isolate 326 and isolate t414 when compared to isolate 366 and isolate 428 respectively.

Mutation type	326 and t414	366 and 428	Effect	Gene	Product	Known for biofilm formation
snp	C	T	missense_variant c.95C>T p.Ser32Leu	*aroP*	Aromatic amino acid transport protein AroP	Unknown
snp	G	A	missense_variant c.409G>A p.Ala137Thr	*bm3R1*	HTH-type transcriptional repressor Bm3R1	Unknown
snp	A	G	missense_variant c.730T>C p.Ser244Pro	*bsdC*	Phenolic acid decarboxylase	Unknown
snp	T	A	missense_variant c.913T>A p.Leu305Met	*buk2*	Butyrate kinase 2	Unknown
snp	G	A	missense_variant c.562C>T p.Pro188Ser	*cheB_1*	Protein-glutamate methylesterase/protein-glutamine glutaminase	(Huang et al., 2019)
snp	C	T	missense_variant c.130G>A p.Val44Ile	*copZ*	Copper chaperone CopZ	Unknown
snp	T	C	missense_variant c.512A>G p.Glu171Gly	*ddl_1*	D-alanine--D-alanine ligase	Unknown
snp	G	A	stop_gained c.665G>A p.Trp222*	*epr*	Minor extracellular protease Epr	Unknown
snp	T	G	missense_variant c.469A>C p.Lys157Gln	*fadA*	3-ketoacyl-CoA thiolase	Unknown
snp	G	A	missense_variant c.1205C>T p.Ala402Val	*htrB*	Serine protease Do-like HtrB	Unknown
snp	G	T	missense_variant c.169G>T p.Val57Leu	*maf*	dTTP/UTP pyrophosphatase	Unknown
snp	C	A	missense_variant c.1237G>T p.Ala413Ser	*murJ_2*	Lipid II flippase MurJ	Unknown

Mutation type	326 and t414	366 and 428	Effect	Gene	Product	Known for biofilm formation
snp	T	A	stop_gained c.212T>A p.Leu71*	*pseB*	UDP-N-acetylglucosamine 4,6-dehydratase (inverting)	Unknown
snp	A	C	missense_variant c.850A>C p.Met284Leu	*ptsG*	PTS system glucose-specific EIICBA component	Unknown
snp	G	T	missense_variant c.117C>A p.Ser39Arg	*qoxD*	Quinol oxidase subunit 4	Unknown
snp	T	G	missense_variant c.185A>C p.Lys62Thr	*rimM*	Ribosome maturation factor RimM	Unknown
snp	G	A	missense_variant c.1130C>T p.Thr377Ile	*speA_2*	Arginine decarboxylase	(Burrell et al., 2010)
snp	A	T	missense_variant c.185T>A p.Ile62Asn	*tuaH*	Putative teichuronic acid biosynthesis glycosyltransferase TuaH	Unknown
ins	C	CCGTCAGGCTTAATG	frameshift_variant c.1101_1114dupCATTAAGCCTGACG p.Gly372fs	*uup*	ABC transporter ATP-binding protein uup	Unknown
snp	G	T	missense_variant c.215C>A p.Ala72Asp	*ybdM*	putative serine/threonine-protein kinase YbdM	Unknown
snp	C	T	missense_variant c.1142C>T p.Pro381Leu	*yknZ*	putative ABC transporter permease YknZ	(Xu et al., 2016)
snp	C	T	missense_variant c.796G>A p.Ala266Thr	*yodF*	putative symporter YodF	Unknown

Hypothetical proteins with non-synonymous mutations found	10 x
Percent of the genome not included in alignment between 326 and t414	0.2%
Percent of the genome not included in alignment between 366 and 428	0.3 %

Table C-2 Location and effect of mutations found between B. subtilis isolate 334 towards both 332 and 365.

Type	t507	332	Effect	Gene	Product	Known for biofilm formation
snp	A	C	missense_variant c.1473T>G p.Asp491Glu	*gluP*	Rhomboid protease GluP	Unknown
Hypothetical proteins with non-synonymous mutations found						1
Percent of the genome not included in alignment between 334 towards 332 and 365 respectively						0.2% & 0.3 %

Table C-3 Location and effect of mutations found between B. pumilus isolate 367 towards 414.

Type	367	414	Effect	Gene	Product	Known for biofilm formation
del	TG	T	frameshift_variant c.1158delG p.Val389fs	*tetA_1*	Tetracycline resistance protein, class C	Unknown
Hypothetical proteins with non-synonymous mutations found						2
Percent of the genome not included in alignment						0.2 %

Table C-4 Location and effect of mutations found between B. pumilus isolate 367 towards w264.

Type	367	w264	Effect	Gene	Product	Known for biofilm formation
del	GCGATTT	G	conservative_inframe_deletion c.1243_1248delTCGATT p.Ser415_Ile416del	*alsT_2*	Amino-acid carrier protein AlsT	Unknown
del	CAGAGG	C	frameshift_variant c.1351_1355delGAGGA p.Glu451fs	*aspA_2*	Aspartate ammonia-lyase	(Wang et al., 2020)
snp	C	A	missense_variant c.89G>T p.Gly30Val	*sasA_4*	Adaptive-response sensory-kinase SasA	Unknown
Hypothetical proteins with non-synonymous mutations found						1
Percent of the genome not included in alignment						1.2 %

Table C-5 Location and effect of mutations found between B. licheniformis isolates t507 towards t505 which are not also present between t507 towards t423.

Type	t507	t505	Effect	Gene	Product	Known for biofilm formation
snp	G	A	missense_variant c.329C>T p.Pro110Leu	*acsA_2*	Acetyl-coenzyme A synthetase	(Hathroubi et al., 2020)
snp	G	A	missense_variant c.745C>T p.Leu249Phe	*bcrA_2*	Bacitracin transport ATP-binding protein BcrA	Unknown
snp	C	A	missense_variant c.280G>T p.Asp94Tyr	*gerBA_1*	Spore germination protein B1	Unknown
snp	C	T	missense_variant c.466G>A p.Gly156Arg	*glsA2*	Glutaminase 2	Unknown
snp	C	G	missense_variant c.325C>G p.Leu109Val	*mutS2*	Endonuclease MutS2	Unknown
snp	T	A	missense_variant c.116T>A p.Ile39Asn	*pcaK*	4-hydroxybenzoate transporter PcaK	Unknown
snp	C	T	missense_variant c.346G>A p.Ala116Thr	*pccB*	Propionyl-CoA carboxylase beta chain	Unknown
snp	G	C	missense_variant c.25G>C p.Glu9Gln	*rapI_2*	Response regulator aspartate phosphatase I	(Christensen et al., 2021)
snp	C	A	missense_variant c.235G>T p.Gly79Cys	*rpsC*	30S ribosomal protein S3	Unknown
snp	G	A	missense_variant c.433G>A p.Glu145Lys	*ttr*	Acetyltransferase	Unknown
snp	A	T	missense_variant c.545A>T p.Glu182Val	*xylA_2*	Xylose isomerase	Unknown
snp	T	A	missense_variant c.1511T>A p.Val504Glu	*yeeF_2*	Putative ribonuclease YeeF	Unknown

Type	t507	t505	Effect	Gene	Product	Known for biofilm formation
snp	A	G	missense_variant c.1484T>C p.Val495Ala	*yitJ*	Bifunctional homocysteine S-methyltransferase/5,10-methylenetetrahydrofolate reductase	Unknown
del	CT	C	frameshift_variant c.917delA p.Gln306fs	*yjeH*	L-methionine/ branched-chain amino acid exporter YjeH	Unknown
snp	T	A	missense_variant c.935A>T p.Lys312Ile	*ylbL*	putative protein YlbL	Unknown

Hypothetical proteins with non-synonymous mutations found	13
Percent of the genome not included in alignment between t507 and t505	0.3 %
Percent of the genome not included in alignment between t507 and t423	1.3 %

Table C-6 Location and effect of mutations found between B. licheniformis isolates g213 towards t423 and t507.

Type	g213	t423 and t507	Effect	Gene	Product	Lipase secretion related
snp	T	A	missense_variant c.1349T>A p.Val450Asp	*bglF_1*	PTS system beta-glucoside-specific EIIBCA component	Unknown
snp	C	T	missense_variant c.551C>T p.Ser184Leu	*birA*	Bifunctional ligase/repressor BirA	Unknown
snp	A	G	missense_variant c.386A>G p.Asp129Gly	*bshB2*	putative N-acetyl-alpha-D-glucosaminyl L-malate deacetylase 2	Unknown
snp	A	G	missense_variant c.248T>C p.Ile83Thr	*comA_2*	Transcriptional regulatory protein ComA	Unknown
snp	T	C	missense_variant c.230T>C p.Leu77Ser	*comN*	Post-transcriptional regulator ComN	Unknown
ins	G	GAT	frameshift_variant c.484_485in	*leuD1*	3-isopropylmalate	Unknown

Type	g213	t423 and t507	Effect	Gene	Product	Lipase secretion related
			sAT p.Ala162fs		dehydratase small subunit 1	
snp	C	G	missense_variant c.1052G>C p.Gly351Ala	*leuS*	Leucine--tRNA ligase	Unknown
snp	A	G	missense_variant c.807A>G p.Ile269Met	*mntH*	Divalent metal cation transporter MntH	Unknown
snp	A	C	stop_lost&splice_region_variant c.448T>G p.Ter150Gluext*?	*murP*	PTS system N-acetylmuramic acid-specific EIIBC component	Unknown
snp	T	C	missense_variant c.122T>C p.Leu41Ser	*nadB*	L-aspartate oxidase	Unknown
snp	A	G	missense_variant c.1103T>C p.Val368Ala	*nagZ*	Beta-hexosaminidase	Unknown
snp	C	G	missense_variant c.559G>C p.Glu187Gln	*oppF_4*	Oligopeptide transport ATP-binding protein OppF	Unknown
snp	A	G	missense_variant c.628A>G p.Ile210Val	*pyrG*	CTP synthase	Unknown
snp	C	T	missense_variant c.1256C>T p.Pro419Leu	*recN*	DNA repair protein RecN	Unknown
snp	T	C	missense_variant c.211T>C p.Tyr71His	*thiN*	Thiamine pyrophosphokinase	Unknown
snp	T	C	missense_variant c.896T>C p.Val299Ala	*xlyA*	N-acetylmuramoyl-L-alanine amidase XlyA	Unknown
snp	T	C	missense_variant c.647T>C p.Met216Thr	*yeeF_1*	Putative ribonuclease YeeF	Unknown

Hypothetical proteins with non-synonymous mutations found	5
Percent of the genome not included in alignment between g213 and t423	1.3 %
Percent of the genome not included in alignment between g213 and t507	1.4 %

Table C-7 Location and effect of mutations found between B. licheniformis isolates t507 towards w389.

Type	t507	w389	Effect	Gene	Product	Spore related
snp	T	A	missense_variant c.92T>A p.Met31Lys	*abh_1*	Putative transition state regulator Abh	Unknown
snp	A	C	missense_variant c.76A>C p.Ile26Leu	*adhE*	Aldehyde-alcohol dehydrogenase	Unknown
snp	C	T	missense_variant c.1171C>T p.Arg391Cys	*ahpF_2*	NADH dehydrogenase	Unknown
complex	TT	CG	missense_variant c.117_118delAAinsCG p.Ile40Val	*araD_2*	L-ribulose-5-phosphate 4-epimerase	Unknown
snp	C	T	missense_variant c.358C>T p.Pro120Ser	*atpA*	ATP synthase subunit alpha	Unknown
snp	C	T	missense_variant c.1418C>T p.Ala473Val	*comP*	Sensor histidine kinase ComP	(Comella and Grossman, 2005)
snp	T	G	missense_variant c.574T>G p.Tyr192Asp	*cpdA*	3',5'-cyclic adenosine monophosphate phosphodiesterase CpdA	Unknown
snp	A	G	missense_variant c.35T>C p.Leu12Pro	*dgk*	Deoxyguanosine kinase	Unknown
snp	T	A	missense_variant c.655T>A p.Ser219Thr	*era_2*	GTPase Era	(Minkovsky et al., 2002)
snp	A	G	missense_variant c.838A>G p.Ser280Gly	*fadA*	3-ketoacyl-CoA thiolase	Unknown
snp	C	T	missense_variant c.86C>T p.Ala29Val	*feuB_2*	Iron-uptake system permease protein FeuB	(Nagler et al., 2016)
snp	A	C	missense_variant c.1540A>C p.Asn514His	*gerAA_2*	Spore germination protein A1	(Ramirez-Guadiana et al., 2017)
ins	T	TC	frameshift_variant c.879_880in	*glgC*	Glucose-1-phosphate adenylyltransferase	Unknown

Type	t507	w389	Effect	Gene	Product	Spore related
			sG p.Asn294fs			
snp	T	C	missense_variant c.872A>G p.Glu291Gly	*gmuC*	PTS system oligo-beta-mannoside-specific EIIC component	Unknown
snp	C	G	missense_variant c.230G>C p.Gly77Ala	*hslO*	33 kDa chaperonin	(Runde et al., 2014)
snp	A	G	missense_variant c.247A>G p.Lys83Glu	*iolS_1*	Aldo-keto reductase IolS	Unknown
snp	T	A	missense_variant c.1166T>A p.Leu389His	*iolT*	Major myo-inositol transporter IolT	Unknown
del	AC	A	frameshift_variant c.591delC p.Ala198fs	*lysN*	2-aminoadipate transaminase	Unknown
snp	G	A	missense_variant c.196G>A p.Gly66Ser	*metN_1*	Methionine import ATP-binding protein MetN	Unknown
snp	T	G	missense_variant c.1669A>C p.Lys557Gln	*mnmG*	tRNA uridine 5-carboxymethylaminomethyl modification enzyme MnmG	(Meeske et al., 2016)
ins	A	ATT	frameshift_variant c.637_638dupTT p.Phe215fs	*nikA_1*	Nickel-binding protein NikA	Unknown
snp	C	A	missense_variant c.1110C>A p.Ser370Arg	*norG*	HTH-type transcriptional regulator NorG	Unknown
snp	C	T	missense_variant c.650C>T p.Ser217Leu	*opuCB*	Glycine betaine/carnitine/choline transport system permease protein OpuCB	Unknown
snp	A	T	missense_variant c.1575T>A p.Asp525Glu	*pbpF*	Penicillin-binding protein 1F	(McPherson et al., 2001)
snp	T	C	missense_variant c.619T>C p.Phe207Leu	*pckA*	Phosphoenolpyruvate carboxykinase (ATP)	Unknown

Type	t507	w389	Effect	Gene	Product	Spore related
snp	G	A	missense_variant c.481G>A p.Glu161Lys	*pepF1_2*	Oligoendopeptidase F, plasmid	(Chao et al., 2006)
snp	C	T	missense_variant c.475C>T p.Pro159Ser	*pglF*	UDP-N-acetyl-alpha-D-glucosamine C6 dehydratase	Unknown
snp	G	T	missense_variant c.443C>A p.Ala148Glu	*sleB*	Spore cortex-lytic enzyme	(Zheng et al., 2016)
snp	A	C	missense_variant c.226T>G p.Ser76Ala	*speA_2*	Arginine decarboxylase	Unknown
snp	T	C	missense_variant c.470T>C p.Val157Ala	*SMU_747c*	Putative two-component membrane permease complex subunit SMU_747c	Unknown
snp	A	C	missense_variant c.468T>G p.His156Gln	*tenA_2*	Aminopyrimidine aminohydrolase	Unknown
snp	G	A	intergenic_region n.37121G>A	*tRNA-Asn*	Aspartate—tRNA(Asn) ligase	Unknown
snp	T	C	missense_variant c.146T>C p.Val49Ala	*yfmT*	Benzaldehyde dehydrogenase YfmT	Unknown
snp	G	A	missense_variant c.1240G>A p.Ala414Thr	*ywqJ*	Putative ribonuclease YwqJ	(Marchadier et al., 2011)
Hypothetical proteins with non-synonymous mutations found					7	
Percent of the genome not included in alignment					4 %	

Table C-8 Location and effect of mutations found between B. licheniformis isolates t501 towards w337.

Type	t501	w337	Effect	Gene	Product	Spore Related
snp	T	A	missense_variant c.76A>T p.Met26Leu	*aadK*	Aminoglycoside 6-adenylyltransferase	Unkonwn
snp	T	A	missense_variant c.231A>T p.Leu77Phe	*abgB*	p-aminobenzoyl-glutamate	Unkonwn

Type	t501	w337	Effect	Gene	Product	Spore Related
					hydrolase subunit B	
snp	A	G	missense_variant c.364A>G p.Lys122Glu	*aspA_2*	Aspartate ammonia-lyase	Unkonwn
snp	A	T	missense_variant c.612A>T p.Glu204Asp	*bioF*	8-amino-7-oxononanoate synthase 2	Unkonwn
snp	G	A	missense_variant c.775G>A p.Gly259Ser	*bpr_1*	Bacillopeptidase F	Unkonwn
snp	A	G	missense_variant c.316A>G p.Lys106Glu	*cheY_2*	Chemotaxis protein CheY	(Nagler et al., 2016)
snp	A	G	missense_variant c.1225A>G p.Ser409Gly	*codB*	Cytosine permease	Unkonwn
del	TA	T	frameshift_variant c.1453delA p.Arg485fs	*eglS_2*	Endoglucanase	Unkonwn
snp	C	T	missense_variant c.508G>A p.Ala170Thr	*gloB_5*	Hydroxyacylglutathione hydrolase	Unkonwn
snp	T	G	missense_variant c.285T>G p.Ser95Arg	*glvR_1*	HTH-type transcriptional regulator GlvR	Unkonwn
snp	A	G	missense_variant c.76T>C p.Tyr26His	*gmuB_1*	PTS system oligo-beta-mannoside-specific EIIB component	Unkonwn
snp	G	A	missense_variant c.710C>T p.Ala237Val	*hemL1*	Glutamate-1-semialdehyde 2,1-aminomutase 1	Unkonwn
complex	CAG	TAT	missense_variant c.1545_1547delCAGinsTAT p.Arg516Ile	*hemZ*	Oxygen-independent coproporphyrinogen-III oxidase-like protein HemZ	Unkonwn
snp	T	C	missense_variant c.341T>C p.Met114Thr	*hisF*	Imidazole glycerol phosphate synthase subunit HisF	Unkonwn
snp	G	T	missense_variant c.582C>A	*lexA_4*	LexA repressor	Unkonwn

Type	t501	w337	Effect	Gene	Product	Spore Related
			p.Asp194Glu			
snp	C	T	missense_variant c.528G>A p.Met176Ile	*lexA_4*	LexA repressor	Unkonwn
snp	G	T	missense_variant c.458C>A p.Ala153Glu	*lexA_4*	LexA repressor	Unkonwn
complex	CAAT	TAGA	missense_variant c.360_363delATTGinsTCTA p.Lys120Asn	*lexA_4*	LexA repressor	Unkonwn
complex	GGGGAGGTGGTCT	TGGTAGTTGATCA	missense_variant c.339_351delAGACCACCTCCCCinsTGATCAACTACCA p.His115Gln	*lexA_4*	LexA repressor	Unkonwn
complex	TAT	CAC	missense_variant c.325_327delATAinsGTG p.Ile109Val	*lexA_4*	LexA repressor	Unkonwn
complex	AAT	CAC	missense_variant c.253_255delATTinsGTG p.Ile85Val	*lexA_4*	LexA repressor	Unkonwn
snp	A	T	missense_variant c.238T>A p.Leu80Ile	*lexA_4*	LexA repressor	Unkonwn
complex	TGC	GGA	missense_variant c.217_219delGCAinsTCC p.Ala73Ser	*lexA_4*	LexA repressor	Unkonwn
snp	G	A	missense_variant c.11C>T p.Thr4Ile	*lexA_4*	LexA repressor	Unkonwn
snp	A	G	missense_variant c.1241T>C p.Val414Ala	*lysA*	Diaminopimelate decarboxylase	Unkonwn
snp	T	C	missense_variant c.1352T>C p.Leu451Ser	*mcpB_2*	Methyl-accepting chemotaxis protein McpB	Unkonwn
snp	A	C	missense_variant c.368A>C p.Asn123Thr	*mcpC*	Methyl-accepting chemotaxis protein McpC	Unkonwn
snp	A	T	missense_variant	*mdtD_1*	Putative multidrug resistance	Unkonwn

Type	t501	w337	Effect	Gene	Product	Spore Related
			c.382A>T p.Ile128Phe		protein MdtD	
snp	A	T	missense_variant c.209T>A p.Leu70Gln	*mrpE*	Na(+)/H(+) antiporter subunit E	Unkonwn
snp	T	C	missense_variant c.1022A>G p.Glu341Gly	*oxdD*	Oxalate decarboxylase OxdD	(Costa et al., 2004)
snp	G	C	missense_variant c.604C>G p.Leu202Val	*pstS1*	Phosphate-binding protein PstS 1	Unkonwn
snp	C	G	missense_variant c.625G>C p.Ala209Pro	*qorB*	Quinone oxidoreductase 2	Unkonwn
snp	A	G	missense_variant c.473A>G p.Glu158Gly	*rapG_1*	Response regulator aspartate phosphatase G	Unkonwn
snp	A	G	missense_variant c.769A>G p.Asn257Asp	*rapI_2*	Response regulator aspartate phosphatase I	Unkonwn
snp	G	A	missense_variant c.577G>A p.Asp193Asn	*sacA*	Sucrose-6-phosphate hydrolase	Unkonwn
snp	A	G	missense_variant c.1505T>C p.Ile502Thr	*sdcS_2*	Sodium-dependent dicarboxylate transporter SdcS	Unkonwn
snp	T	C	missense_variant c.119T>C p.Leu40Pro	*sdhB*	L-serine dehydratase, beta chain	Unkonwn
snp	C	A	missense_variant c.1288C>A p.Leu430Ile	*tuaE*	Teichuronic acid biosynthesis protein TuaE	Unkonwn
snp	A	C	missense_variant c.351A>C p.Leu117Phe	*xerC_3*	Tyrosine recombinase XerC	Unkonwn
complex	GCC	ACT	missense_variant c.867_869delGGCinsAGT p.Ala290Val	*xerC_8*	Tyrosine recombinase XerC	Unkonwn
snp	T	G	missense_variant c.856A>C p.Lys286Gln	*xerC_8*	Tyrosine recombinase XerC	Unkonwn

Type	t501	w337	Effect	Gene	Product	Spore Related
snp	C	T	missense_variant c.835G>A p.Asp279Asn	*xerC_8*	Tyrosine recombinase XerC	Unkonwn
snp	G	A	missense_variant c.767C>T p.Ala256Val	*xerC_8*	Tyrosine recombinase XerC	Unkonwn
snp	C	T	missense_variant c.505G>A p.Ala169Thr	*xerC_8*	Tyrosine recombinase XerC	Unkonwn
complex	CGC	AGA	missense_variant c.235_237delGCGinsTCT p.Ala79Ser	*xerC_8*	Tyrosine recombinase XerC	Unkonwn
snp	T	C	missense_variant c.160A>G p.Lys54Glu	*xerC_8*	Tyrosine recombinase XerC	Unkonwn
snp	G	T	missense_variant c.374G>T p.Gly125Val	*yflN*	putative metallo-hydrolase YflN	(Yamamoto et al., 2000)
snp	C	T	missense_variant c.404G>A p.Gly135Asp	*yheD_1*	Endospore coat-associated protein YheD	(van Ooij et al., 2004).
snp	G	T	missense_variant c.47C>A p.Thr16Lys	*ytlR*	Putative lipid kinase YtlR	Unkonwn
Hypothetical proteins with non-synonymous mutations found					49	
Percent of the genome not included in alignment					2 %	

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