Survey of naturally fermented raw milk in Bhutan for the prevalence of *Bacillus cereus*, *Escherichia coli* and *Staphylococcus aureus*

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

Master

in

Food Technology

at Massey University, Manawatū

New Zealand

Tashi Zangmo

2019

ABSTRACT

In Bhutan, naturally fermented raw milk (NFRM) is an ethnic dairy product consumed as a nutritional and refreshing beverage. It is produced by spontaneously fermenting raw milk at ambient temperatures. Across the globe, raw milk and raw milk products are considered as high-risk foods that may contain human pathogens. However, no survey has been conducted to study the presence of pathogens in NFRM in Bhutan. Therefore, in the present survey, 114 NFRM samples from 19 milk outlets and stalls in Thimphu, Phuntsholing and along the Thimphu-Phuntsholing NH (NH) in Bhutan were screened for the presence of *Escherichia coli, Bacillus cereus* and *Staphylococcus aureus* using culture-independent (high throughput sequencing (HTS)) and culture-dependent methods (most probable number (MPN) and selective plating). The culture isolates were confirmed by the PCR using species-specific primers. HTS identified the *Escherichia* and *Staphylococcus* genera. The culturing methods found more than 90 % and 100 % of NFRM samples contaminated with *E. coli* and *S. aureus* respectively. 45 % of NFRM samples contained more than 3.04 $\log_{10}$ cfu/ml *E. coli* based on MPN estimates. 61 % of NFRM samples contained more than 4 $\log_{10}$ cfu/ml *S. aureus* and is an immediate food safety concern. *B. cereus* was suspected since HTS detected *Bacillus anthracis* which is closely related to *B. cereus. Shigella* and *Vibrio* genera, and *Streptococcus agalactiae* and *Streptococcus parauberis* were also identified by HTS. Ideally, fermented milk products including NFRM should be prepared from pasteurised milk and fermented using commercial starter cultures since pasteurisation is the most effective method to inactivate the vegetative pathogens and ensure the safety of the product. Hygienic practices and control measures during NFRM production should also be adopted to minimise bacterial contamination in NFRM in Bhutan.

ACKNOWLEDGEMENTS

It is my honour to express my gratitude to many people for their kind support and help during my research.

First and foremost, I would like to thank my supervisor, Professor Steve Flint, School of Food and Advanced Technology, Massey University whose expertise, constant encouragement, guidance and support throughout my research enabled me to successfully complete my research.

I am indebted to the Ministry of Foreign Affairs and Trade (MFAT), New Zealand for providing me the scholarship opportunity to pursue my master degree at Massey University. I am also obliged to the Massey Institute of Food Science and Technology (MIFST) and the Massey University Foundation for providing me the grants for my project.

I am also grateful to Ann-Marie Jackson, Haoran Wang, Kylie Evans and Baizura Zain for their patience and support in the microbiology laboratory.

I would also like to thank my friends Soon, Gloria, Fitry, Norma, Mahmoud, Jiby, and Yiying for their friendship and support.

Lastly, I would like to thank my parents and family for their love and encouragement throughout my study.

TABLE OF CONTENTS

ABSTRACT i

ACKNOWLEDGEMENTS iii

TABLE OF CONTENTS v

LIST OF TABLES ix

LIST OF FIGURES xi

LIST OF ABBREVIATIONS xiii

CHAPTER 1. INTRODUCTION 1

1.1 Research questions 1

1.2 Hypotheses 1

1.3 Aim 2

1.4 Objectives 2

CHAPTER 2. LITERATURE REVIEW 3

2.1 RM, RMP and NFRM definitions 3

2.1.1 History of natural fermentation of milk 5

2.2 Microbiological risk associated with the consumption of RM and RMP across the globe 7

2.2.1 Prevalence of human pathogens in RM and RMP 8

2.2.2 Historical perspective 8

2.2.3 Current perspective 10

2.2.4 Reported human cases and outbreaks due to RM and RMP consumption 10

2.3 RM and NFRM in Bhutan 14

2.3.1 Background 14

2.3.2 Method of production 16

2.4 The select bacteria in this survey: *B. cereus*, *E. coli* and *S. aureus* 18

2.4.1 *B. cereus* 21

2.4.2 *E. coli* 22

2.4.3 *S. aureus* 25
2.5 Growth and survival of *B. cereus, E. coli* and *S. aureus* in RM and RMP 27
2.5.1 Intrinsic factors 27
2.5.2 Extrinsic factors 32
2.6 pH and acidity of NFRM in Bhutan 34
2.7 Detection of *B. cereus, E. coli* and *S. aureus* by PCR 35
CHAPTER 3. MATERIALS AND METHODS 37
3.1 Materials used 37
3.1.1 Bacterial profile analysis 37
3.1.2 *E. coli, B. cereus* and *S. aureus* enumeration 37
3.1.3 Chemical analysis 38
3.2 Methods 39
3.2.1 Survey areas 39
3.2.2 NFRM production 39
3.2.3 Sources selection 41
3.2.4 Sample collection 41
3.2.5 Bacterial profile analysis by HTS 42
3.2.6 *B. cereus, E. coli* and *S. aureus* enumeration 43
3.2.7 Laboratory trials 46
3.2.8 Chemical analysis 46
3.2.9 pH 46
3.2.10 Acidity 46
3.2.11 Statistical analysis 47
CHAPTER 4. RESULTS 48
4.1 Bacterial profile analysis using HTS 48
4.1.1 Overall bacterial abundance and diversity 48
4.1.2 Bacterial composition of NFRM 49

4.1.3 Differences in the bacterial composition of NFRM of Thimphu, Phuntsholing and NH 51
4.2 *B. cereus* 53
4.3 *E. coli* 53
4.3.1 Confirmation by PCR 53
4.3.2 Prevalence and MPN estimates 54
4.4 *S. aureus* 55
4.4.1 Confirmation by PCR 55
4.4.2 Prevalence and viable counts 55
4.5 pH and acidity 56
5.0 DISCUSSION 57
6.0 CONCLUSIONS AND RECOMMENDATIONS 66
6.1 Conclusions 66
6.2 Recommendations for future work 67
REFERENCES 68
APPENDICES 91
Appendix A. Microbiological criteria of FSANZ 91
Appendix A1. Interpreting results for testing of pathogenic microorganisms in RTE food 92
Appendix A2. Interpreting results for testing of indicator organisms in RTE foods 94
Appendix B. Taxonomy tree of NFRM in Bhutan 95
Appendix C. Venn diagram showing common and unique OTUS in NFRM in Thimphu, Phuntsholing and NH 96
Appendix D. UPGMA tree 96
Appendix E. Post-milking cooling curves 97
Appendix E1. Post- milking cooling curve to meet FSANZ guidelines for RM 97

Appendix E2. Post- milking curve to meet EC requirements for heat treated milk products.....97

LIST OF TABLES

Table 1. Examples of NFRM in developing countries of Africa and Asia 5

Table 2. Time and temperature combinations for milk pasteurisation 9

Table 3. Examples of reported human cases and outbreaks due to the consumption of RM and RMP in the USA, Europe and New Zealand 11

Table 4. Factors which lead to supressed immune functions 14

Table 5. Temperature variations in Bhutan 17

Table 6. Pathogens identified in NFRM in Africa 19

Table 7. Minimum pH values required for the growth of some pathogens including *B. cereus*, *E. coli* and *S. aureus* 28

Table 8. Effect of LAB culture broths on the growth of pathogens including *B. cereus*, *E. coli* and *S. aureus*[1] 30

Table 9. Applications of bacteriocins in dairy products 31

Table 10. Minimum water activity required by pathogens including *B. cereus*, *E. coli* and *S. aureus* 32

Table 11. Growth temperatures for mesophilic pathogens including *B. cereus* (mesophilic strains), *E. coli* and *S. aureus* 33

Table 12. Growth temperatures for psychrotropic pathogens including *B. cereus* (psychrotrophic strains), *E. coli* and *S. aureus*. 33

Table 13. Sample details 41

Table 14. Pool sample details 42

Table 15. Species specific primer details 45

Table 16. PCR amplification protocols 46

Table 17. Alpha diversity details 48

Table 18. Relative abundance of phyla and top 10 genera in NFRM 51

Table 19. AMOVA of differences among NFRM samples 52

Table 20. *E. coli* prevalence and counts based on MPN estimates 54

Table 21. *S. aureus* viable counts 56

LIST OF FIGURES

Figure 1. Production trend of milk and dairy products in Bhutan (2013-2017) 16

Figure 2. Process flow for ethnic dairy products production in Bhutan 17

Figure 3. Ten commonest milk-borne pathogens in RM in Bhutan..... 23

Figure 4. Maintenance recycling of the pathogens on dairy farms 24

Figure 5. *E. coli* in naturally soured milk..... 24

Figure 6. Prevalence of mastitis in cattle in Bhutan..... 26

Figure 7. Factors affecting microbial growth..... 27

Figure 8. Map of Bhutan showing the survey areas..... 39

Figure 9. PET bottles used for NFRM fermentation and marketing..... 40

Figure 10. (a) Milk outlets in Thimphu and Phuntsholing, (b) Milk stalls in Gedu 40

Figure 11. Single colonies of suspicious *E. coli* on BHI agar..... 44

Figure 12. Single colonies of suspicious *S. aureus* on BHI agar 45

Figure 13. Top 10 phyla identified by HTS 49

Figure 14. Top 10 genera identified by HTS 50

Figure 15. PCoA scores based on the relative abundances of OTUs in NFRM samples (Based on the weighted UniFrac distance)..... 52

Figure 16. No typical *B. cereus* colonies observed on MYP agar 53

Figure 17. (a) *E. coli* colonies on EMB agar and (b) *E. coli* bands on agarose gel. 53

Figure 18. % of NFRM samples with *E. coli* counts categorised according to the FSANZ limits..... 54

Figure 19. (a) *S.* aureus with clear zones (lipolytic and proteolytic strains), (b) *S. aureus* colonies without clear zones (non-lipolytic strains) and (c) *S. aureus* bands on agarose gel. 55

Figure 20. % of samples with *S. aureus* counts categorised according to the FSANZ limits 56

LIST OF ABBREVIATIONS

CAC	Codex Alimentarius Commission
CDC	Centers for Disease Control and Prevention
EFSA	European Food Safety Authority
FBD	Foodborne diseases
FDA	Food and Drug Administration, USA
FSANZ	Food Standards Australia New Zealand
HTS	High throughput sequencing
MPI	Ministry for Primary Industries, New Zealand
MPN	Most probable number
NFRM	Naturally fermented raw milk
NH	Thimphu-Phuntsholing national highway
NZFS	New Zealand Food Safety
PCR	Polymerase chain reaction
PET	Polyethylene terephthalate
RM	Raw milk
RMP	Raw milk product
SEs	Staphylococcal enterotoxins
USA	United States of America
WHO	World Health Organisation

CHAPTER 1. INTRODUCTION

In Bhutan, naturally fermented raw milk (NFRM) is a popular ethnic product processed from raw milk (RM). It is consumed as a nutritional and refreshing beverage. Across the globe, RM and raw milk products (RMP) are considered as high-risk foods that may contain pathogens (Angulo, LeJeune, & Rajala-Schultz, 2009; Oliver, Boor, Murphy, & Murinda, 2009) which cause a wide range of diseases in humans from minor conditions like vomiting and diarrhoea to more severe conditions like meningitis, miscarriages, paralysis and kidney problems (Centers for Disease Control and Prevention (CDC, 2018b)). In developing countries, hygiene practices are not adopted during the production of milk and traditional milk products (Wanjala, Nduko, & Mwende, 2018). There are also no microbial standards for NFRM. Therefore, NFRM has been reported to contain various pathogens and of poor hygiene quality in developing countries including Africa (Agyei, Owusu-Kwarteng, Akabanda, & Akomea-Frempong, 2019; Maikai & Madaki, 2018). There are also no standardised production methods (specified fermentation time and temperature) for NFRM. Hence, NFRM is processed by spontaneously fermenting RM for 2-3 days depending mainly on the ambient temperature and the viscosity desired by the manufacturers resulting in variation in the pH of NFRM (Akabanda, Owusu-Kwarteng, Glover, & Tano-Debrah, 2010; Okiki, Adeniji, Oyetunji, Yusuf, & Peters, 2018). pH is one of the main factors which determines the safety of fermented food (Adams & Nicolaides, 1997).

However, there has been no research on the prevalence of pathogens in NFRM and on the pH of NFRM in Bhutan. Therefore, this survey was performed with the following research questions, hypotheses, aim, and objectives.

1.1 Research questions

1. Are *B. cereus*, *E. coli* and *S. aureus* present in NFRM in Bhutan?
2. What are the viable counts of *B. cereus*, *E. coli* and *S. aureus* in NFRM in Bhutan?
3. What are the pH and acidity levels in NFRM in Bhutan?

1.2 Hypotheses

1. NFRM in Bhutan is contaminated with *B. cereus, E. coli* and *S. aureus*.
2. The pH of NFRM in Bhutan is not adequate to inhibit the growth of *B. cereus, E. coli* and *S. aureus*.

1.3 Aim

The aim is to survey NFRM in Bhutan for the prevalence of *B. cereus* and *S. aureus* in NFRM in Bhutan and to determine the pH of NFRM in Bhutan.

1.4 Objectives

The objectives are to:

1. Study the bacterial profile of NFRM by high-throughput amplicon sequencing
2. Screen NFRM for the presence of *E. coli* by the most probable number method and confirm using PCR
3. Screen NFRM for the presence of *B. cereus* and *S. aureus* by selective plating methods and confirm using PCR
4. Determine the *E. coli* counts in NFRM based on MPN estimates
5. Determine the viable counts of *B. cereus* and *S. aureus* in NFRM
6. Determine the pH and acidity of NFRM.

CHAPTER 2. LITERATURE REVIEW

This chapter defines RM, RMP and NFRM. It describes the history of the natural fermentation of milk. It presents the global scenario of the microbiological risk associated with the consumption of RM and RMP. It also presents the most common pathogens in RM and RMP and examples of reported foodborne diseases (FBD) and outbreaks caused by them. The unavailability of the reported cases in developing countries is discussed. This chapter also presents the background on the consumption of RM and RMP including NFRM in Bhutan and their production methods. The survey of the prevalence of *B. cereus, E. coli* and *S. aureus* in NFRM in Bhutan is highlighted along with the diseases caused by them, sources of contamination and the factors affecting their survival in fermented food. The advantages of using PCR over the conventional methods are presented.

2.1 RM, RMP and NFRM definitions

RM is defined as the milk from animals such as cows, goats, sheep, and others that "has not been heated beyond 40 ºC" nor been subjected to other heat treatment or any other treatment which would produce an equivalent effect (CAC, 2004; Oliver & Murinda, 2011). RMP are processed from RM and NFRM is a ready-to-drink RMP processed through natural fermentation of RM using traditional techniques (Cogan et al., 1997). RMP including NFRM are not subjected to any heat treatment before consumption.

Although NFRM is consumed in many countries across the world (Narvhus & Gadaga, 2003), it is more popular in the developing countries of South-East Asia (R. Rai, Shangpliang, & Tamang, 2016; Tamang, 2010) and Africa (Narvhus & Gadaga, 2003). In developing countries, it is mostly prepared at household level from cow's milk in the rural communities where the animals are reared mainly for milk (Narvhus & Gadaga, 2003). Therefore, it is regarded as a "small-scale product" (Jatmiko, Howarth, & Barton, 2018). Like any other fermented milk, NFRM has been consumed for a long time and it constitutes an essential part of the traditional human diet (Panesar, 2011). It is consumed as a refreshing beverage (Tamang, 2010) and nutritional drink or as a relish on staple food (Mutukumira, Narvhus, & Abrahamsen, 1995).

"Nono" is an example of NFRM which has traditionally been produced and consumed by the Fulani and Hausa in Nigeria. It is also known as "nunu" in some parts of Africa. Similar products like "amasi" and 'mafi" are consumed in other parts of Africa (Agyei et al., 2019; Beukes, Bester, & Mostert, 2001; Eka & Ohaba, 1977; Maikai & Madaki, 2018; Osvik et al., 2013; Simatende, Gadaga, Nkambule, & Siwela, 2015). Likewise, "dahi" is ethnic to many Asian countries like Bangladesh, Bhutan, Nepal, Pakistan, and India which is the local name for the curd processed by the natural fermentation of milk (Dewan & Tamang, 2007). In these countries, although dahi is mostly prepared from boiled milk, there are some regions like the Eastern Himalayan regions of Bhutan, India and Nepal where it is prepared from RM (R. Rai et al., 2016). Examples of NFRM in Africa and Asia are listed in Table 1.

NFRM is processed by spontaneously fermenting RM at ambient temperature by the lactic acid bacteria (LAB) naturally present in milk (Wouters, Ayad, Hugenholtz, & Smit, 2002) until the desired level of fermentation (yogurt-like consistency and mild sour flavour) is achieved. The fermentation time largely depends on the temperature and can vary between 1 to 2 days at 28 ± 2 ºC or 2 to 4 days at lower temperatures (Dewan & Tamang, 2007; Okiki et al., 2018). Geographic location, elevation, climate, sunlight and the manufacturers' desired viscosity also determine the fermentation time (Mo et al., 2019; Sun et al., 2014; Sun et al., 2010; Watanabe et al., 2008). Therefore, the pH of NFRM varies (Akabanda et al., 2010; Beukes et al., 2001; Okiki et al., 2018; Okonkwo, 2011). Although Dewan and Tamang (2007) suggested that the traditional production method produced a consistent product with a consistent pH, their result was based on the pH results of only ten NFRM samples.

The microbiological criteria for "Ready-to-eat" (RTE) food of Food Standards Australia and New Zealand (FSANZ, 2018a) serve as a guide for microbiological standards. The criteria categorise RTE food as "satisfactory" when microbiological results are within the expected levels, "marginal" when the results are within the expected levels but on the higher range, "unsatisfactory" when the results are beyond the expected levels and "potentially hazardous" when the results are beyond the expected levels and poses an immediate food safety concern (Appendix A).

Product name	Country	References
Amasi	Zimbhawe and South Africa	Agyei et al. (2019), Beukes et al. (2001), Osvik et al. (2013), Simatende et al. (2015)
Dahi	Bhutan, India and Nepal	R. Rai et al. (2016)
Ergo	Ethiopia	Agyei et al. (2019)
Nono/ Nunu	Ghana and Nigeria	Agyei et al. (2019), Akabanda et al. (2010), Maikai and Madaki (2018)
Mafi	South Africa	Agyei et al. (2019), Beukes et al. (2001), Simatende et al. (2015)
Rayeb	Arab	Samet-Bali, Felfoul, Lajnaf, Attia, and Ayadi (2016)

Table 1. Examples of NFRM in developing countries of Africa and Asia

2.1.1 History of natural fermentation of milk

Food fermentation is one of the oldest known uses of biotechnology (Campbell-Platt, 1994) and the oldest food preservation method after drying (Prajapati & Nair, 2003). The history of food fermentation dates back before 10,000 – 15,000 years ago when humans began to produce food instead of gathering and started to domesticate lactating animals (Geigl, 2008; Jakobsen, Heggebø, Sunde, & Skjervheim, 2011; Pederson, 1971; Zeder & Hesse, 2000). Since then, humans started keeping the milk in containers for storage and consumption. They later discovered fermented milk when the milk had turned sour. By then, humans had already learnt to appreciate foods with pleasant flavours, aroma and textures (Steinkraus, 2004). It was by mere accident that they experienced the flavour and taste of fermented milk and found it pleasant (Prajapati & Nair, 2003). Thus, NFRM was one of the first fermented foods discovered by humans since they became agriculturists. Humans depended on fermented foods including fermented milks for survival (Steinkraus, 2004) and food fermentation started getting popular as it preserved food preventing spoilage and putrefaction, and provided different forms, tastes and sensory sensations (Prajapati & Nair, 2003; Steinkraus, 2018).

During earlier times, the fundamentals of the milk turning sour and containing the pleasant aromas were probably not understood. The fermentation of RM started naturally. It was fermented by a wide range of micro-organisms including bacteria, yeast and moulds which could grow rapidly on the nutrients available in the milk and suited the environmental conditions (Campbell-Platt, 1994; Tamang & Kailasapathy, 2010). These micro-organisms may be present in or on the raw material, containers, utensils and the environment (Steinkraus, 1997). They easily grow in milk since it contains all essential

nutrients like fat, proteins, carbohydrates, and minerals that are excellent substrates for their growth (Steinkraus, 2018).

Over time, through trial and error, the technical parameters during fermentation were controlled through a back-slopping technique in which the residue of a previous batch of fermented milk is used as an inoculum for the following batch. With this technique, the micro-organisms which initiated and accelerated the fermentation process to produce desirable changes in the taste and flavour were preserved (Holzapfel, 1997). Eventually, only certain bacteria, yeast and moulds that could adapt to the substrates were selected over others (Steinkraus, 1997).

Since then, much research has been conducted and literature published on the role and the type of micro-organisms responsible for fermentation. Now it is widely accepted that the wild starter cultures naturally present in milk or the indigenous microflora of milk are mostly responsible for fermenting milk (Jatmiko et al., 2018). These microflora have been identified as LAB which are a dominant Gram-positive mesophilic population of bacteria "naturally present in milk as adventitious contaminants" (Wouters et al., 2002). LAB have a prominent role during milk fermentation. They grow rapidly above 20 ºC and mainly produce lactic acid from lactose thus lowering the milk pH to 4.0 to 4.6 in fermented milk products like cheese, kefir and yogurt. This brings in the desired changes of the milk structure and also imparts the mildly sour taste to the fermented products (König & Fröhlich, 2017; Niamsiri & Batt, 2009). In NFRM too, the desired consistency is achieved by the pH drop (Panesar, 2011; Puniya, Kumar, Puniya, & Malik, 2015; Steinkraus, 2018). LAB also generate desirable flavours and aromas (Axelsson, 2004).

In milk, the most common LAB are the *Lactococcus, Lactobacillus, Leuconostoc, Enterococcus* and *Streptococcus* genera (Quigley, O'sullivan, et al., 2013). In dahi and nunu, the predominant LAB identified were *Lactobacillus helveticus* and *Lactococcus lactis* (Akabanda et al., 2010; Shangpliang, Rai, Keisam, Jeyaram, & Tamang, 2018). Additionally, in dahi, acetic acid bacteria including *Acetobacter lovaniensis* and *Acetobacter pasteurianus* (Shangpliang et al., 2018) and in nunu, yeasts including *Saccharomyces cerevisiae* and *Candida kefyr* (Akabanda et al., 2010) have also been identified.

These days, fermentation is achieved through controlled growth of microbes (Marco et al., 2017) under set conditions with the addition of specified LAB as starter cultures

(Robinson & Tamime, 2006). In the dairy industry, the most common commercial LAB used are the mesophilic and thermophilic strains of *Lactococcus, Lactobacillus* and *Streptococcus* species (Donkor, Henriksson, Vasiljevic, & Shah, 2007).

Although in the earlier times fermentation was primarily used to preserve the food for storage and to improve the shelf life (Adams & Mitchell, 2002; Campbell-Platt, 1987), fermentation has other advantages too. It improves the taste and digestibility of milk, enables the processing of milk into several other dairy products like cheese and yoghurt (Robinson & Tamime, 2006) and most importantly, it also enhances the safety of the products. Through fermentation, risky raw materials like RM are transformed into products with a lesser risk of causing illnesses by lowering the pH and inhibiting the growth of human pathogens (Adams & Mitchell, 2002) and most fermented foods are regarded as safe to eat (Campbell-Platt, 1994). Therefore, fermentation renders the food less risky than non-fermented RMP. However, fermentation must be carried out properly to achieve the required safeness since the survival of bacterial pathogens depends on many factors as discussed in section 2.5.

2.2 Microbiological risk associated with the consumption of RM and RMP across the globe

It is well known that RM is a good source of all nutrients required for growth and survival of micro-organisms (Steinkraus, 2018) and can contain a diverse and complex population of micro-organisms including milk-borne bacterial pathogens (Quigley et al., 2011). Consequently, RM and RMP products including NFRM are considered as "high-risk foods" of animal origin (Nyachuba, 2010; West, 2008). CDC (2017) considered RM as the riskiest of all the foods that can pose a similar risk to humans. The prevalence of pathogens in RM is important since RMP including NFRM are prepared exclusively from RM and are not heat-treated for the inactivation of these pathogens before consumption. Therefore, it is highly likely that the pathogens present in RM will naturally be present in such products (Verraes et al., 2015).

The bacterial pathogens are the main hazards which pose one of the greatest threat to food safety presenting various disease risks and causing severe health consequences in humans (Alegbeleye, Guimarães, Cruz, & Sant'Ana, 2018; Verraes et al., 2015). The bacterial pathogens have also often been reported to cause significant numbers of FBD as compared to other microbiological hazards like viruses (Nyachuba, 2010). The World

Health Organization (WHO, 2015) reported that in 2010, bacterial pathogens were the most frequent cause of diarrhoeal disease which then led to the most frequent cause of death due to food poisoning. Similarly, in the USA, they caused 73 outbreaks of FBD due to the consumption of RM and RMP (Langer et al., 2012) and also caused more than half (55 %) of the total number of FBD outbreaks (CDC, 2018c).

2.2.1 Prevalence of human pathogens in RM and RMP

Across the globe, numerous studies, surveys and reviews have been published internationally on the prevalence of bacterial pathogens in RM that are capable of causing various diseases in humans. Based on such review documents by Claeys et al. (2013), EFSA (2015b) and Oliver and Murinda (2011), the following bacteria have been identified as the most prevalent bacterial pathogens in RM and RMP:

- *B. cereus*
- *Brucella abortus*
- *Campylocater jejuni*
- *Clostridium botulinum*
- *Corynebacterium* spp.
- *E. coli* O157:H7
- Non-O157:H7 Shiga toxin-producing *E. coli*
- *Listeria monocytogenes*
- *Salmonella* spp.
- *S. aureus*

2.2.2 Historical perspective

In the earliest times of human civilisation, the main concern was milk adulteration which primarily compromised the nutritional and microbiological content of milk. Addition of water is the oldest form of milk adulteration and materials like chalk were usually added to milk to conceal such adulteration. Incidence of melamine addition to infant formula in China which resulted in illness in 2008 is one variation of this practice. Therefore, various strategies were implemented to prevent this practice. Some cultures made it illegal and some focused on the religious precepts of handling milk. Some cultures also had the traditional practice of boiling milk for consumption (Motarjemi, Moy, Jooste, & Anelich, 2014). These measures were successful and although during that time humans were probably not concerned with the risk of human pathogens and milk-borne diseases associated with consumption of RM and RMP, such measures also indirectly might have reduced those risks.

However, in the early 1800s, dairy production was industrialised and there was an increase in the production, distribution and use of milk and milk products. Also, the consumption of RM was popular till the mid-20th century. The disease risk increased and consequently, numerous milk-borne diseases and outbreaks occurred (Leedom, 2006; Motarjemi et al., 2014).

It was only in the 1860s that the foundation was laid for the microbial safety of milk and milk products when the germ theory was established by Louis Pasteur and its practical application in the food industry was demonstrated. Thus, in the 1890s, milk pasteurisation was officially introduced in many states in the USA and some countries in Europe (Motarjemi et al., 2014). Pasteurisation is the "process of heating every particle of milk or milk product, in properly designed and operated equipment to one of the temperatures outlined in Table 2 and held continuously at or above that temperature for at least the corresponding specified time" (FDA, 2017). It reduces the microbial load of milk and specifically limits the number of spoilage microorganisms as well as the pathogens thus improving the safety (Oliver & Murinda, 2011; Quigley, O'sullivan, et al., 2013).

When pasteurisation was introduced, the main aim was to destroy *Mycobacterium tuberculosis* var. *bovis* that caused bovine and human tuberculosis. Later, in the late 1950s, pasteurisation was mainly aimed at destroying *Coxiella burnetii* that caused Q fever. Simultaneously, the risk of other milk-borne diseases was also mitigated (Motarjemi et al., 2014).

Table 2. Time and temperature combinations for milk pasteurisation

Batch (vat) pasteurisation	
Temperature	Time
63ºC (145ºF)	30 minutes
Continuous flow (HTST and HHST) pasteurisation	
Temperature	Time
72ºC (161ºF)	15 seconds
89ºC (191ºF)	1.0 seconds
90ºC (194ºF)	0.5 seconds
94ºC (201ºF)	0.1 seconds
96ºC (204ºF)	0.05 seconds
100ºC (145ºF)	0.01 seconds

HTST: High Temperature Short Time, HHST: Higher Heat Shorter Time

From 'Grade "A" pasteurised milk ordinance' by FDA (2017) (https://www.fda.gov/media/114169/download). In the public domain.

2.2.3 Current perspective

Nevertheless, the food safety risk still prevails today especially in developing countries and also in developed countries like the USA and European Union where people consume RM and RMP (EFSA, 2015b; Motarjemi et al., 2014; Oliver et al., 2009). Although the sale of RM and RMP is prohibited in these countries, they are readily available through various distribution channels. In the USA, RM may be obtained as "animal or pet food" (Oliver et al., 2009) and in Europe, while it is prohibited in countries like Spain, it can be purchased directly from the farms in Germany and the Netherlands (EFSA, 2015b). In Asia and Africa, NFRM is an ethnic product commonly consumed by many people (Mutukumira et al., 1995; Tamang, 2010).

A detailed report based on scientific literature claiming that RM is unsafe is provided by the USA Food and Drug Administration (FDA, 2011). However, there are many reasons why people consume RM and RMP. The RM consumers believe that it is superior to pasteurised milk as it is more nutritious (Amagliani et al., 2012) and has health benefits like curing lactose intolerance, treating allergy and asthma, preventing osteoporosis and contains beneficial bacteria for gastrointestinal health (FDA, 2011; French, Benschop, & Marshall, 2013; Oliver et al., 2009). Similarly, since RM has a more diverse bacterial population that aids in the development of flavours, RMP like cheese, are perceived to have an "enhanced and more intense flavour" (Beuvier & Buchin, 2004). Moreover, an increasing trend for the consumption of RM and RMP is observed these days. RM is promoted as a "health food" (Zastempowska, Grajewski, & Twarużek, 2016) and consumers' demands are rising for unprocessed or minimally processed foods (Verraes et al., 2015), healthier and more natural animal products (Egger-Danner et al., 2015). The concept of "produce, sell and buy local" is a growing trend among the consumers (Oliver et al., 2009). On the other hand, some of the consumers are probably not aware of the ill health consequences of such products and thus continue to consume them (Motarjemi et al., 2014).

2.2.4 Reported human cases and outbreaks due to RM and RMP consumption

There are several reports compiled by many researchers for example by Motarjemi et al. (2014) and Verraes et al. (2015) on disease incidences and outbreaks due to the consumption of RM and RMP (mostly cheese) in the developed countries including the

USA, New Zealand and Europe which have caused numerous infections, few hospitalisations and deaths. Some examples are provided in Table 3. An outbreak is the "occurrence of > 2 cases of a similar illness resulting from ingestion of a common food" (Mungai, Behravesh, & Gould, 2015).

In the USA where the consumption of RM is quite common, the majority of the FBD has been attributed to RM for a very long time. Oliver et al. (2009) summarised the milk-borne diseases for 2000 - 2008. Since then, there have been many other outbreaks. During 2009 to 2014, while 760 illnesses and 22 hospitalisations were reported due to the consumption of dairy products, 96 % were attributed to the consumption of RM and RMP (Costard, Espejo, Groenendaal, & Zagmutt, 2017). Moreover, such outbreaks increased from 30 during 2010 - 2012 to 51 during 2007 - 2009 (Mungai et al., 2015). In 2016, dairy was one of the most common single food categories implicated in FBD. Of all the outbreaks, 11 % were due to RM and RMPs resulting in 19 outbreaks and 252 illnesses (CDC, 2018c). Likewise, during 2009 to 2017, RM caused 32 outbreaks, 232 illnesses, 31 hospitalisations and 1 death, and RM cheeses caused 6 outbreaks, 142 illnesses, 30 hospitalisations and 3 deaths (CDC, 2018b). In the USA it is assumed that the milk borne outbreak-related illnesses will increase by 96 % when the consumption of RM and RMP doubles. RM and RMP are reported to cause 840 times more illnesses and 45 times more hospitalisations compared to the pasteurised milk products (Costard et al., 2017). In New Zealand, from 2009 to 2016, RM alone caused 46 outbreaks (NZFS, 2018) while in Europe from 2007 to 2013, it caused 27 outbreaks (EFSA, 2015a). There were no details on the illnesses, hospitalisations and deaths caused.

Table 3. Examples of reported human cases and outbreaks due to the consumption of RM and RMP in the USA, Europe and New Zealand

Pathogens	Product	Country	Year	Cases	Reference
C. jejuni, STEC, *S. enterica*, *L. monocytogenes*,	RM	US	2009-2017	32 outbreaks, 232 illnesses, 31 hospitalisations, 1 death	CDC (2018b)
C. jejuni, STEC, *S. enterica*, *L. monocytogenes*	RM cheeses	US	2009-2017	6 outbreaks, 142 illnesses, 30 hospitalisations, 3 deaths	CDC (2018b)
Campylobacter, *Salmonella*, STEC	RM	Europe	2007-2013	27 outbreaks	EFSA (2015a)
S. aureus	RM cheese	Switzerland	2014	1 outbreak, 14 illnesses	Johler et al. (2015)

Campylobacter, STEC, *Cryptosporidium*	RM	New Zealand	2009-2016	46 outbreaks,	NZFS (2018)

2.2.4.1 Unavailability of human cases and outbreak reports in developing countries

Although the pathogens and disease risk may be higher in developing countries due to unhygienic practices and lack of refrigeration, human cases and outbreak reports due to the consumption of RM and RMP are rare in developing countries (Motarjemi et al., 2014). One explanation is that in developing countries, there are no surveillance and outbreak investigations. In contrast, in developed countries, like the USA, the reporting and monitoring of the FBD and outbreaks have been in place for 80 years (CDC, 2006). These are administered by the CDC which track and monitor FBD reports through several surveillance systems including the Foodborne Disease Active Surveillance Network (FoodNet), Foodborne Disease Outbreak Surveillance System (FDOSS) and National Outbreak Reporting System (NORS) (CDC, 2018a). Even so, there are several factors to be considered for the recognition of FBD and outbreaks in any country. For example, similar to the consumers in developing countries, the consumers in the developed countries are unlikely to report diseases that do not cause symptoms, serious illness and hospitalisations and not leading to death. Hence, the USA reports might not represent the true numbers of such incidences and the milk-borne related illness might be under-reported even in the USA (CDC, 2006).

Similarly, in New Zealand, the Ministry for Primary Industries (MPI) monitors and controls the incidences of FBD through its human health surveillance programme (MPI, 2019). In Europe, the European Food Safety Authority (EFSA) is responsible for monitoring FBD (EFSA, 2019).

Apart from the lack of surveillance and outbreak investigations in developing countries, disease outbreak reports may be absent for other reasons. Firstly, such incidences may not have occurred at all. Secondly, consumers may not be aware of such diseases and therefore not interested in reporting. Thirdly, even if the consumers were aware, since most of the diseases caused by RM and RMP including NFRM are vomiting and diarrhoea which do not lead to serious illnesses, hospitalisations and deaths, they may not be interested in reporting (CDC, 2006); CDC (2018b).

2.2.4.2 Vulnerable population

In humans, the risk of the pathogens in RM and RMP causing disease and serious consequences differ from one individual to another because of different immune conditions (Acheson, 2013). Vulnerable people are more likely to be affected than the healthy ones (Lund, 2015) as their immune function is suppressed due to many factors including immunodeficiency as a result of genetic defects, age, disease, pharmacologic therapy and reproductive status (Table 4). In developed countries, 15 to 20 % of the population comprises the vulnerable group (Arqués, Rodríguez, Langa, Landete, & Medina, 2015). Children and the elderly are most susceptible to disease (Acheson, 2013). The WHO (2015) reported that all FBD can be deadly in children who are less than 5 years old and that they account for 1/3 of deaths resulting from FBD. Immune compromised people and those already suffering from diseases like leukemia and HIV/AIDS are more susceptible too (Acheson, 2013).

For example, for listeriosis, Goulet et al. (2011) reported that people with chronic lymphocytic leukemia were 1000 fold at greater risk of getting listeriosis. Listeriosis affects pregnant women more than non-pregnant women. People of 45 - 59 years old are also at higher risk (Pouillot, Hoelzer, Jackson, Henao, & Silk, 2012). Malnourished people are more prone to infection due to their poor diet (Kalyoussef & Feja, 2014). Ethnicity is another risk factor. For example, Pouillot et al. (2012) reported that the rate of listeriosis was higher for Hispanics compared with non-Hispanics.

Apart from the factors relating to the host, disease susceptibility is also affected by factors relating to the consumption of contaminated food including time, amount, frequency and the average serving size. For example, people who consume the contaminated food on an empty stomach, like at breakfast, are at higher risk of listeriosis as this facilitates faster transit of food through the stomach (Adams & Mitchell, 2002).

People who consume food that contains the “minimum infective dose” of disease-causing pathogens are at higher risk too. This concept of “minimum infective dose” indicates that there is a minimum threshold required by a microorganism to cause disease. However, this may be most applicable to pathogens which require a certain population to produce toxins to cause any disease as discussed for *B. cereus* and *S. aureus* in section 2.4. Although the chances are lower, even a single cell of an infectious pathogen could also

be capable of initiating a disease and should not be neglected completely (Adams & Mitchell, 2002).

Table 4. Factors which lead to supressed immune functions

Primary immunodeficiency, caused by a genetic defect in some component of the immune system
Secondary immune deficiencies:
Immunosuppressive drugs in organ transplantation
Leukaemia
HIV/AIDS
Chemotherapy for cancer
Radiotherapy for cancer
Treatment with corticosteroids
Treatment with inhibitors of tumour necrosis factor e.g. for rheumatoid arthritis, Crohn's disease
Diabetes, primary and secondary
Pregnancy
Age < 5 years
Age > 65 years
Other factors:
Malnutrition, involving protein, calories, vitamins or trace metals
Use of acid-suppressing medication, particularly proton pump inhibitors

From 'Microbiological food safety for vulnerable people' by Lund (2015), *International Journal of Environmental Research and Public Health*, 12, p. 10120. In the public domain.

2.3 RM and NFRM in Bhutan

2.3.1 Background

Livestock plays an essential role in peoples' lives in Bhutan (Ministry of Agriculture and Forests (Bhutan), 2018) since it is one of the main sources of income for rural households. Dairy farming is a major rural activity and farmers generate 50 – 90 % of their income from dairying. Bhutanese households practise small scale dairy farming. They mostly rear cattle for milk, manure production and as assets (National Stastics Bureau of Bhutan, 2018; Phanchung, Dorji, Sonam, & Pelden, 2002).

Bhutanese consume milk and dairy products as staple items in their diets. In the early days when there was no easy access to roads and transportations, they produced milk mostly for home consumption only. At present, with easy access to roads that offer better marketing opportunities, milk is produced for vending purposes and fetches a good income (Phanchung et al., 2002). Thus, milk production in the country has shown an increasing trend over time (Renewable Natural Resources (Bhutan), 2018).

It is not common for the majority of the Bhutanese to consume fresh milk. Therefore, most of the milk is processed into various dairy products which are ethnic to Bhutan (Phanchung et al., 2002; R. Rai et al., 2016; Tamang, 2010). Most of the ethnic dairy products are produced from RM. These ethnic RMP are dominated by soft/cottage cheese, locally known as "datshi" and butter ("mar") (Renewable Natural Resources (Bhutan), 2018; Wangdi, Dema, Karma, & Bhujel, 2014). Other ethnic RMP are NFRM ("dahi") and hard cheese "chugo". NFRM is consumed in most parts of Bhutan. It is a popular product and is readily available in the markets. However, its production has not been reported in the national statistics. At times, the terminology used for NFRM in Bhutan can be confusing since people also call it "curd". Dahi/curd is similar to the western yoghurt (Sarkar, 2008).

The production of milk and milk products has steadily increased over the last 5 years (Renewable Natural Resources (Bhutan), 2018) (Figure 1) which further highlights the importance of dairy in people's lives in Bhutan. Production of NFRM is cheaper too since natural fermentation is highly energy efficient with no heating or cooking required. It saves labour and time and keeps the costs of production low. However, few Bhutanese don't purchase the ethnic dairy products due to the perceived poor quality and hygiene of the products (Phanchung et al., 2002).

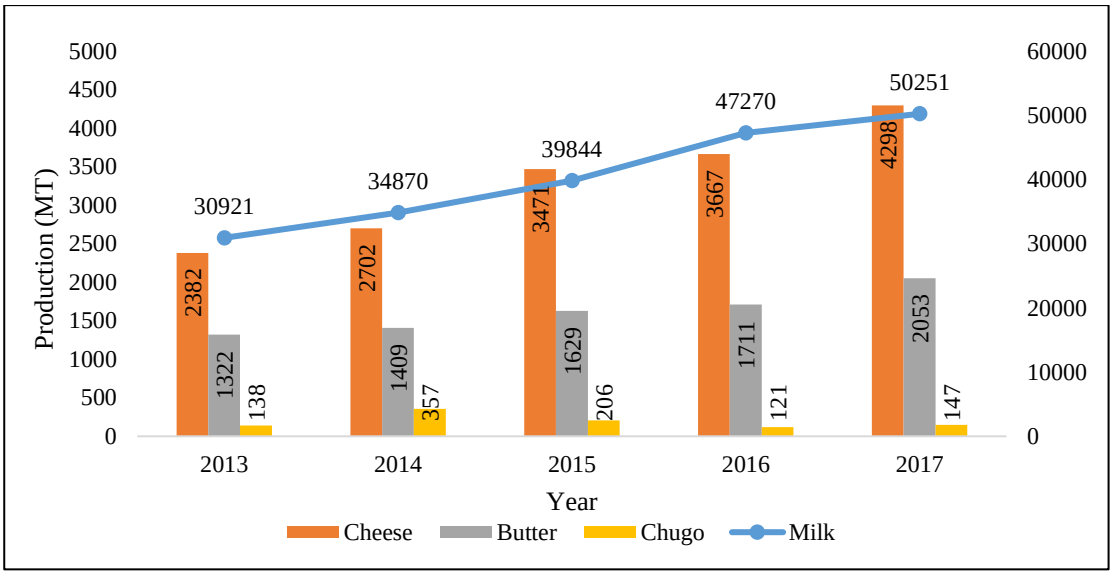


Figure 1. Production trend of milk and dairy products in Bhutan (2013-2017)

From *Statistics on dairy animal population and production trend from 2013 to 2017* (p. 12) by Renewable Natural Resources (Bhutan) (2018), Thimphu, Bhutan: Renewable Natural Resources Statistics Division. Copyright 2018 by Renewable Natural Resources Statistics Division (RSD). Adapted with permission.

2.3.2 Method of production

NFRM is the first intermediate product during the preparation of any other ethnic dairy product in Bhutan. RM is naturally fermented into NFRM and used to produce butter and buttermilk. The buttermilk is further processed into soft and hard cheese (R. Rai et al., 2016) (Figure 2). Although dahi/curd is prepared from both raw and boiled milk (R. Rai et al., 2016), it is exclusively prepared from RM by spontaneous fermentation in some districts like Thimphu and Chukkha in Bhutan. Therefore, as fermentation depends mainly on the fermentation temperature (Wouters et al., 2002), the fermentation period varies. R. Rai et al. (2016) reported that dahi in Bhutan is prepared by fermenting raw or boiled milk for 15 days. Shangpliang, Sharma, Rai, and Tamang (2017) reported that it is prepared from boiling the milk and fermenting for 2 - 3 days using the back-sloping technique. However, it is not a common practice in Bhutan to boil milk for preparing dahi and it is also not fermented for as long as 15 long days, especially when it is produced as a ready-to-drink product. It is usually fermented up to 3 days, depending on the fermentation temperature.

In Bhutan, there are huge variations in temperatures in different places. For example, in the southern region of Bhutan, the temperature during winter (November - January) ranges from 10 ºC to 27 ºC and the temperature during summer (June - August) ranges from 24 ºC to 31 ºC. In the northern mountainous areas, the temperatures during winter

and summer range from -1 ºC to 18 ºC and 15 ºC to 25 ºC respectively (Climates to travel, 2019) (Table 5). During winter, milk is sometimes kept near fireplaces to accelerate fermentation. As in Africa, since there are no standardised methods for NFRM production in Bhutan, the processors determine the fermentation time based on their previous experiences and looking at the thickness and consistency of the curd. Depending on the facilities availability, after the fermentation, NFRM is either stored under refrigeration or simply left at room temperature in certain places with cold weather.

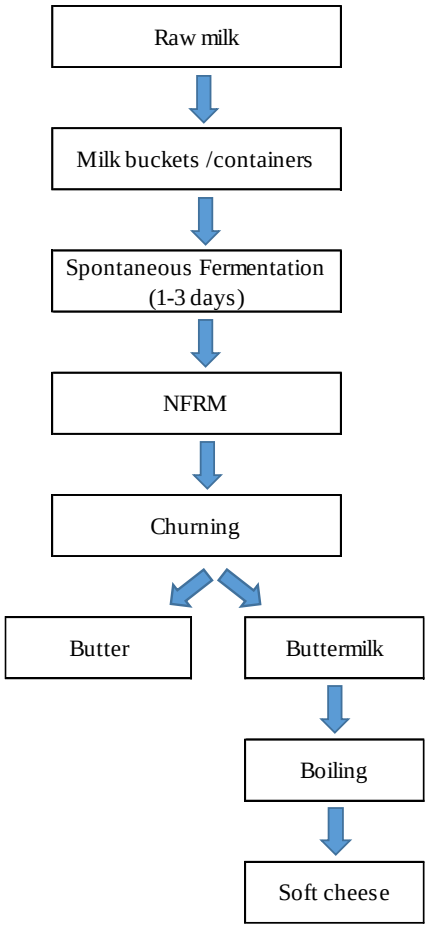


Figure 2. Process flow for ethnic dairy products production in Bhutan

Table 5. Temperature variations in Bhutan

Region	Jan	Feb	Mar	Apr	May	June	July	Aug	Sept	Oct	Nov	Dec
Flat southern region (Min ºC)	10	12	15	20	22	24	25	25	24	21	15	11
Hilly areas (Min ºC)	-3	1	4	7	13	15	15	16	15	10	5	-1
Flat southern region (Max ºC)	23	25	29	32	32	31	31	31	31	30	27	25
Hilly areas (Max ºC)	12	14	16	20	23	24	25	25	23	22	18	15

2.4 The select bacteria in this survey: *B. cereus, E. coli* and *S. aureus*

The prevalence of human pathogens in Africa (**Error! Reference source not found.**) is an indication of the possibility of human pathogens present in NFRM in Bhutan as well because of the natural habitats of these microorganisms and similar processing environment including lack of hygiene practices during NFRM processing and lack of refrigeration. Contamination with *B. cereus, E. coli* and *S. aureus* is inevitable in foods due to various sources of contamination during the "production-to-consumption continuum". Generally, the primary source of contamination in RM is attributed to the pre-harvest and post-harvest contamination (Angulo et al., 2009). Pre-harvest contamination occurs due to the commensal microflora naturally present in the teat canals or on the teat skin of the animal since farm animals, including healthy cattle are a major reservoir of pathogens that are likely to be transferred to milk (Arqués et al., 2015). Pre-harvest contamination also occurs from the unhealthy cattle that are suffering from infectious diseases like mastitis. The pathogens causing such diseases are directly shed into the milk (Motarjemi et al., 2014; Murphy & Boor, 2000).

Post-harvest contamination occurs at the time of collection and during processing, distribution, and storage of RM from external sources (cattle, surfaces of milk handling and storage equipment, milking personnel) (Angulo et al., 2009; Murphy & Boor, 2000). This contamination occurs in milk from cattle which are free from systemic diseases and/or any intra-mammary infections (Angulo et al., 2009). During and post-processing contamination mainly occur from NFRM processing personnel and manufacturing environment including the processing equipment and utensils. In Bhutan, since the manufacturing process of NFRM is simple, contamination may occur mostly during the pre-harvest and post-harvest, and may be minimal during and post-processing.

The prevalence study of *B. cereus, E. coli* and *S. aureus* in Bhutan will be useful in understanding the risk of the consumption of NFRM and identifying potential sources of contamination to prevent and minimise disease risk. The results of this survey can also be used to devise strategies to improve the safety of the products, enhancing the development of the dairy industry in Bhutan.

1 *Table 6. Pathogens identified in NFRM in Africa*

Name of the product	Place	Pathogens identified	References
Naturally soured RM	Zimbabwe	*E. coli*	Gran, Mutukumira, Wetlesen, and Narvhus (2002)
Naturally soured RM	Zimbabwe	*E. coli* and *S. aureus*	Gran, Wetlesen, Mutukumira, Rukure, and Narvhus (2003)
Nono	Bauchi, Nigeria	*B. cereus, E. coli, S. aureus, Enterobacter* spp. and *Streptococcus* spp.	Adebesin, Amusa, and Fagade (2001).
Nono	Nigeria	*B. cereus, E. coli, Enterobacter aerogenes, Pseudomonas aeruginosa* and *S. aureus*	Obi and Ikenebomeh (2007)
Nono	Makurdi, Nigeria	*Mycobacterium bovis* and *Mycobacterium africanum*	Ofukwu, Oboegbulem, and Akwuobu (2008)
Nono	Northern Nigeria	*E. coli. S. aureus, Shigella and Salmonella* spp.	Okonkwo (2011)
Nono	Makurdi, Benue State, Nigeria	*Bacillus* spp., *E. coli, Klebsiella* spp, *Salmonella* spp., *S. aureus, Staphylococcus* spp. and *Streptococcus* spp.	Obande and Azua (2013)
Nono	Nasarawa State, Nigeria	STEC O157:H7	Reuben, Okolocha, Bello, and Tanimu (2013)
Nono	Ogun state, Nigeria	STEC O157	Ivbade, Ojo, and Dipeolu (2014)
Nono	Zaria, Kaduna State, Nigeria	*Salmonella* spp.	Tamba, Bello, and Raji (2016)
Nono	Mangu Local Government Area of Plateau State, Nigeria	*Coliforms, E. coli, Salmonella* spp., *Shigella* spp., *P. aeruginosa and S. aureus*	Dafur, Iheukwumere, Azua, and Dafur (2018)

Nono	Samura, Kaduna state, Nigeria	*E. coli*, *Enterobacter* spp, *Klebsiella* spp, *Citrobacter* spp. and *Proteus* spp	Maikai and Madaki (2018)
Nunu	Ghana	*Enterobacter*, *E. coli*, *Klebsiella* , *Proteus vulgaris* and *Shigella*	Akabanda et al. (2010)
Raw fermented milk	Nigeria	*E. coli* and *STEC O157:H7*	Yakubu, Shuaibu, Ibrahim, Hassan, and Nwachukwu (2018)
Rayeb	Tunisia	*S. aureus* and *coliforms*	Samet-Bali et al. (2016)
Traditionally fermented raw milk	South Africa and Namibia	*S. aureus*	Beukes et al. (2001)
Traditionally fermented raw milk	Musanze District, Rwanda	*E. coli*, *S. aureus* and *Providencia alcalifaciens*, ,	Nzabuheraheza and Nyiramugwera (2016)
Traditional yogurt	Borana pastoral area, southern Ethiopia	*E. coli* and *S. aureus*	Amenu, Grace, Nemo, and Wieland (2019)

1

2.4.1 *B. cereus*

B. cereus is one of the most frequent spore-forming pathogens encountered in RM and RMP (Christiansson, Naidu, Nilsson, Wadström, & Pettersson, 1989; Gopal et al., 2015). It also produces enzymes that spoil dairy products and shorten shelf life. Therefore, its presence in food is of both safety and quality concern (Porcellato, Narvhus, & Skeie, 2016). *B. cereus* has been reported in NFRM in Nigeria (**Error! Reference source not found.**).

Bacillus is commonly present on the skin of the teat and epithelial lining of the teat canal as commensal microflora (Isaac et al., 2017). *B. cereus* produces endospores to survive in a low nutrient environment like soil (Kotiranta, Lounatmaa, & Haapasalo, 2000). These endospores are very resistant (Schoeni & Wong, 2005) and can survive harsh environments like dehydration and are therefore widespread in the environment (MPI, 2016). Although *B. cereus* is most abundant in soil, it can also be present in the dust, water and air (MPI, 2016; Schoeni & Wong, 2005). *B. cereus* enters the milk through the udders in contact with soil and grass (Granum & Toril, 2013). It may also be present on the plastic bottles made up of polyethylene terephthalate (PET) that are used for fermenting NFRM since the empty PET bottles are stored in the open-air without lids and used without washing or sterilising. Some *B. cereus* strains are also psychrotrophic (Schoeni & Wong, 2005). Hence, since *B. cereus* easily contaminates food including milk and milk products (Hwang & Park, 2015; Zhang, Feng, et al., 2016), it is very difficult to prevent the contamination of *B. cereus* during food processing (Kotiranta et al., 2000).

B. cereus causes two types of food poisoning, the emetic and diarrheal syndromes by producing toxins (De Jonghe et al., 2010; Ghelardi et al., 2002). The emetic syndrome is caused by the consumption of preformed toxins in the food. It is characterised by vomiting, nausea and occasionally diarrhoea. The diarrhoeal syndrome is caused when contaminated food containing live *B. cereus* is consumed and *B. cereus* grows in the gut and produces the diarrhoeal toxins. It is characterised by watery diarrhoea and abdominal pain. Diarrhoeal illness is often associated with foods such as milk and milk products (MPI, 2015a).

Generally, 5 to 8 $\log_{10}$ cfu/ml *B. cereus* is required to produce sufficient toxins to cause food poisoning (MPI, 2015a). The FSANZ (2018a) categorises food containing more than 5 $\log_{10}$ cfu/ml *B. cereus* as potentially hazardous. However, lower counts or the absence

of *B. cereus* in food may not completely render the food safe since sufficient toxins to cause illnesses may have already been produced before the unfavourable growth conditions in the food killed the pathogen or reduced its counts (Bennett, Hait, & Tallent, 2013).

2.4.2 *E. coli*

E. coli has most commonly been reported in NFRM in many countries in Africa including Zimbabwe, Nigeria and Ghana (**Error! Reference source not found.**). It has also been reported at 33 % prevalence in the top 10 most common bacteria present in RM in Bhutan (S. B. C. Rai et al., 2018) (Figure 3). *E. coli* is naturally found in the intestinal tract of humans and other warm-blooded animals and its presence in food indicates a direct or an indirect faecal contamination (Krumperman, 1983). Thus *E. coli* "has been the traditional indicator of faecal contamination" (Miskimin et al., 1976). Its presence also indicates the general hygiene during milk production and processing (Yucel & Ulusoy, 2006). Generally, in developing countries, hygienic practices are not adopted during the handling and processing of milk and milk products (Wanjala et al., 2018). In Bhutan too, the quality and hygiene of the RM and RMP are poor due to which few Bhutanese don't purchase RM and RMP (Phanchung et al., 2002).

Faecal contamination is considered the primary source of pathogens in milk. During the milking process, the primary source of contamination of milk is directly linked to faecal contamination which maintains and recycles the pathogens on the dairy farm. These pathogens are then transmitted through the major (manure, lagoons, beddings, and equipment) and minor (birds, rats, insects and on-farm pets) pathways (Oliver, Jayarao, & Almeida, 2005) (Figure 4). Thus milk is contaminated when it comes in direct contact with the faeces or contaminated sources in the dairy environment (Oliver et al., 2005). Additionally, *E. coli* has been found in the rivers in Bhutan (Rinzin, 2017) which are one of the main sources for drinking water (National Environment Commission (Bhutan), 2016).

Although most *E. coli* strains are harmless, there are pathogenic strains like the Vero-cytotoxin-producing *E. coli (*VTEC) a.k.a Shiga toxin-producing *E. coli* (STEC) which are commonly associated with diseases due to the consumption of contaminated food including RM (Baylis, 2009; CDC, 2014). The occurrence of STEC in RM is especially high as cattle are one of the main animal reservoirs of STEC (Karmali, Gannon, &

Sargeant, 2010). They easily adhere to the skin and the udder too. Some strains of STEC including *E. coli* O157:H7 which is generally the most common STEC that causes human illnesses, was also reported in NFRM (Ivbade et al., 2014; Yakubu et al., 2018). When the *E. coli* found in naturally soured milk in Zimbabwe was tested for pathogenicity, 50 % were reported to be enterotoxigenic *E. coli* (ETEC) (Gran et al., 2003) (Figure 5). ETEC is a pathogenic *E. coli* which is commonly found in water in developing countries (MPI, 2015b). Therefore, there is a high possibility that *E. coli* may be present in NFRM in Bhutan.

STEC causes illnesses ranging from mild diarrhoea and vomiting to more serious conditions including Haemorrhagic Colitis (HC) and Haemolytic Uraemic Syndrome (HUS) (MPI, 2018). FSANZ (2018a) states food containing more than 2 $\log_{10}$ cfu/ml *E. coli* as unsatisfactory and indicates possible faecal contamination and poor hygiene mainly during primary food production. FSANZ (2018a) also categorises food as potentially hazardous if STEC is detected in 25g of food.

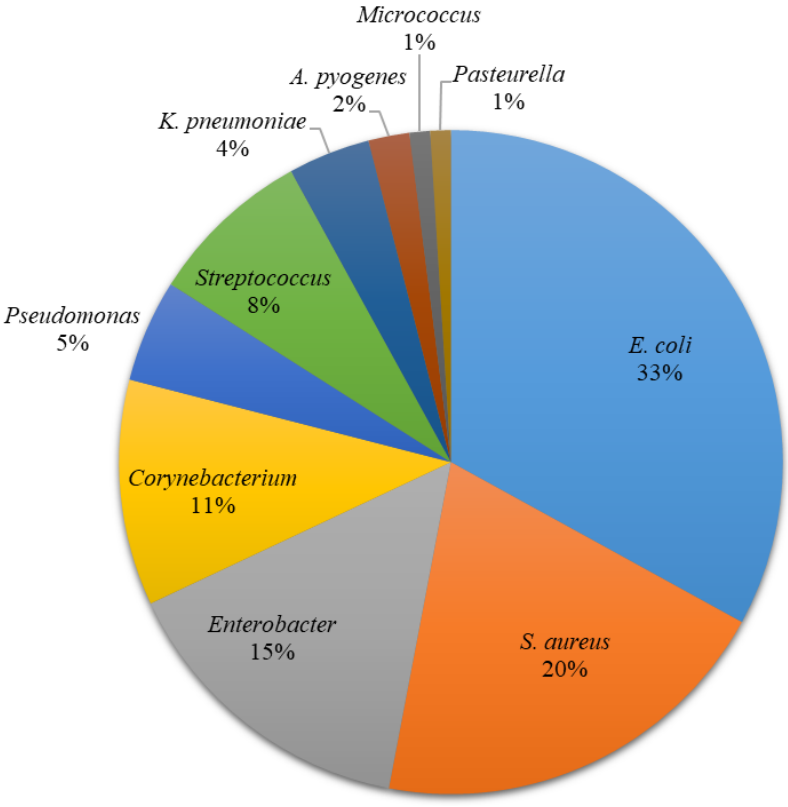


Figure 3. Ten commonest milk-borne pathogens in RM in Bhutan

From "Microbiological quality of raw milk in Bhutan" by S. B. C. Rai et al. (2018), *Bhutan Journal of Animal Science*, 2, p. 78. Copyright 2018 by Department of Livestock. Adapted with permission.

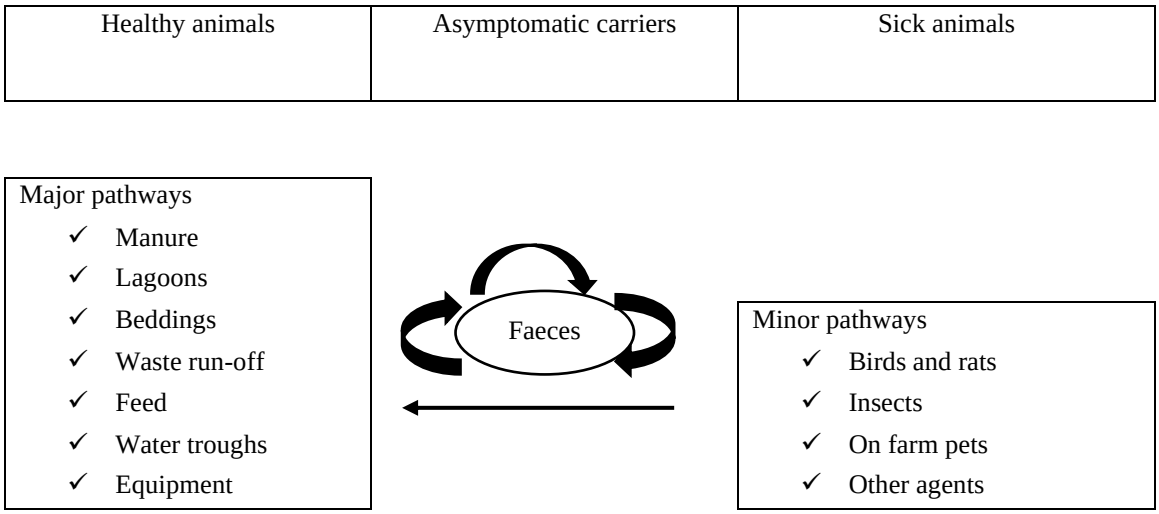


Figure 4. Maintenance recycling of the pathogens on dairy farms

From 'Foodborne pathogens in milk and the dairy farm environment: Food safety and public health implications' by Oliver et al. (2005), *Foodborne Pathogens and Disease*, 2, p. 120. Copyright 2005 by Mary Ann Liebert, Inc. Adapted with permission.

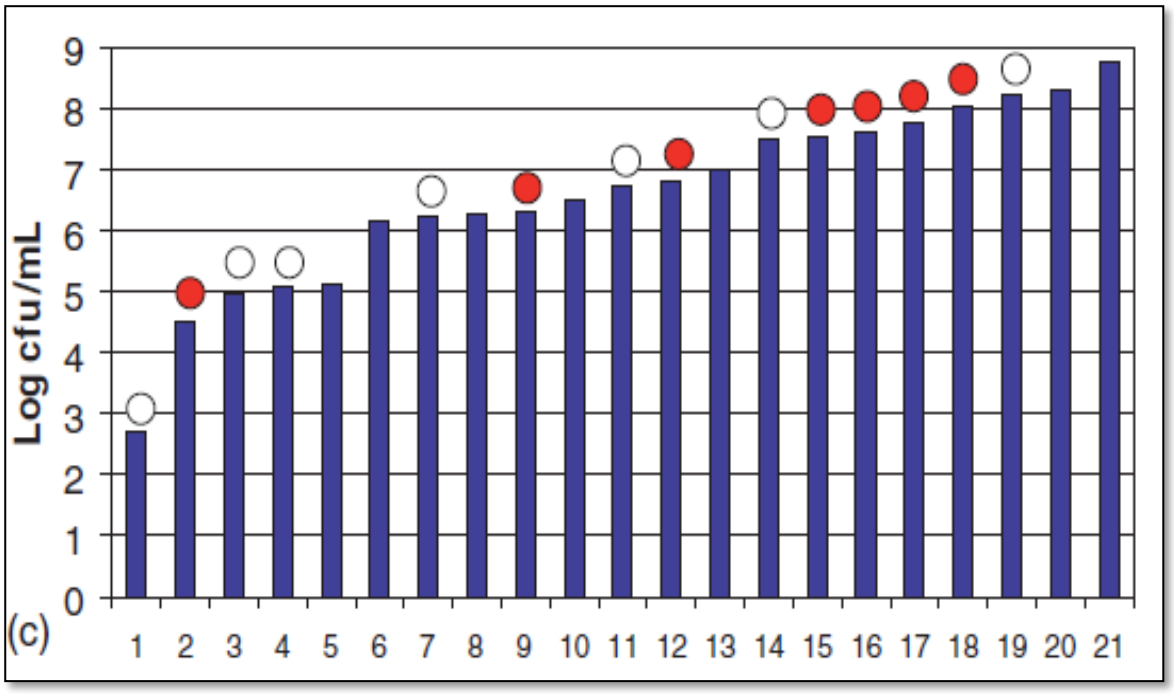


Figure 5. *E. coli* in naturally soured milk.
***E. coli* strains tested for pathogeny are marked with circle. Pathogenic strains are shown with (●) while non-pathogenic strains are shown with (○).**

From "Occurrence of pathogenic bacteria in raw milk, cultured pasteurised milk and naturally soured milk produced at small-scale dairies in Zimbabwe" by Gran et al. (2003), *Food Control*, 14, p. 541. Copyright 2003 by Elsevier Ltd. Adapted with permission.

2.4.3 *S. aureus*

S. aureus has been identified as one of the most common causes of food poisoning outbreaks (Zakary, Nassif, & Mohammed, 2011). Like *E. coli*, *S. aureus* is commonly reported in NFRM in Africa (**Error! Reference source not found.**). It has been reported at 20 % prevalence in the top 10 most common bacteria present in RM in Bhutan (S. B. C. Rai et al., 2018). It is a ubiquitous pathogen and can contaminate food from many sources (Rosengren, Fabricius, Guss, Sylvén, & Lindqvist, 2010) including RM from cattle infected with mastitis and from humans (Hennekinne, De Buyser, & Dragacci, 2012).

Mastitis is the inflammation of the mammary gland (Kuang et al., 2009). It is a common infection in cattle and the "single disease that has the most significant impact on milk quality" (Angulo et al., 2009). It is estimated that up to 90 % of dairy farms have *S. aureus* (Zakary et al., 2011). Although mastitis is caused by more than 150 different microorganisms including bacteria and yeast (Kuang et al., 2009), the most common bacteria isolated from cattle with mastitis are *Staphylococcus* species (Makovec & Ruegg, 2003; Wilson, Gonzalez, & Das, 1997; Zadoks, Gonzalez, Boor, & Schukken, 2004) and *S. aureus* is the most predominant species causing mastitis in cattle (Chen, Tang, Hu, Zhao, & Tang, 2018). In Bhutan, 83.9 % and 89 % mastitis have been reported in cattle belonging to government cattle breeding farms and dairy farmers (S. B. C. Rai et al., 2018; Tshering & Gyem, 2015). Two types of mastitis are usually observed in the cattle, clinical and subclinical. When the cattle suffer from clinical mastitis, they produce milk with an altered appearance. In such cases, the milk usually has different colour and contains clots of blood and flakes. Such milk is deemed unfit for human consumption and discarded thus lowering the chance of milk contamination. However, when the cattle suffer from sub-clinical mastitis, there is a higher chance of milk from infected cattle entering the food chain as there is no visible change in the milk appearance (Angulo et al., 2009; EFSA, 2015b). In Bhutan, the prevalence of sub-clinical mastitis has been reported at 67 % and the prevalence of clinical mastitis has been reported at 20.7 % (S. B. C. Rai et al., 2018) (Figure 6).

Like *Bacillus*, *Staphylococcus* is commonly present on the skin of the teat and epithelial lining of the teat canal as commensal microflora (Isaac et al., 2017). *S. aureus* may also

be introduced into milk from milking personnel as it is found on the skin, hair and nasal passages of humans (Tong, Davis, Eichenberger, Holland, & Fowler, 2015). Humans are the chief source of staphylococcal enterotoxin (SEs) producing strains which cause staphylococcal food poisoning (FSANZ, 2018a) and in Bhutan, cattle are commonly milked by hand. NFRM is also handled directly by hand. Therefore, there is some risk that *S. aureus* may also be present in NFRM in Bhutan.

The symptoms of staphylococcal food poisoning are vomiting, nausea and general weaknesses. The illness is rarely fatal. Nevertheless, it can result in severe dehydration and shock (Zakary et al., 2011).

Generally, toxins less than 1.0 µg can cause illness and this level is reached when more than 5 $\log_{10}$ cfu/ml *S. aureus* is present in food (MPI, 2001). The FSANZ (2018a) categorises food containing more than 4 $\log_{10}$ cfu/ml *S. aureus* as potentially hazardous. However, similar to *B. cereus*, lower counts or the absence of *S. aureus* in food may not render the food safe since sufficient toxins to cause illnesses may have already been produced before the unfavourable growth conditions in the food killed the pathogen or reduced its counts (Bennett et al., 2013).

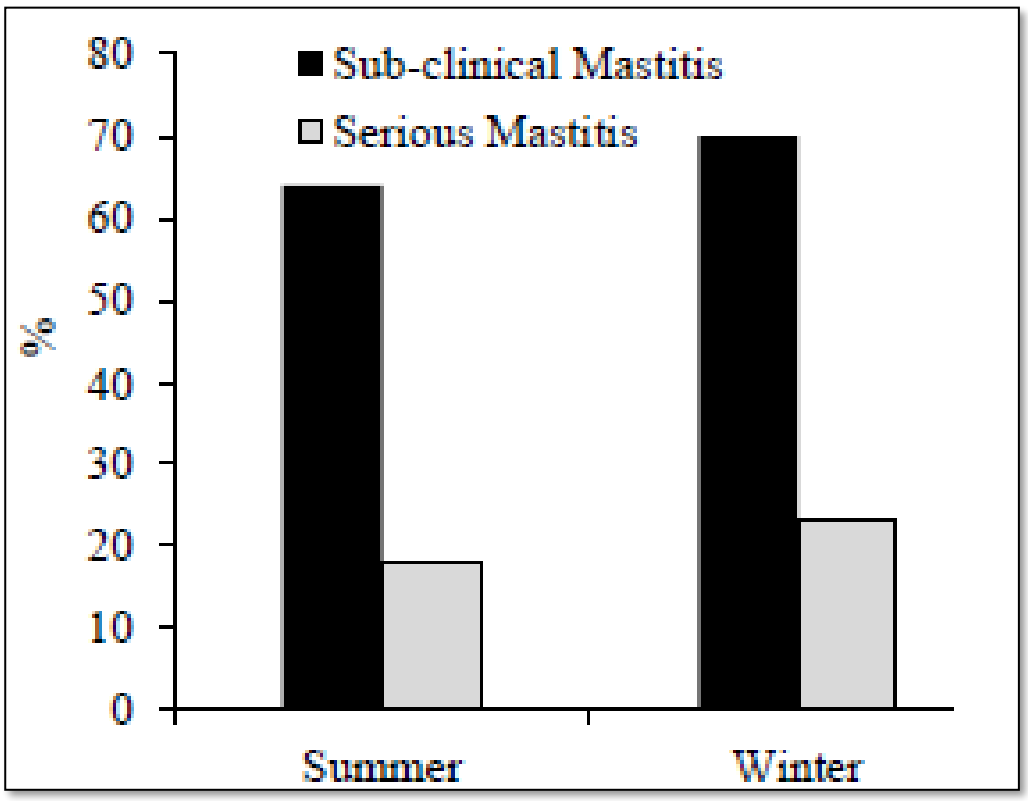


Figure 6. Prevalence of mastitis in cattle in Bhutan

From “Microbiological quality of raw milk in Bhutan” by S. B. C. Rai et al. (2018), *Bhutan Journal of Animal Science,* 2, p. 79. Copyright 2018 by Department of Livestock. Adapted with permission

2.5 Growth and survival of *B. cereus*, *E. coli* and *S. aureus* in RM and RMP

There are many factors that affect the survivability of *E. coli*, *B. cereus* and *S. aureus* (Koutsoumanis, Lianou, & Gougouli, 2016) (Figure 7). These factors are described under intrinsic and extrinsic factors (Choi, Lee, Lee, Kim, & Yoon, 2016).

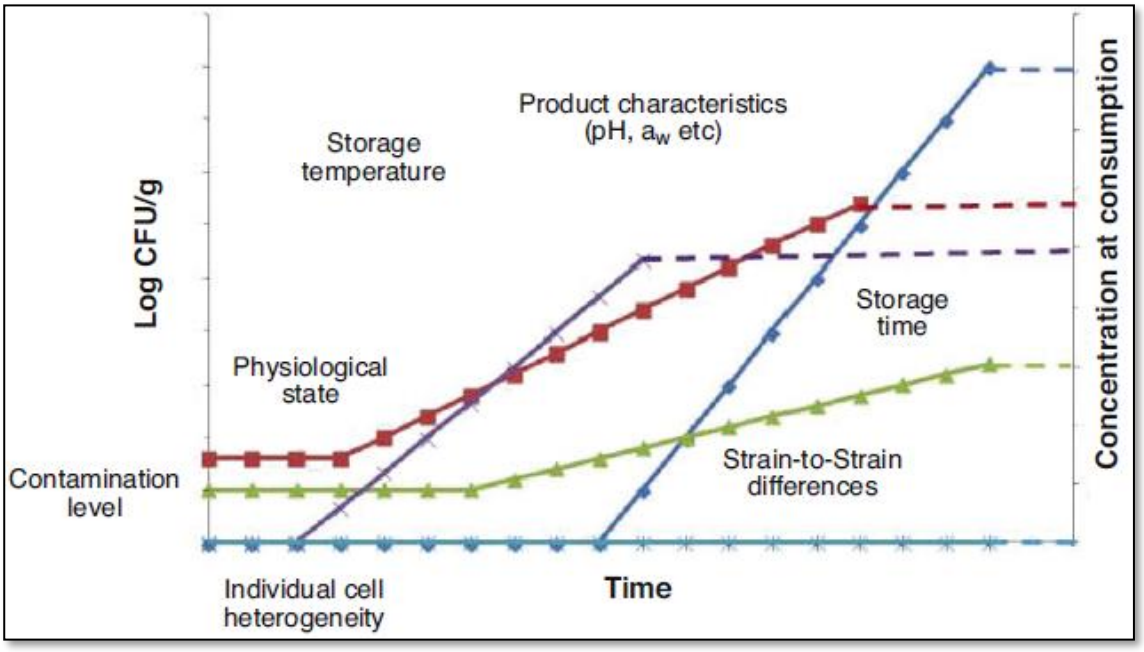


Figure 7. Factors affecting microbial growth

From 'Latest developments in foodborne pathogens modeling' by Koutsoumanis et al. (2016), *Current Opinion in Food Science*, 8, p. 92. Copyright 2016 by Elsevier Ltd. Adapted with permission.

2.5.1 Intrinsic factors

These are the factors present in the food matrix of NFRM itself (CAC, 2004).

2.5.1.1. pH

pH is considered as one of the main factors affecting the survival, growth and inactivation of the bacteria in fermented food (Verraes et al., 2015). It is a critical control parameter for the production of safe fermented dairy products including NFRM (Adams & Nicolaides, 1997). Generally, pathogens have a narrow pH range for growth and an acid shock from fermentation retards or inhibits their growth (Davidson, Critzer, & Taylor, 2013). Pathogens exhibit different sensitivity to pH and require different minimum pH values for growth (Table 7). For example, if the pH of the food is 4.0, *S. aureus* may grow while *B. cereus* and *E. coli* will not grow. Most pathogenic bacteria are unable to grow from pH 4 to 6 (Horn & Bhunia, 2018), so this helps in reducing the risk of food safety

issues in RMP and NFRM which has pH ranging from 3.7 to 5.7 (Obi & Ikenebomeh, 2007; Okiki et al., 2018; Okonkwo, 2011; Shangpliang et al., 2017). The growth of *S. aureus* was reported completely inhibited when the pH decreased to 4.4 - 4.5 by lactic acid (Charlier, Even, Gautier, & Loir, 2008). *B. cereus* was also inhibited at pH 4.9 to 5.0 (Røssland, Langsrud, & Sørhaug, 2005). Similarly, after 24 h of co-fermentation, the pH of the fermented milk was reduced to about 5.0 by *Lb. plantarum* and the concentration *of B. cereus* was reduced by 1 order of magnitude (Zhang, Tao, Shah, & Wei, 2016). Nevertheless, even at pH 5.2 - 5.5 in RM cheeses, the survival of *S. aureus* and has been reported (Bellio et al., 2016; Gould, Mungai, & Behravesh, 2014).

Table 7. Minimum pH values required for the growth of some pathogens including *B. cereus, E. coli* and *S. aureus*

Micro-organism	Minimum pH
Escherichia coli O157:H7	4.5
Salmonella spp.	3.8-4.05
Campylobacter jejuni/coli	4.9
Vibrio parahaemolytics	4.8
Yersinia enterocolitica	4.2
Clostridium botulinum	4.6-5.0
Clostridium perfringens	5.0
Staphylococcus aureus	4.0
Listeria monocytogenes	4.1-4.5
Bacillus cereus	4.9

From 'Factors affecting the growth of microorganisms in food' by Hamad (2012) in R. Bhat, A. K. Alias, & G. Paliyath (Eds.), *Progress in Food Preservation* (p. 410). West Sussex, UK: John Wiley & Sons Ltd. Copyright 2012 by John Wiley & Sons Ltd. Adapted with permission.

2.5.1.2 LAB and secondary antimicrobials

In a mixed population, micro-organisms that have a higher growth rate in the conditions extant compete the best for the nutrients available in the food matrix and can grow rapidly, thus dominating the population (Hamad, 2012). Bacteria that ferment dairy products dominate through the production of acid which prevents the growth of bacteria that are not acid-tolerant (Verraes et al., 2015). In fermented foods including NFRM, LAB are likely to dominate as they grow rapidly in milk at ambient temperatures and can tolerate acid and pH changes (Axelsson, 2004; König & Fröhlich, 2017; Niamsiri & Batt, 2009; Steinkraus, 2018). They may outcompete other micro-organisms including *B. cereus, E. coli* and *S. aureus* (Gutiérrez, Martínez-Blanco, Rodríguez-Aparicio, & Ferrero, 2016). This is another important factor affecting the survival of these bacteria in fermented milk

like NFRM (Verraes et al., 2015). However, for the LAB to dominate over the growth of the pathogens, their counts need to be greater than the counts of the pathogens present (Adams & Mitchell, 2002).

Organic acids including lactic acid produced by the LAB also inhibit the growth of the pathogenic bacteria independent of the effect of pH. Charlier et al. (2008) reported that during the early stages of milk fermentation (24 h), *L. lactis* inhibited the growth of *S. aureus* by 3 logs without any change in the pH. This was due to lactic acid produced by *L. lactis*.

Apart from lactic acid, some LAB also produce other secondary antimicrobial substances such as bacteriocins, hydrogen peroxides and ethanol that inhibit bacterial pathogens and render foods safe (Adams & Nicolaides, 1997; Campbell-Platt, 1994; Fusco, Oguntoyinbo, & Franz, 2017; Gutiérrez et al., 2016; Hamad, 2012; Niamsiri & Batt, 2009). Although the factors directly involved in these effects were not analysed, Gutiérrez et al. (2016) demonstrated the inhibition of pathogens by LAB culture broths. They reported that *L. lactis* C660 and *Lb. rhamnosus* inhibited the growth of *E. coli* by 31 % and 12 % respectively. *L. casei* inhibited the growth of *S. aureus* by up to 35 % (Table 8). These may be due to the secondary antimicrobial substances.

Bacteriocins are bioactive peptides that are ribosomally synthesized by the LAB and have antimicrobial activity (bacteriostatic or bactericidal effect) against related or nonrelated bacteria. They are considered as natural bio preservatives (Arqués et al., 2015; Prudêncio, Dos Santos, & Vanetti, 2015) since they naturally inhibit the growth of most Gram-positive bacteria. Thus the growth of *B. cereus* and *S. aureus* can easily be inhibited by bacteriocins. However, they do not inhibit the growth of the Gram-negative bacteria due to an outer layer on the cell membrane of Gram-negative bacteria which acts as a natural barrier (Gyawali & Ibrahim, 2014). Nevertheless, bacteriocins are able to inhibit Gram-negative bacteria including *E. coli* after the destabilization of their outer membrane (Belfiore, Castellano, & Vignolo, 2007; Stevens, Sheldon, Klapes, & Klaenhammer, 1991). Bacteriocins are generally regarded as safe (GRAS) and are used in improving food safety (Arqués et al., 2015).

Examples of bacteriocins produced by LAB used in food preservation include nisin, pediocin and reuterine (Horn & Bhunia, 2018). Nisin was the first bacteriocin to be discovered (Chikindas, Weeks, Drider, Chistyakov, & Dicks, 2018) and the first

bacteriocin to be commercially produced for adding to food. Since it is the only bacteriocin approved by the FDA as a food preservative, it is the most widely used bacteriocin (Gharsallaoui, Oulahal, Joly, & Degraeve, 2016). Nisin is naturally produced *in situ* when *L. lactis* is used as a starter culture for the manufacture of fermented dairy products like camembert cheese. It is effective against Gram-positive bacteria like *L. monocytogenes* and *Staphylococcus* (Arqués et al., 2015; Gharsallaoui et al., 2016) and spore-forming bacteria like *Bacillus* and *Clostridium*. (Venema, Venema, & Kok, 1995). It is effective against *E. coli* when used in combination with heat treatment, freezing or chelators (Belfiore et al., 2007). Some more examples of the bacteriocins applied in dairy products are provided in Table 9.

Table 8. Effect of LAB culture broths on the growth of pathogens including *B. cereus, E. coli* and *S. aureus*[1]

Strain (%)	***Escherichia coli* K92**	***Bacillus cereus***	***Listeria innocua***	***Pseudomonas fluorescens***	***Pseudomonas putida***	***Staphylococcus aureus***	***Staphylococcus epidermidis***
Lactococcus lactis C660	31	-[2]	-	19	-	-	76
L. lactis ATCC 11454	-	-	-	-	-	-	90
Lactobacillus casei	-	-	-	25	-	35	76
Lactobacillus paracasei	-	-	-	-	-	-	-
Lactobacillus rhamnosus	12	-	28	-	-	-	76

[1]Percent inhibition is the difference between the optical density at the point of maximal inhibition by the LAB broth and control thereof cultured in trypticase soy broth.
[2]Nondetectable inhibition effect of growth.

From 'Effect of fermented broth from lactic acid bacteria on pathogenic bacteria proliferation' by Gutiérrez et al. (2016), *Journal of Dairy Science*, 99, p. 2658. Copyright 2016 by American Dairy Science Association. Adapted with permission.

Table 9. Applications of bacteriocins in dairy products

Bacteriocin	Bacteriocin producing culture	Application	Pathogen	Product
Lacticin 3147	*Lc. lactis* DPC 3147	Spray-dried powder	*L. monocytogenes*	Cottage cheese
Pediocin	*P. acidilactici*	Dry powder	*L. monocytogenes*	Cottage cheese and yogurt
Pediocolin 126	*C. piscicola* JG 126	Concentrated supernatant	*L. monocytogenes*	Camembert cheese
Enterocin CRL35	*E. faecium* CRL 35	Concentrated supernatant	*L. monocytogenes*	Goat milk cheese
Nisin	*Lc. lactis* CNRZ 150	Starter culture	*L. monocytogenes*	Camembert cheese
Nisin	*Lc. lactis* TAB 50	Starter culture	*L. monocytogenes*	Semihard cheese
Lacticin 481	*Lc. lactis* TAB 24	Starter culture	*L. monocytogenes*	Semihard cheese
Lacticin 3147	*Lc. lactis* DPC 4275	Starter culture	*L. monocytogenes*	Cottage cheese
Enterocin AS-48	*E. faecalis* TAB 28	Starter culture	*L. monocytogenes*	Semihard cheese
Enterocin AS-48	*E. faecalis* INIA 4	Starter or adjunct culture	*L. monocytogenes*	Manchego cheese
Pediocin	*Lc. lactis* MM 217	Starter culture	*L. monocytogenes*	Cheddar cheese
Pediocin	*Lb. plantarum*	Surface sprayed cell suspension	*L. monocytogenes*	Munster cheese
Pediocin	*Lc. lactis* CL1	Adjunct culture	*L. monocytogenes*	Semihard cheese
Pediocin	*Lc. lactis* CL1	Adjunct culture	*S. aureus*	Semihard cheese
Nisin	*Lc. lactis* ESI 515	Adjunct culture	*S. aureus*	Semihard cheese

From 'Antimicrobial activity of lactic acid bacteria in dairy products and gut: effect on pathogens by (Arqués et al., 2015), *BioMed Research International*, p. 3. In the public domain.

2.5.1.3 Pathogen microbiota

The initial microbial load in food is a primary factor that determines the effectiveness of any treatment given to the food for inactivating pathogens and rendering the products safe (Adams & Mitchell, 2002; Syed, Buffa, Guamis, & Saldo, 2016). The pathogens may survive the treatment if present in high numbers in the food matrix (Syed et al., 2016). For NFRM, since the source of the microbes is mainly the RM, if the RM is contaminated with high initial counts of pathogens, it is likely that the decrease in pH might not be able to inactivate all pathogens leaving survivors with potential for food safety issues. As discussed, there is also a concern with the production of toxins from high numbers of pathogens like *S. aureus* and *B. cereus* (Adams & Mitchell, 2002). *E. coli* O157:H7 survives fermentation when present in high counts in the raw material (Getty, Phebus, Marsden, Fung, & Kastner, 2000). The presence of Gram-positive or Gram-negative

bacteria also affects the risk of pathogen survival (Syed et al., 2016). Fermentation is not effective against Gram-negative bacteria until their cell wall is destabilised. The physiological state of the pathogens also determines the success of inactivation or inhibition of micro-organisms in food (Adams & Mitchell, 2002).

2.5.1.4 Others

Other factors involved in the growth of pathogenic micro-organisms are the moisture and water, and nutrient content. High moisture foods with a water activity above 0.85 like NFRM supports the growth of most bacteria since they require minimal water activity of 0.88 to 0.91 for their growth and survival (Table 10). Similarly, as NFRM contains all nutrients required for pathogens to survive and grow, *B. cereus*, *E. coli* and *S. aureus* are highly likely to grow well in NFRM (Ray, 2004).

Table 10. Minimum water activity required by pathogens including *B. cereus, E. coli* and *S. aureus*

Micro-organism	Minimal a_w required
Escherichia coli	0.94-0.97
Clostridium botulinum	0.90-0.98
Staphylococcus aureus	0.83-0.92
Vibrio parahaemolyticus	0.94-0.98
Salmonella	0.93-0.96
Bacillus cereus	0.92-0.95

From 'Factors affecting the growth of microorganisms in food' by Hamad (2012) in R. Bhat, A. K. Alias, & G. Paliyath (Eds.), *Progress in Food Preservation* (p. 409). West Sussex, UK: John Wiley & Sons Ltd.

2.5.2 Extrinsic factors

These are the factors related to the storage environment for NFRM (CAC, 2004; Hamad, 2012).

2.5.2.1 Transportation and storage temperature

The transportation temperature for RM and storage temperature for NFRM determine the type of pathogens that will survive and grow since all micro-organisms including the pathogens require an optimum, maximum and minimum temperature for their growth. Micro-organisms are grouped according to the temperatures at which they grow (Hamad, 2012). Most of the pathogens are either psychrotrophic or mesophilic. During the transportation of RM, pathogens may also grow if the cold chain is not maintained

(Motarjemi et al., 2014). Similarly, if food is stored at favourable temperatures for bacterial growth, pathogens are likely to grow (Table 11 and Table 12).

Table 11. Growth temperatures for mesophilic pathogens including *B. cereus* (mesophilic strains), *E. coli* and *S. aureus*

Micro-organism	Minimal (ºC)	Optimum (ºC)	Maximum (ºC)
Escherichia coli	7	35-40	46
Salmonella spp.	5-10	35-37	45-49
Staphylococcus aureus	5-10	35-40	44-48
Clostridium perfringens	12-20	30-47	45-51
Clostridium botulinum (proteolytic strains)	12.5	35	50
Campylobacter jejuni	30	42-45	47
Vibrio parahaemolyticus (mesophilic strains)	13	35-37	42-44
Vibrio cholerae	10	37	43
Bacillus cereus (mesophilic strains)	10-15	35-40	47-55
Shigella	7	37	45-47

From 'Factors affecting the growth of microorganisms in food' by Hamad (2012) in R. Bhat, A. K. Alias, & G. Paliyath (Eds.), *Progress in Food Preservation* (p. 419). West Sussex, UK: John Wiley & Sons Ltd. Copyright 2012 by John Wiley & Sons Ltd. Adapted with permission.

Table 12. Growth temperatures for psychrotropic pathogens including *B. cereus* (psychrotrophic strains), *E. coli* and *S. aureus.*

Micro-organism	Minimal (ºC)	Optimum (ºC)	Maximum (ºC)
Bacillus cereus (psychrotrophic strains)	4-5	28-35	30-35
Yersinia enterocolitica	-1 to 4	28-30	37-42
Listeria monocytogenes	0-4	30-37	45
Aeromonas hydrophila	0-4	28-35	42-45
Clostridium botulinum (non-proteolytic strains)	3.0-3.3	30	45
Vibrio parahaemolyticus (psychrotrophic strains)	3-5	30-37	40-42

From 'Factors affecting the growth of microorganisms in food' by Hamad (2012) in R. Bhat, A. K. Alias, & G. Paliyath (Eds.), *Progress in Food Preservation* (p. 419). West Sussex, UK: John Wiley & Sons Ltd. Copyright 2012 by John Wiley & Sons Ltd. Adapted with permission.

2.5.2.2 Implicit factors

These factors are those related to the pathogens themselves (Hamad, 2012). Generally, pathogens have certain requirements for survival. Some pathogens grow faster than others and deplete the nutrients in a food matrix which are necessary for the growth of other bacteria while others grow slower and are not able to utilize the nutrients efficiently. For example, coliforms generally grow more rapidly than Staphylococci, depriving them of amino acids and thus prohibiting their growth. Some pathogens are better at tolerating

stress like low water activity, high acidic conditions, high or low temperature and the presence or absence of oxygen (Hamad, 2012).

2.5.2.3 Stress adaptation and acid tolerance response (ATR)

Stress refers to "any deleterious factor or condition that adversely affects microbial growth or survival" including high and low temperatures, low pH, preservatives and others that exist during food production, processing, storage and distribution. Pathogens can sense their surroundings and then respond by adapting to the stress through the production of proteins to repair the damage or remove the stress agent. Pathogens develop tolerance and may be able to survive under lethal conditions. This phenomenon is called stress adaptation or stress hardening (Yousef & Courtney, 2003).

The stress adaptation in acidic conditions plays an important role in the survival and growth of the pathogens in fermented food. Acid tolerance response (ATR) is the most studied stress adaptation (Begley & Hill, 2015). When bacteria are exposed to mildly acidic conditions with low pH, changes in gene expression and protein synthesis make them more tolerant to extremely acidic conditions with low pH. Sub lethal stress results in a stress response that protects against subsequent exposure to lethal stress. This stress response also enhances virulence. This phenomenon is known as ATR (Horn & Bhunia, 2018; Yousef & Courtney, 2003). ATR is likely to contribute to the rising number of foodborne illnesses (Horn & Bhunia, 2018) as the stress-adapted pathogens are likely to survive the normally lethal stresses during food production, storage, distribution, and preparation for consumption (Begley & Hill, 2015; Yousef & Courtney, 2003). The ATR of the bacterial pathogens commonly found in milk products has been studied. For example, an increased ATR for *E. coli* O157:H7 was induced at 4.0 – 5.5 and the maximum ATR was induced at pH 5. 0 (Koutsoumanis & Sofos, 2004).

2.6 pH and acidity of NFRM in Bhutan

The pH of NFRM in Africa has been reported between 3.4 to 4.6 (Akabanda et al., 2010; Beukes et al., 2001; Okiki et al., 2018; Okonkwo, 2011). The pH of NFRM of different places of Bhutan, Eastern Nepal and Darjeeling hills, Sikkim in India has been reported at 4.2 ± 0.3 (Dewan & Tamang, 2007) and the pH of NFRM in Bhutan in another report was 3.7 ± 0.17 (Shangpliang et al., 2017). Although these results were generated from only ten and four NFRM samples respectively, they provide a rudimentary data on pH in NFRM in Bhutan. No data is available on the acidity of NFRM.

2.7 Detection of *B. cereus, E. coli* and *S. aureus* by PCR

Conventional broth or agar culture methods have widely been adopted as the standard methods for the detection of pathogens in food. These methods identify and confirm pathogens based on culturing on selective media followed by various chemical tests and immunological assays (Quigley, McCarthy, et al., 2013). These methods are cheap, sensitive and provide both the quantitative (number) and qualitative (type) of the pathogen (Zhao, Lin, Wang, & Oh, 2014). However, these methods require several days to obtain results, are laborious and at times inconclusive (Quigley et al., 2011).

To supplement or replace the conventional methods, more advanced, rapid and sensitive methods have been developed (Zhao et al., 2014) including the novel molecular techniques that are based on the DNA analysis of the pathogens in food. PCR, real-time quantitative PCR (qPCR) and multiplex PCR are some of the DNA-based pathogen detection assays (Chiang et al., 2012). These methods are highly specific and rapid (Zhao et al., 2014). PCR has been recognized as "one of the most promising rapid microbiological methods for the detection and identification of bacteria in a wide range of foods" (Cancino-Padilla, Fellenberg, Franco, Ibáñez, & Vargas-Bello-Pérez, 2017). It has been widely used to detect *E. coli* O157:H7, *Salmonella, L. monocytogenes* and *S. aureus* in food (Cancino-Padilla et al., 2017). PCR is also combined with traditional methods to confirm the presence of pathogens. For example, PCR was used to confirm an MPN test for *L. monocytogenes* in raw and RTE foods in Malaysia (Marian et al., 2012).

The conventional methods are also culture-dependent. Thus, pathogens present at subdominant level or which are not able to grow easily in the laboratory may not be detected and identified. Therefore, culture-independent methods which are DNA-based like the HTS are used which provide a complete bacterial profile of the food analysed. HTS is a novel and an advanced sequencing technology that rapidly sequences hundreds to millions of DNA molecules of the bacterial species per sample (Sekse et al., 2017). HTS provides a "more in-depth insight into the diversity and dynamics of entire microbial communities" (Quigley, McCarthy, et al., 2013). This technology has been used for the study of the bacterial profile of milk (Quigley, McCarthy, et al., 2013). In their study, HTS identified several bacterial genera for the first time in RM (Quigley, McCarthy, et al., 2013). Recently, it was also used to study the bacterial community of NFRM products including dahi (Shangpliang et al., 2018). In this study, PCR will be used to confirm the

presence of *B. cereus*, *E. coli* and *S. aureus* and the HTS will be used to study the overall bacterial community of NFRM in Bhutan.

CHAPTER 3. MATERIALS AND METHODS

3.1 Materials used

The following materials and media were used.

3.1.1 Bacterial profile analysis

- Presto™ Mini gDNA Bacteria Kit (Geneaid)
- Primer 27F
- Primer 1492R
- Colibri Microvolume Spectophotometer (Titertek Berthold)
- Pro Flex PCR system (Applied biosystems)
- 1 kb DNA ladder (Biolabs Inc.)
- E-Gel® EX 2 % Agarose (Invitrogen)
- E-Gel®iBase™ Power System (Invitrogen)

3.1.2 *E. coli, B. cereus* and *S. aureus* enumeration

3.1.2.1 Sample preparation and serial dilutions

- GranuCult™ Buffered peptone water (BPW) (Merck KGaA), 225 ml bottles and 9 ml tubes
- BagMixer Smasher stomacher (AES Laboratories)

3.1.2.2 *B. cereus* enumeration

- Mannitol Egg Yolk Polymyxin (MYP) agar (Fort Richard Laboratories)
- Incubation chamber set to 30 ºC
- BHI agar
- BHI broth

3.1.2.3 *E. coli* enumeration (MPN method)

- BBL™ Lauryl Sulphate Tryptose broth with MUG (Becton, Dickison and company),10 ml tubes with Durham tubes
- *E.coli* (EC) broth with MUG (Oxiod),10 ml tubes with Durham tubes
- Eosin Methylene Blue (EMB) agar (Merck Merck KGaA)
- Difco™ Brain Heart Infusion (BHI) agar (Becton, Dickison and company)
- Bacto™ Brain Heart Infusion broth (Becton, Dickison and company), 5 ml bottles

- Incubation chamber set to 37 ºC
- Water bath (Lauda Alpha) set to 42 ºC

3.1.2.4 *S. aureus* enumeration

- Baird Parker (BP) agar (Fort Richard Laboratories)
- Incubation chamber set to 37 ºC
- BHI agar
- BHI broth

3.1.2.5 Molecular identification

- QIAamp BiOstic Bacteremia DNA Kit (Qiagen)
- Species specific primers
- RNAse/DNase free water (Invitrogen)
- Platinum Green (2XMM) Master mix (Invitrogen)
- 1 kb DNA ladder (Biolabs Inc.)
- Colibri Microvolume Spectophotometer (Titertek Berthold)
- Pro Flex PCR system (Applied Biosystems)
- E-Gel® EX 2 % Agarose (Invitrogen)
- E-Gel®iBase™ Power System (Invitrogen)

3.1.3 Chemical analysis

3.1.3.1 pH

- Pocket Pro+ pH meter (Hach)

3.1.3.2 Acidity

- 0.1 N sodium hydroxide solution
- Phenolphthalein indicator

3.2 Methods

3.2.1 Survey areas

The survey areas were Thimphu city (Thimphu district), Phuntsholing city (Chukha district) and the Thimphu-Phuntsholing national highway in Bhutan (NH) (Figure 8). Along the NH, the products were available in Gedu (Chukha district) only.

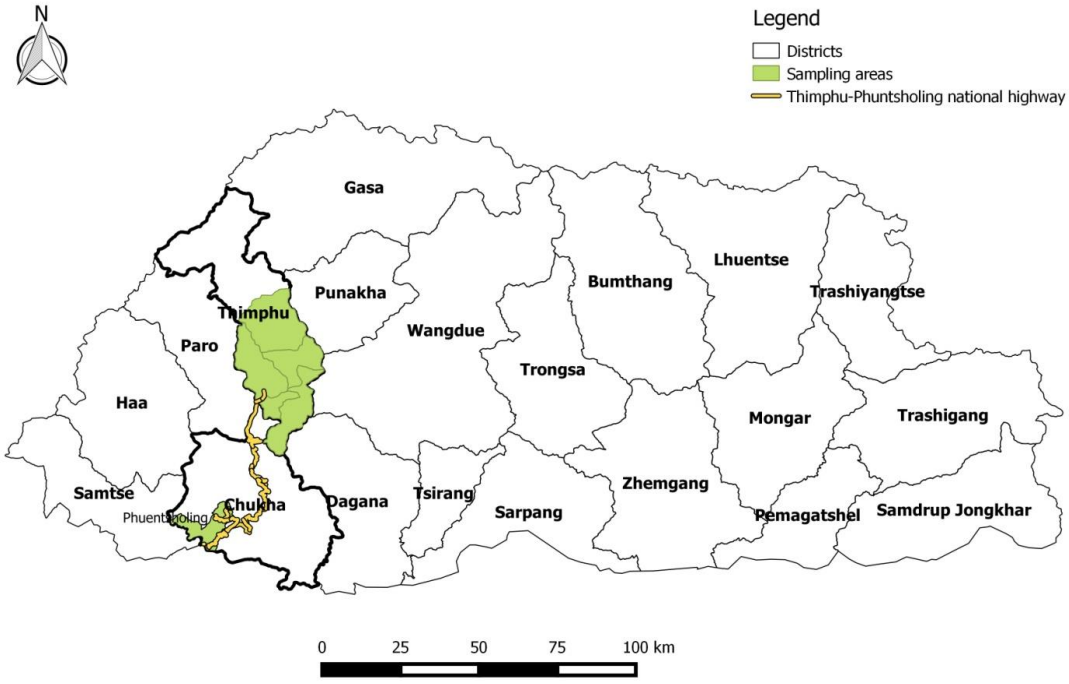


Figure 8. Map of Bhutan showing the survey areas

3.2.2 NFRM production

There are several dairy outlets in Thimphu and Phuntsholing where NFRM is processed and sold. RM is collected from the dairy farmers and delivered to these outlets by the milk vendors. The milk vendors take up to an hour to gather RM from the individual farmers and pool together in milk cans each day. They deliver a major portion of RM to Thimphu outlets from Paro which is located 1.5 h from Thimphu. Farmers in Phuntsholing live within 1 h from the milk outlets. There are no cold chain facilities during the collection and transportation of RM. Then the RM is filled into PET bottles (Figure 9) and left at ambient temperatures at 8 ºC to 10 ºC for spontaneous fermentation for up to 3 days in Thimphu and Gedu. In Phuntsholing, the RM is fermented at 20 ºC for less than a day. NFRM is then stored under refrigeration at 6 ºC to 7 ºC for selling in Thimphu and Phuntsholing. In Gedu, the dairy farmers themselves produce the RM and process NFRM. They sell NFRM in temporary milk stalls without refrigeration (Figure 10).

Figure 9. PET bottles used for NFRM fermentation and marketing

Figure 10. (a) Milk outlets in Thimphu and Phuntsholing, (b) Milk stalls in Gedu

3.2.3 Sources selection

All the milk outlets and stalls selling NFRM in PET bottles were selected. There were 9 outlets in Thimphu, 6 outlets in Phuntsholing and 4 stalls in Gedu (Table 13).

3.2.4 Sample collection

From each source, 2 sample bottles were purchased between 10 am to 1 pm on 3 alternative days during October - November, 2018. A total of 114 samples were purchased.

From each sample bottle, 50 ml was aseptically collected into sterile containers, sealed, labelled and immediately frozen at -20 ºC. The samples were then packed in double-layered ice-gel packs in a thermocol box for shipping to the microbiology laboratory at the School of Food and Advanced Technology, Massey University, Palmerston North for analysis. The samples were kept frozen at -20 ºC until further analysed.

Some researchers do not recommend freezing and storing milk samples before studying the bacterial counts for risk of misdiagnosis since the bacterial counts are adversely affected after freezing. When raw milk samples were frozen at -18ºC, *E. coli* counts significantly declined over time while *S. aureus* counts increased after 7 days of storage and decreased thereafter. However, the decrease in *S. aureus* counts was not significant after 21 days of storage (Alrabadi, 2015; Hubáčková & Ryšánek, 2007). Therefore, in the present study, *E. coli* counts may be lower than the actual counts in NFRM at the time of consumption.

Before the samples were brought to Massey, a trial was done to determine that the milk samples remained frozen for 24 h to enable them to reach the Massey University laboratory without any quality deterioration.

Table 13. Sample details

Place	Outlets/ stalls (nos)	Samples (nos)
Thimphu	9	54
Phuntsholing	6	36
National highway	4	24
Total	19	114

3.2.5 Bacterial profile analysis by HTS

3.2.5.1 Sample preparation

Samples were thawed at room temperature and mixed thoroughly. Nine pooled milk samples were prepared in sterile containers by aseptically transferring 2 g of the samples from each day of collection from each location and labelled accordingly (Table 14).

Table 14. Pool sample details

Sample name	Place	Collection day
Tphu1	Thimphu	First
Tphu2	Thimphu	Second
Tphu3	Thimphu	Third
Pling1	Phuntsholing	First
Pling2	Phuntsholing	Second
Pling3	Phuntsholing	Third
Nh1	NH	First
Nh2	NH	Second
Nh3	NH	Third

3.2.5.2 DNA extraction and PCR amplification

Genomic DNA was extracted from the pooled samples using the Presto™ Mini gDNA Bacteria Kit as per the supplier's protocol (Geneaid Biotech Ltd, 2019). The DNA quantity was measured with a spectrophotometer and was observed to be more than 30 ng/µL for all DNA samples. The DNA samples were then sent to Novogene (HK) Co., Ltd, China for 16s rRNA high throughput amplicon sequencing based on the IonS5^{TM} XL platform and for bioinformatics services.

The company assigned the sequences with ≥ 97 % sequence similarity to the same Operational Taxonomic Units (OTUs) and performed the OTUs clustering and filtering. All the species were annotated at each taxonomic rank (kingdom, phylum, class, order, family, genus, and species) along with their relative abundances and were presented in taxonomy trees and histograms. Alpha-diversity was analysed using rarefaction curve, Chao-1 index, Shannon index, Goods coverage and rank abundance curve, and beta-diversity was analysed using Principal Component Analysis (PCA), Principal Co-ordinate Analysis) and unweighted pair-group methods with arithmetic means (UPGMA). The multivariate analysis was performed by Analysis of Molecular Variance (AMOVA).

3.2.6 *B. cereus, E. coli* and *S. aureus* enumeration

3.2.6.1 Sample preparation

Tenfold serial dilutions (1:10) were prepared according to Midura and Bryant (2001). The first dilution (10^{-1}) was prepared by adding 25 g of the sample to 225 ml of sterile buffered peptone water. It was then homogenised in a sterile stomacher plastic bag in the stomacher for 45 seconds. Up to three serial dilutions were prepared by transferring 1 ml from a subsequent dilution to 9 ml BPW.

3.2.6.2 *B. cereus*

The enumeration was performed according to the plate count method of the APHA as described by Bennett and Beley (2001) with modification.

Inoculation: 0.1 ml of the first, second and third dilutions was spread on the MYP agar plates in duplicate.

Incubation and colony count: The MYP plates were incubated aerobically at 30 °C for 24 h and examined for typical *B .cereus* colonies (pink to violet colonies surrounded by a typical lecithinase reaction band of precipitate). Colonies were counted for plates containing 10 - 100 colonies.

Confirmation: Two typical *B. cereus* colonies from each MYP agar plate were streaked onto BHI agar plate and incubated at 30 °C for 24 h. A single colony was grown in BHI broth for 18 h for DNA extraction and confirmation by PCR.

3.2.6.3 *E. coli*

The enumeration was performed according to the Most Probable Number (MPN) method of the American Public Health Association (APHA) as described by Swanson, Petran, and Hanlin (2001) with modification.

Three 1 ml of the first, second and third dilutions were inoculated into three test tubes containing 10 ml LST (single strength) broth.

Presumptive test: The LST tubes were incubated at 37 °C for 24 h and examined for the turbidity increase of the broth and gas production indicated by a bubble in the Durham tube. Negative tubes were re-incubated and examined after 48 h.

Confirmed test (1): A loopful of suspension from each presumptive positive culture was transferred to 10 ml EC broth tubes and incubated at 44 °C for 24 h. The tubes were examined for turbidity increase and gas production.

Confirmation test (2) as described by Kornacki and Johnson (2001): A loopful of suspension from each EC positive culture was streaked onto EMB agar plates and incubated at 37 °C for 24 h. The plates were examined for suspicious *E. coli* colonies (nucleated, dark centered colonies with or without green sheen).

Confirmation test (3): Two suspicious *E. coli* colonies from each EMB agar plate were streaked onto BHI agar and incubated at 37 °C for 24 h (Figure 11). A single colony was grown in BHI broth for 18 h for DNA extraction and confirmation by PCR.

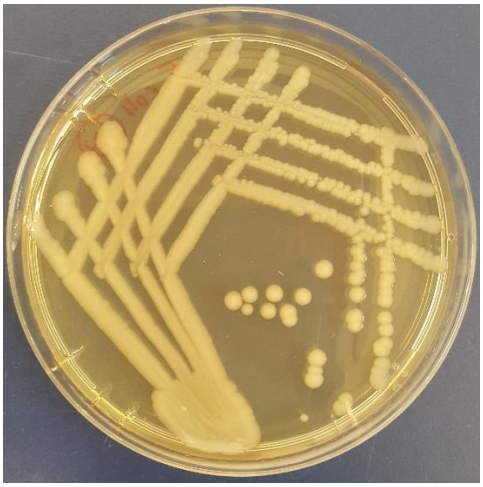


Figure 11. Single colonies of suspicious E. coli on BHI agar

***E .coli* MPN calculation**: An MPN table was used to determine the *E. coli* MPN.

3.2.6.4 *S. aureus*

The enumeration was performed according to the plate count method of the APHA as described by Lancette and Bennett (2001) with modification.

Inoculation: 0.1 ml of the first, second and third dilutions was spread on the BP agar plates in duplicate.

Incubation and colony count: The BP plates were incubated aerobically at 37 °C for 48 h and examined for typical *S. aureus* colonies (small, maximum 2 - 3 mm diameter, black or grey, circular, smooth, 2 - 3 mm diameter with an opaque halo frequently surrounded by an outer clear zone (lipolytic strains), and without the halo and clear zones (non-lipolytic strains)). Colonies were counted for plates containing 20 - 200 colonies.

Confirmation: Two typical *S. aureus* colonies from each BP agar plate were streaked onto BHI agar plate and incubated at 37 °C for 24 h (Figure 12). A single colony was grown in BHI broth for 18 h for DNA extraction and confirmation by PCR.

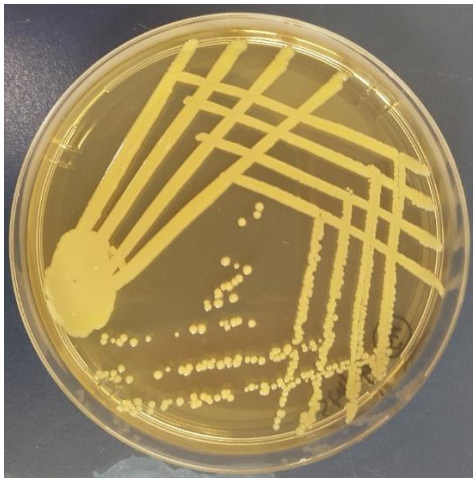


Figure 12. Single colonies of suspicious *S. aureus* on BHI agar

3.2.6.5 DNA extraction

The DNA was extracted from the BHI broths using the QIAamp BiOstic Bacteremia DNA Kit as per the supplier's protocol (Qiagen, 2019).

3.2.6.6 PCR amplification

The DNA sample (3 µL) was mixed into a 50 µL reaction mixture which contained 32.5 µL RNA/DNAase free water, 12.5 µL master mix, and 1 µL each of the reverse and forward primers. The primers used were species-specific (Table 15). The reaction mixture was subjected to PCR amplification cycles set according to the references (Table 16).

Table 15. Species specific primer details

Bacteria	Primer	Sequence (5'- 3')	Reference
B. cereus	BCFomp2	-CGCCTCGTTGGATGACG-	Oliwa-Stasiak, Kolaj-Robin, and Adley (2011)
	BCRomp2	-GATATACATTCACTTGACTAATACCG-	
E. coli	ECA75F	-GGAAGAAGCTTGCTTCTTTGCTGAC-	Sabat, Rose, Hickey, and Harkin (2000)
	ECR619R	-AGCCCGGGGATTTCACATCTGACTTA-	
S. aureus	Primer 1	-GCGATTGATGGTGATACGGTT-	Brakstad, Aasbakk, and Maeland (1992)
	Primer 2	-AGCCAAGCCTTGACGAACTAAAGC-	

Table 16. PCR amplification protocols

Bacteria	Start	Cycles	Denaturation	Annealing	Extension	Final extension	References
B. cereus	94°C, 5 min	35	95°C, 10 sec	59°C, 40 sec,	72°C, 1 sec	72°C, 7 min	Oliwa-Stasiak et al. (2011)
E. coli	94°C, 5 min	40	94°C, 45 sec	50°C, 245 sec	72°C, 1.5 min	72°C, 5 min	Sabat et al. (2000)
S. aureus	94°C, 5 min	37	94°C, 1 min	55°C, 30 sec	72°C, 1.5 min	72°C, 3.5 min	Brakstad et al. (1992)

3.2.6.7 Agarose gel electrophoresis

The PCR product (10 µL each) was loaded into a 2 % agarose gel. The E-Gel Electrophoresis system was run for 15 mins and observed for the presence of DNA fragments as bands in the gel.

3.2.7 Laboratory trials

Before the sample analysis, the DNA extraction kit and species-specific primers were tested in the laboratory. Positive cultures of *B. cereus*, *E. coli* and *S. aureus* from the laboratory stocks were grown in BHI broths and DNA was extracted using the kit. The quantity of the DNA was measured in the spectrophotometer. The reaction mixture was prepared with the DNA and the species-specific primers. Then PCR was run at the specific thermal cycles. The gel electrophoresis was run for 15 mins.

The yield of all extracted DNA was more than 20 ng/µL and DNA fragments were observed as bands in the gel for all the PCR products.

3.2.8 Chemical analysis

3.2.9 pH

10 ml of the sample was aseptically transferred to a 25 ml glass beaker and the pH was measured using a portable pH meter.

3.2.10 Acidity

10 ml of the sample was aseptically transferred to a 25 ml glass beaker and titrated against 0.1 N sodium hydroxide using the phenolphthalein indicator to an endpoint of pH 8.3 (Troller & Scott, 1992).

The acidity was calculated as:

$$\% \text{ LA} = \frac{\text{No. of 0.1 N NaOH solution required for neutralization}}{\text{Weight of the sample}} \times 100$$

3.2.11 Statistical analysis

The statistical analysis was conducted in R (R Core Team, 2017) and Microsoft excel to calculate the percentage of samples positive for the bacteria and determine the mean values of the viable counts, pH and acidity.

CHAPTER 4. RESULTS

4.1 Bacterial profile analysis using HTS

4.1.1 Overall bacterial abundance and diversity

A total of 694,862 high-quality bacterial sequences reads were generated from the 9 pooled NFRM samples with an average of 77207 sequences read per sample. The total number of unique and classifiable representative OTU was 795 (average= 88 OTUS ± 12.5 per sample, range = 67 - 106) with a high threshold of ≥ 97 % sequence similarity level.

The Good's coverage indicated good completeness of sampling with levels of 99.9 to 100 % (Table 17).The rarefaction graphs did not reach the saturation phase which indicated that new phylotypes were likely to be identified with additional sequencing. However, the Shannon diversity curves for all the samples reached the saturation phase and indicated that most of the phylotypes present in NFRM had already been captured in the current analysis. The alpha diversity analysis indicated abundant microbial richness (Chao 1 value and observed species value) and high diversity (Shannon index value) in all NFRM samples. The samples of Phuntsholing had the richest bacterial community and the Thimphu samples had the most diverse bacterial community.

Table 17. Alpha diversity details

Sample	Sampling location	Number of OTUs	Observed species	Shannon index	Simpson index	Chao1 index	Goods cover age
NH3	NH	97.0	78.0	3.3	0.9	106.9	0.999
NH2		67.0	60.0	2.7	0.8	70.5	0.999
NH1		84.0	67.0	3.1	0.8	86.1	0.999
Mean (NH samples)		82.7 ± 15.04	68.3 ± 9.07	3.0 ± 0.33	0.8 ± 0.04	87.8 ± 18.25	
Pling3	Phuntsholing	77.0	69.0	2.9	0.8	78.0	1
Pling2		100.0	91.0	3.5	0.9	96.4	1
Pling1		106.0	93.0	3.7	0.9	100.0	0.999
Mean (Pling samples)		94.3 ± 15.31	84.3 ± 13.32	3.4 ± 0.39	0.9 ± 0.04	91.5 ± 11.79	
Tphu3	Thimphu	96.0	80.0	3.6	0.9	87.0	1
Tphu2		88.0	77.0	3.6	0.9	96.0	0.999
Tphu1		80.0	72.0	3.7	0.9	72.5	1
Mean (Tphu samples)		88.0 ± 8.00	76.3 ± 4.04	3.6 ± 0.07	0.9 ± 0.003	85.2 ± 11.86	

4.1.2 Bacterial composition of NFRM

The bacteria present in NFRM were classified at different taxonomic levels from the phylum to the genus (Appendix B). A total of 8 phyla were identified. Overall, the predominant phyla were Firmicutes (93.80 %) and Proteobacteria (6.20 %). Phylum Firmicutes was represented by 16 families dominated by *Streptococcaceae, Lactobacillaceae, Enterococcaceae, Leuconostocaceae,* and *Staphylococcaea.* Phylum Proteobacteria included 12 families dominated by *Aeromonadaceae, Moraxellaceae, Enterobacteriaceae, Pseudomonadaceae,* and *Acetobacteraceae.* Other phyla identified were Bacteriodetes, Acitenobacteria, Fusobacteria, Tenericutes, Peregrinibacteria, and Saccharibacteria (Figure 13).

At the genus level, a total of 55 bacterial genera were identified. 25 genera belonged to the phylum Firmicutes and this was predominated by *Lactococcus* (91.31 %) which also predominated the whole bacterial diversity. 17 genera belonged to phylum Proteobacteria and this was predominated by *Aeromonas* (4.71 %). Other genera belonging to Firmicutes (*Streptococcus, Lactobacillus, Enterococcus,* and *Leuconostoc)* and Proteobacteria (*Acinetobacter, Cedecea, Pseudomonas* and *Escherichia-Shigella)* were identified in the top 10 phyla (Figure 14).

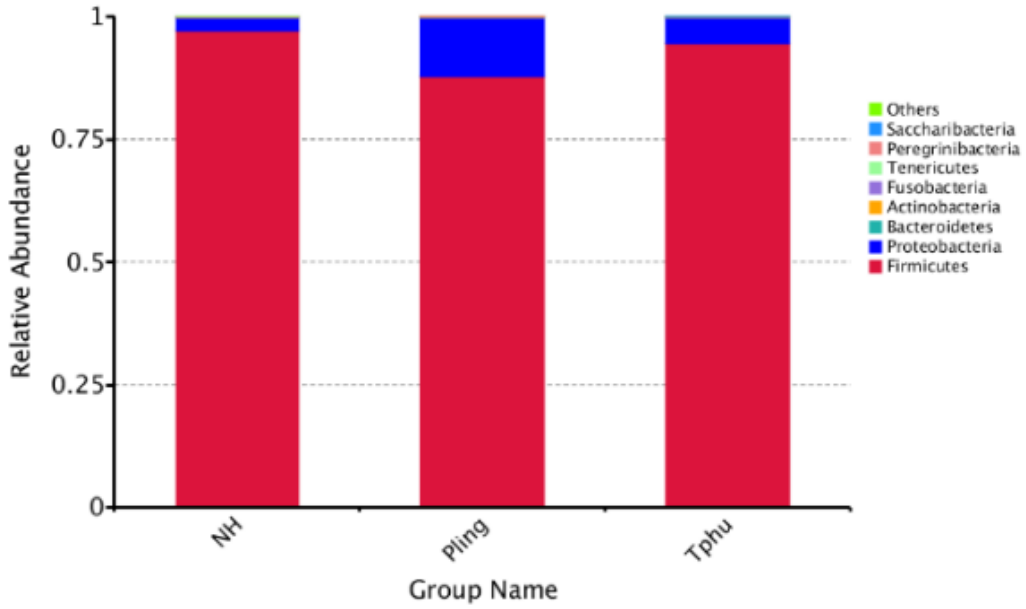


Figure 13. Top 10 phyla identified by HTS

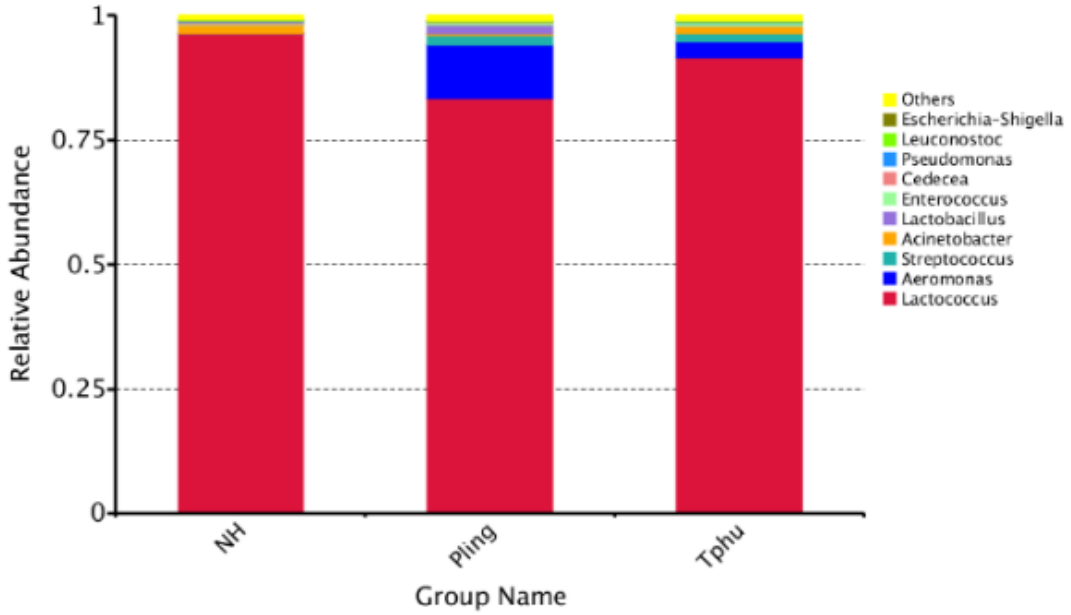


Figure 14. Top 10 genera identified by HTS

4.1.2.1 LAB

LAB species belonging to *Lactobacillus* genus (*Lb. brevis* and *Lb. casei*), *Lactococcus* species (*L. lactis* and *Lactococcus raffinolactis*), *Leuconostoc* (*Leuconostoc mesenteroides)* and *Streptococcus species* (*S. agalactiae* and *S. parauberis*) were identified in all NFRM.

4.1.2.2 Potential pathogens

Genera that may have pathogenic species including *Enterococcus, Escherichia-Shigella, Staphylococcus* and *Vibrio* were identified. At the species level, a presumptive identification of pathogens including *B. anthracis, Pseudomonas aeruginosa, Serratia marcescens, Streptococcus agalactiae,* and *Streptococcus parauberis* was achieved.

4.1.3 Differences in the bacterial composition of NFRM of Thimphu, Phuntsholing and NH

4.1.2.1 Overall microbial abundance

Although the top 10 bacterial composition was similar for NFRM from all 3 places, the abundance was different at each taxonomy level (Table 18).

Table 18. Relative abundance of phyla and top 10 genera in NFRM

	Place		
Bacteria	**National highway**	**Phuntsholing**	**Thimphu**
Phylum			
Firmicutes	97.1944	87.7487	94.514
Proteobacteria	2.7207	12.031	5.3049
Bacteroidetes	0.038	0.1476	0.1588
Actinobacteria	0.0414	0.066	0.0112
Fusobacteria	0.0034	0.0022	0.0056
Tenericutes	0.0022	0.0022	0.0045
Peregrinibacteria	0	0.0022	0
Saccharibacteria	0	0	0.0011
Genus			
Lactococcus	96.4127	83.4994	91.5909
Aeromonas	0.0347	10.7697	3.1937
Streptococcus	0.0291	1.8115	1.7769
Acinetobacter	1.6751	0.5233	1.3799
Lactobacillus	0.0201	1.5834	0.0324
Enterococcus	0.303	0.5144	0.6542
Cedecea	0.2896	0.0425	0.0749
Pseudomonas	0.2069	0.0201	0.1219
Leuconostoc	0.2617	0.1946	0.2292
Escherichia-Shigella	0.0011	0.1029	0.0034
Others	0.766	0.9382	0.9427

4.1.2.3 Multivariate diversities

NFRM from the three places shared 76 core OTUS and 5, 14 and 1 OTUs were unique to NFRM of Thimphu, Phuntsholing and NH respectively (Appendix C).

AMOVA results showed that the bacterial communities differed significantly ($p < 0.05$, AMOVA test, df=6) between the three places (Table 19). On the principal coordinate (PCoA) score plot based on the weighted Unifrac distance which accounted for the 63.49 % (PC1) and 23.56 % (PC2) of the total variance, NFRM samples of Thimphu and NH were clustered to the left of the graph while the Phuntsholing samples were grouped to

the right of the graph along the first principal co-ordinate PC1. The overlapping of the samples of Thimphu and NH on the PCoA indicated that the bacterial communities of these places were relatively similar to each other while that of Phuntsholing was different (Figure 15). A similar pattern was illustrated on the UPGMA tree as well (Appendix D).

Table 19. AMOVA of differences among NFRM samples

vs_group	SS	df	MS	Fs	p-value
NH-Pling-Tphu	0.015593(0.00538395)	2(6)	0.0077965(0.000897324)	8.68861	0.004*

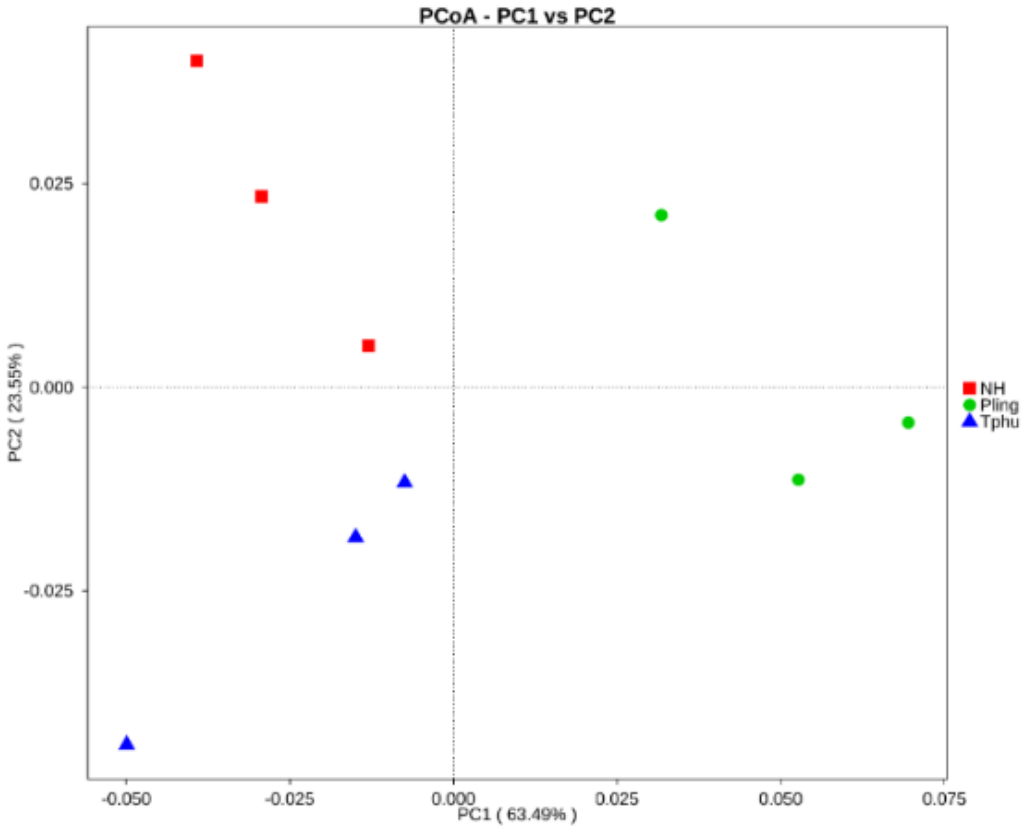


Figure 15. PCoA scores based on the relative abundances of OTUs in NFRM samples (Based on the weighted UniFrac distance).

4.2 *B. cereus*

No typical colonies of *B. cereus* (pink to violet surrounded by a band of precipitate) were observed on the MYP agar for any sample examined (Figure 16).

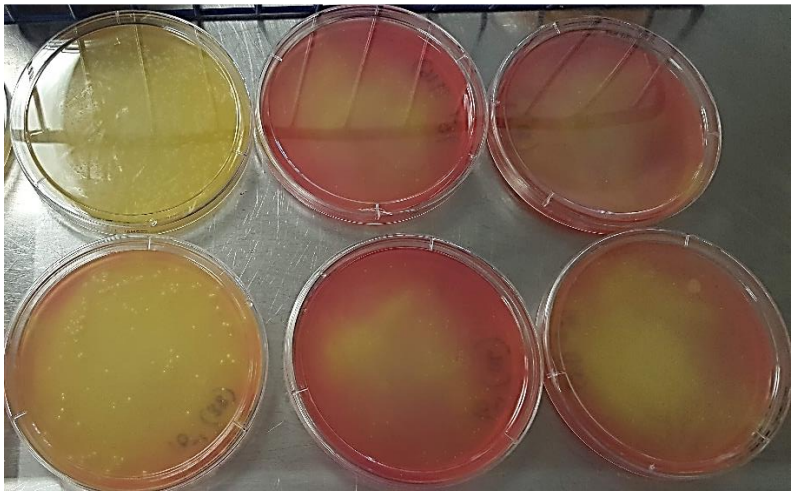


Figure 16. No typical *B. cereus* colonies observed on MYP agar

4.3 *E. coli*

4.3.1 Confirmation by PCR

The suspected *E. coli* colonies (nucleated and dark centered with a green metallic sheen) were observed on the EMB agar (Figure 17 (a)). The colonies were confirmed by the presence of DNA fragments amplified by species-specific PCR, visualised as bands in the agarose gel (Figure 17 (b)).

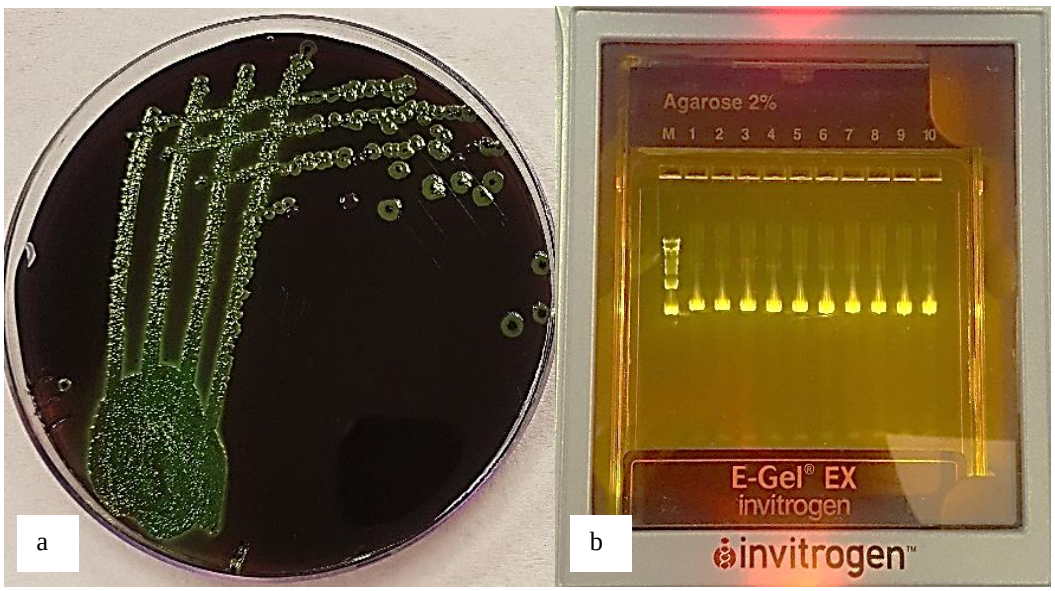


Figure 17. (a) *E. coli* colonies on EMB agar and (b) *E. coli* bands on agarose gel.
From left to right: M (Ladder), 1-10 (sample DNAs)

4.3.2 Prevalence and MPN estimates

Overall, *E. coli* was present in 90.4 % of NFRM samples. The percentage of NFRM that tested positive for *E. coli* and percentage of NFRM containing *E. coli* based on MPN estimates in Thimphu, Phuntsholing and NH are presented in Table 20. *E. coli* MPN estimate results were categorised according to the FSANZ (2018a) limits for *E. coli* in RTE foods as satisfactory (containing < 0.48 $\log_{10}$ cfu/ml *E. coli*), marginal (containing 0.48 – < 2 $\log_{10}$ cfu/ml *E. coli*) and unsatisfactory (containing > 2 $\log_{10}$ cfu/ml *E. coli*). 70 %, 75 % and 63 % of NFRM in Thimphu, Phuntsholing and NH were unsatisfactory respectively (Figure 18).

Table 20. *E. coli* prevalence and counts based on MPN estimates

Place	**Prevalence (% samples positive/samples tested)**	**95 % CI**	**Samples (% samples positive/samples tested) containing $\log_{10}$ cfu/ml *E. coli* counts based on MPN estimates**		
			< 0.48 $\log_{10}$ cfu/ml	0.48-3.04 $\log_{10}$ cfu/ml	> 3.04 $\log_{10}$ cfu/ml
Thimphu	90.7 (49/54)	79.7 - 97.0	9	52	39
Phuntsholing	91.7 (33/36)	77.5 - 98.2	8	36	56
NH	87.5 (21/24)	67.4 - 97.3	13	46	42
Overall	90.4 (103/114)	83.4 - 95.1	10	46	45

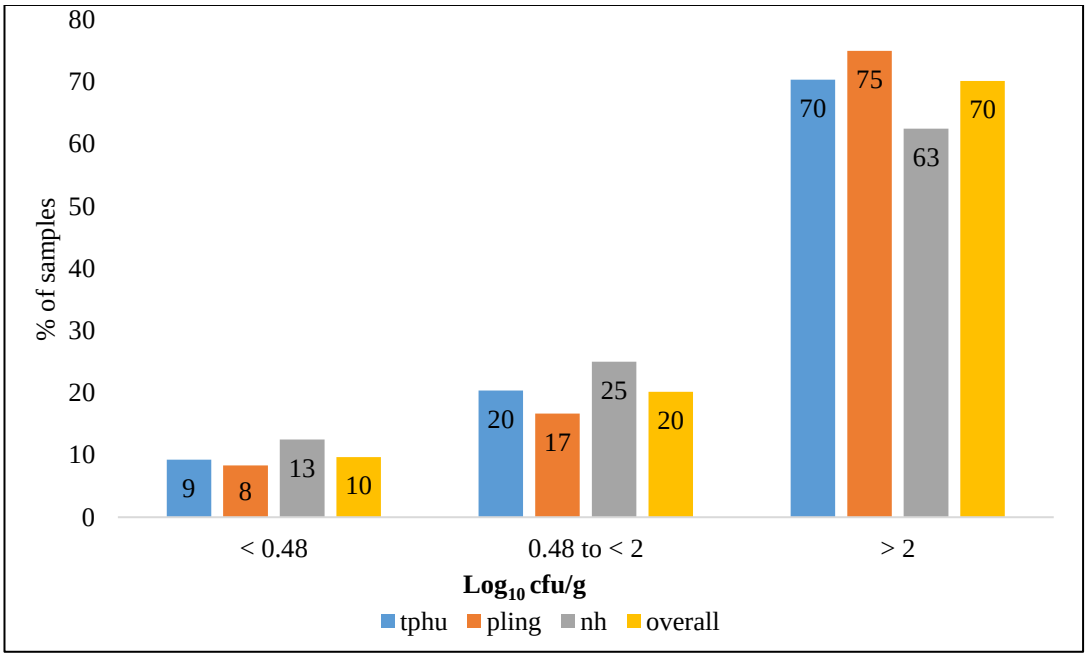


Figure 18. % of NFRM samples with *E. coli* counts categorised according to the FSANZ limits

4.4 *S. aureus*

4.4.1 Confirmation by PCR

Typical colonies of *S. aureus* (small and black with an opaque halo, frequently surrounded by an outer clear zone) were observed on the BP agar (Figure 19 (a) and (b)). The colonies were confirmed by the presence of DNA fragments from species-specific PCR, visualised as bands in the agarose gel (Figure 19 (c)).

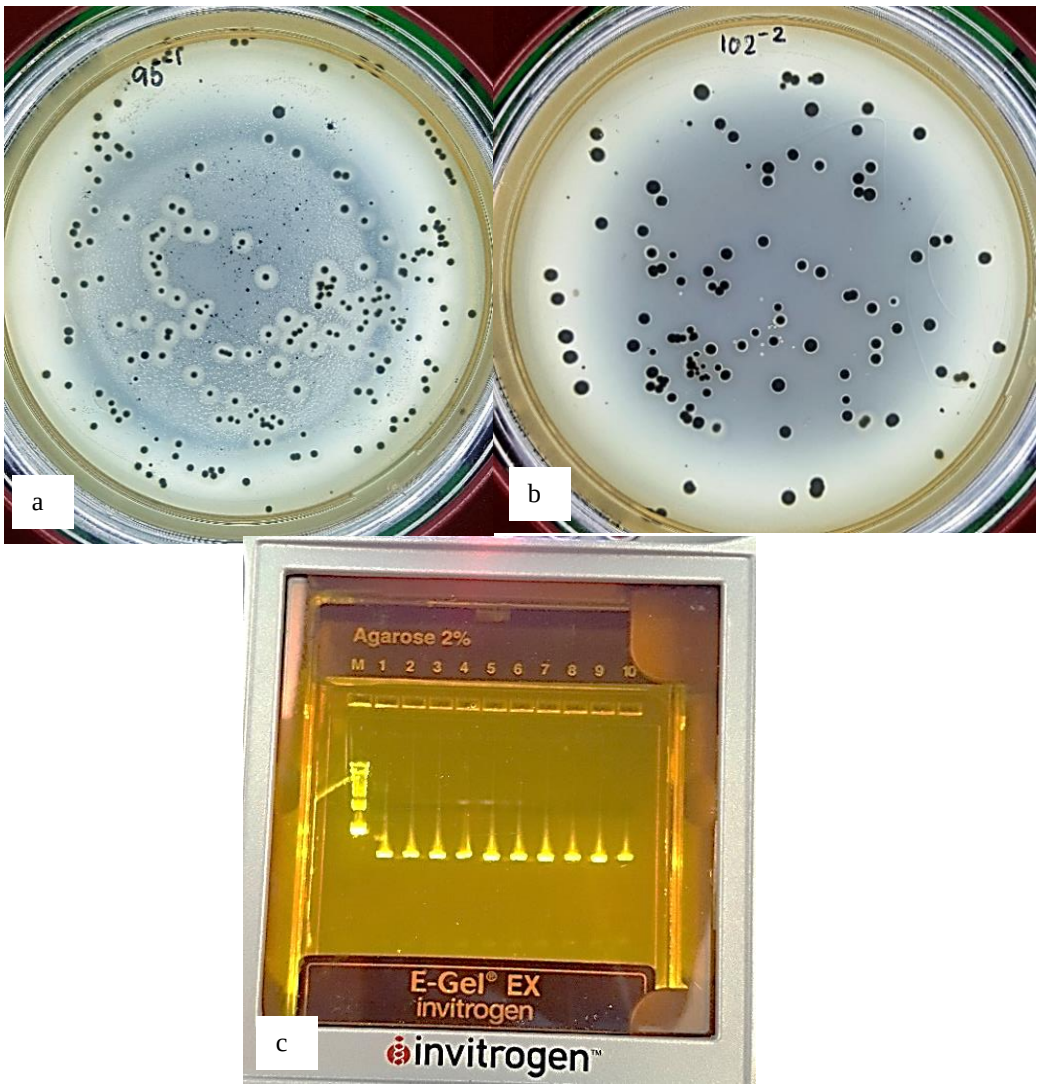


Figure 19. (a) *S. aureus* with clear zones (lipolytic and proteolytic strains), (b) *S. aureus* colonies without clear zones (non-lipolytic strains) and (c) *S. aureus* bands on agarose gel.

From left to right: M (Ladder), 1 - 10 (sample DNAs)

4.4.2 Prevalence and viable counts

S. aureus was present in all the samples examined. Overall, the mean count was 4.18 ± 0.67 $\log_{10}$ cfu/ml and ranged from 1.95 to 5.41 $\log_{10}$ cfu/ml (Table 21).

S. aureus count results were categorised according to the FSANZ (2018a) limits for *S. aureus* in RTE foods as satisfactory (< 2 $\log_{10}$ cfu/ml *S. aureus*), marginal (2 – 3 $\log_{10}$ cfu/ml *S. aureus*), unsatisfactory (3 - ≤ 4 $\log_{10}$ cfu/ml *S. aureus*) and potentially hazardous (> 4 $\log_{10}$ cfu/ml *S. aureus*). 50 %, 69 % and 75 % of NFRM samples in Thimphu, Phuntsholing and NH were potentially hazardous respectively (Figure 20).

Table 21. *S. aureus* viable counts

Place	Prevalence (% samples positive / samples tested)	Minimum ($\log_{10}$ cfu/ml)	Average ($\log_{10}$ cfu/ml)	Maximum ($\log_{10}$ cfu/ml)
Thimphu	100 (54/54)	2.06	4.08 ± 0.75	5.41
Phuntsholing	100 (36/36)	1.95	4.30 ± 0.69	5.02
NH	100 (24/24)	3.29	4.22 ± 0.38	5.02
Overall	100 (114/114)	1.95	4.18 ± 0.67	5.41

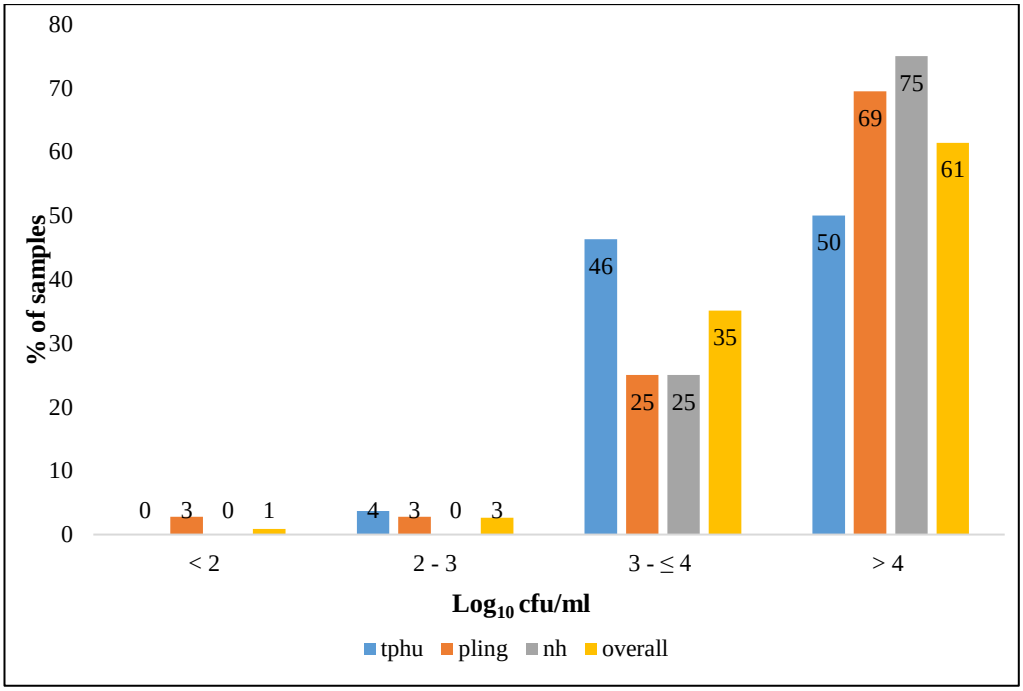


Figure 20. % of samples with *S. aureus* counts categorised according to the FSANZ limits

4.5 pH and acidity

The overall mean pH was 4.51 ± 0.20 and varied from a minimum of 4 to a maximum of 5.10. The overall mean acidity was 0.60 % ± 0.09 % LA. The mean pH (4.5 ± 0.21, 4.41 ± 0.12 and 4.66 ± 0.18) and acidity (0.54 % ± 0.07, 0.64 % ± 0.06 and 0.66 ± 0.11 % LA) for Thimphu, Phuntsholing and NH respectively were similar.

5.0 DISCUSSION

This survey was designed to study the prevalence of *B. cereus, E. coli* and *S. aureus* in NFRM in Bhutan using culture-independent and culture-dependent methods. One of the objectives was to study the bacterial profile of NFRM using HTS which is an effective tool to detect the pathogens in food. The information generated using HTS can be used to justify introducing heat treatment to reduce the risk of food poisoning and prevent FBD and outbreaks (Walsh et al., 2017). HTS identified bacteria at different taxonomy levels in NFRM. Firmicutes and Proteobacteria were the predominant phyla, as reported previously in naturally fermented products prepared from RM (Walsh et al., 2017) and boiled milk (Liu et al., 2015; Mo et al., 2019; Shangpliang et al., 2018). At the genus level, LAB belonging to *Lactococcus, Lactobacillus, Streptococcus, Enterococcus,* and *Leuconostoc* were identified, agreeing with previous research (Liu et al., 2015; Mo et al., 2019; Motato et al., 2017; Shangpliang et al., 2018; Walsh et al., 2017). These genera are the most commonly found LAB in milk as adventitious contaminants (Wouters et al., 2002). The LAB produce lactic acid from lactose during fermentation that lowers the pH which is a critical control parameter for the safety of fermented products (Adams & Nicolaides, 1997). The predominant LAB species identified in the current study were *L. raffinolactics* and *L. lactis.* The predominant LAB species identified in the other studies were *Lb. helveticus* and *Lb. delbrueckii subsp. bulgaricus* (Liu et al., 2015), *S. infantarius* and *L. lactis* (Walsh et al., 2017), *L. lactis* and *Lb. helveticus* (Shangpliang et al., 2018), and *Lb. delbrueckii* and *Lb. helveticus* (Mo et al., 2019). The relative abundance of the bacterial community at each taxonomic level also differed.

In this survey, the lack of a standard processing method with variations in fermentation temperature and time, and viscosity desired by the manufacturers may be the main causes of variations in the bacterial community seen in NFRM of Thimphu, Phuntsholing and NH. The bacterial community structures of NFRM of Thimphu and NH overlapped on the PCoA plot and UPGMA cluster tree. This similarity may be due to similar fermentation conditions at 8º C to 10 ºC fermentation temperature and fermentation period for up to 2-3 days in Thimphu and NH. In contrast, the fermentation conditions (20 ºC for less than a day) in Phuntsholing resulted in a dissimilar bacterial community and also produced the richest bacterial community and the highest number of observed species.

HTS identified *Escherichia* and *Staphylococcus* genera which was consistent with the corresponding culture-dependent analysis of NFRM samples. One of the objectives of this survey was to screen NFRM for the prevalence of *E. coli* and *S. aureus* using the MPN and a plating method respectively and confirm by PCR using the species-specific primers. However, HTS did not identify *E. coli* and *S. aureus* at the species level. This may be because the DNA used for amplification and sequencing by HTS was extracted from the pooled samples. Manter, Weir, and Vivanco (2010) reported that pooling of soil samples for PCR-based estimation of microbial richness and community structure dilutes the rare phylotypes present in the samples and results in missed detection of several bacterial phylotypes. This is because PCR is competitive in nature and does not amplify the species present in lower abundance up to the threshold levels for detection (Siebert & Larrick, 1992). This is a disadvantage in pooling several small samples into fewer large samples to study the bacterial profile of NFRM. In the current survey, since LAB are used to undertake the fermentation in the NFRM, LAB species predominated the microbial diversity. *E. coli* and *S. aureus* were present in relatively lower abundance.

Another objective was to screen NFRM for the prevalence of *B. cereus*. HTS identified the *Bacillus* genus and *B. anthracis* species. The presence of *B. anthracis* was in line with the sporadic anthrax cases reported in cattle in Bhutan (Tenzin, 2018). *B. anthracis* is closely related to *B. cereus*, *B. mycoides* and *B. thuringiensis* with 99 % 16sRNA gene similarity (Ash, Farrow, Dorsch, Stackebrandt, & Collins, 1991; Rasko et al., 2004). So, 16S PCR-based methods may fail to distinguish *B. anthracis* from the other *Bacillus* species (Kim et al., 2008). Therefore, *B. cereus* which is the most common milk-borne pathogen belonging to *Bacillus* genus and other closely related species may be present in NFRM. *Bacillus* species and *B. cereus* have previously been identified in NFRM in Africa (Adebesin et al., 2001; Obande & Azua, 2013; Obi & Ikenebomeh, 2007). In contrast, *B. mycoides* and *B. thuringiensis* have not been reported in NFRM. Although they carry the enterotoxin genes involved in food poisoning, their toxigenic potential is unclear (Johler et al., 2018; Prüß, Dietrich, Nibler, Märtlbauer, & Scherer, 1999) and they have not been associated with any milk-borne diseases and outbreaks.

In the present survey, *B. cereus* was not detected in any of the NFRM samples by the culture method. It is possible that the growth of *B. cereus* was inhibited by the bacterial competition of the dense LAB microflora and the low pH (Tirloni, Ghelardi, Celandroni, Bernardi, & Stella, 2017). A rapid decrease in the pH by the fast-acid producing LAB

like *Lb. casei* 2756 inhibits the growth of *B. cereus* (Røssland et al., 2005). *Lb. casei* was identified in NFRM by HTS that may have commonly inhibited *B. cereus* growth. Hence *B. cereus* in NFRM has only been reported by a few researchers (Adebesin et al., 2001; Obi & Ikenebomeh, 2007). Moreover, although MYP has been widely used for the enumeration of *B. cereus* in Europe and the USA (Bennett & Beley, 2001), there are issues with the MYP plates used for this purpose. MYP is not selective for the growth of *B. cereus* when competitive flora like other *Bacillus* species and *S. aureus* are present as they also produce lecithinase and ferment mannitol. The colonies of *B. cereus* coalesce and precipitation zones overlap which make the enumeration difficult (van Netten & Kramer, 1992).

PCR is more sensitive than culture methods and can detect dead cells or viable but non-cultivable cells (Postollec, Falentin, Pavan, Combrisson, & Sohier, 2011). HTS may have detected non-cultivable cells of *B. cereus* in the present survey. This is important because *B. cereus* produces pre-formed toxins in contaminated food that causes emetic *B. cereus* gastroenteritis (Griffiths & Schraft, 2017; Rajkovic, 2014). *B. cereus* may have produced the toxins in NFRM before it was inhibited by the LAB.

E. coli is a food-quality indicator of poor hygiene (Yucel & Ulusoy, 2006). Researchers have reported poor hygiene in RM and RMP based on the presence of *E. coli* (Amenu et al., 2019; Dafur et al., 2018; De Reu, Grijspeerdt, & Herman, 2004; Maikai & Madaki, 2018; Okonkwo, 2011; Yucel & Ulusoy, 2006). In this survey, *E. coli* was present in 90 % of NFRM samples. *E. coli* has been commonly isolated from NFRM in other developing countries in Africa. However, the prevalence reported was much lower (9 % to 66.7 %) (Akabanda et al., 2010; Amenu et al., 2019; Dafur et al., 2018; Maikai & Madaki, 2018; Nzabuheraheza & Nyiramugwera, 2016; Okonkwo, 2011; Yakubu et al., 2018) compared with the present survey which implies that the hygiene quality of NFRM in Bhutan is poorer than many other developing countries. It also implies that the hygiene measures, if any, employed during the production and processing of NFRM are ineffective in Bhutan (FSANZ, 2018a). The *E. coli* prevalence in the present survey is comparable to 81 % (Gran et al., 2002) and 100 % prevalence (Gran et al., 2003) in Zimbabwe.

The *E. coli* load was high with 45 % of NFRM samples containing more than 3.04 $\log_{10}$ cfu/ml *E. coli* based on MPN estimates. The mean *E. coli* counts of 1.8 ± 2.1 $\log_{10}$ cfu/ml

ranging from 0 to 5.1 $\log_{10}$ cfu/ml (Amenu et al., 2019), 2.21 $\log_{10}$ cfu/ml ranging from 0 to 7.89 $\log_{10}$ cfu/ml (Okonkwo, 2011) and 3.56 ± 4.10 $\log_{10}$ cfu/ml ranging from 2.49 ± 3.86 to 4.66 ± 4.41 $\log_{10}$ cfu/ml (Dafur et al., 2018) have previously been reported in NFRM in different countries in Africa. Higher counts up to 4.76 ± 5.30 $\log_{10}$ cfu/ml and 7.8 ± 8.1 $\log_{10}$ cfu/ml have also been reported by Gran et al. (2002) and Gran et al. (2003) respectively. This further supports that in developing countries, including Bhutan, hygienic practices are not adopted during the handling and processing of milk and milk products (Wanjala et al., 2018).

The microbiological criteria for "Ready-to-eat" (RTE) food of FSANZ (2018a) were used as a guide since there are no microbial standards for NFRM across the globe. According to the criteria, *E. coli* counts for 70 % of NFRM samples were outside the expected microbiological levels and were unsatisfactory while for 20 %, the counts were within expected microbiological levels but were at the upper range and were marginal. Only 10 % of the samples were satisfactory with counts within the expected microbiological levels.

Although most *E. coli* strains are harmless, there are a few pathogenic strains like the STEC which is most commonly associated with foodborne outbreaks (CDC, 2014). STEC causes illnesses ranging from mild diarrhoea and vomiting to more serious conditions including HC and HUS (MPI, 2018) and has caused several outbreaks due to the consumption of RM and RMP. Some strains of STEC including *E. coli* O157:H7 that is generally the most common STEC that causes human illnesses, was reported in NFRM in Nigeria (Ivbade et al., 2014; Yakubu et al., 2018). 50 % of the *E. coli* strains were identified as ETEC producing heat-stable enterotoxin strains in Zimbabwe (Gran et al., 2003). *E. coli* contamination also indicates the possible presence of other enteric pathogens including *Campylobacter* and *Salmonella* carried in the intestinal tract of cattle (Baylis, 2009; Oliver et al., 2005) and commensal pathogens including *Bacillus, Corynebacterium, Micrococcus, Staphylococcus,* and *Streptococcus* present on the skin of the teat and epithelial lining of the teat canal (Isaac et al., 2017) which are also common milk-borne pathogens (Alegbeleye et al., 2018). *Salmonella* spp. and *Streptococcus* spp. were reported in NFRM in Nigeria (Adebesin et al., 2001; Dafur et al., 2018; Tamba et al., 2016). The presence of *E. coli*, therefore, is a food safety concern.

S. aureus was present in all NFRM samples in the present survey. The prevalence is higher than the prevalence (5.6 % to 28 %) reported in NFRM in developing countries in Africa (Amenu et al., 2019; Dafur et al., 2018; Okonkwo, 2011; Samet-Bali et al., 2016). The present results are comparable to the 95 % prevalence reported by Gran et al. (2003) in Zimbabwe. In the present survey, the average count was 4.18 ± 0.67 $\log_{10}$ cfu/ml and varied over a wide range (1.95 to 5.41 $\log_{10}$ cfu/ml).This is lower than the average count of 7.8 $\log_{10}$ cfu/ml (varying from < 1 to 8.9 $\log_{10}$ cfu/ml) (Gran et al., 2003) in NFRM in Zimbabwe but higher than 1.30 $\log_{10}$ cfu/ml (0 to 1.95 $\log_{10}$ cfu/ml) (Dafur et al., 2018) and 1.51 $\log_{10}$ cfu/ml (0 to 6.60 $\log_{10}$ cfu/ml) (Okonkwo, 2011) in NFRM in Nigeria. *S. aureus* has also been reported in NFRM without the mean counts being reported (Amenu et al., 2019; Beukes et al., 2001; Nzabuheraheza & Nyiramugwera, 2016; Obande & Azua, 2013; Obi & Ikenebomeh, 2007). In the present survey, both the lipolytic and non-lipolytic strains of *S. aureus* were observed with and without clear zones around the colonies on BP agar respectively. Both lipolytic and non-lipolytic strains have been reported in milk (Silva, Destro, Landgraf, & Franco, 2000) and NFRM (Okonkwo, 2011) and were further confirmed with additional tests like API-Staph-system and coagulase tests respectively. The non-lipolytic strains may be from cattle with mastitis (Elliott, Clark, Lewis, Lundbeck, & Olson Jr, 1978).

S. aureus is one of the most important food-borne pathogens (Sahebekhtiari et al., 2011) and a common milk-borne pathogen (Oliver & Murinda, 2011) associated with *S. aureus* food poisoning (Srinivasan et al., 2006). *S. aureus* causes vomiting, diarrhoea and nausea by producing toxins like SEs in contaminated food (Hu & Nakane, 2014; Oliver & Murinda, 2011; Schelin, Wallin-Carlquist, Cohn, Lindqvist, & Barker, 2011). Doses of SEs to cause illness are reached when *S. aureus* grows to levels of 5 to 8 $\log_{10}$ cfu/ml (FSANZ, 2018a). Generally, less than 1.0 µg toxin causes illness (MPI, 2001). However, even if the counts are lower than 5 to 8 $\log_{10}$ cfu/ml due to unfavourable growth conditions in the food, there may still be sufficient toxins already produced which can cause illness (Bennett et al., 2013). Similarly, higher *S. aureus* counts than 5 to 8 $\log_{10}$ cfu/ml may not cause illness since some of the *S. aureus* do not produce enterotoxins Thus it is important to test the enterotoxigenicity of the *S. aureus* isolates (Bennett et al., 2013). Although fatalities are rare, staphylococcal food poisoning has caused fatalities especially in children and elderly (MPI, 2001). Overall, 61 % of NFRM samples in the present survey contained more than 4 $\log_{10}$ cfu/ml *S. aureus* and therefore the product was deemed

potentially hazardous presenting “an immediate food safety concern”. 35 % were unsatisfactory, 3 % were marginal and only 1 % were satisfactory (FSANZ, 2018a).

HTS identified other common milk-borne pathogens including *Shigella* and *Streptococcus agalactiae* which cause shigellosis and dysentery, and sore throat respectively (Alegbeleye et al., 2018). *Shigella* spp. have previously been reported in NFRM in Nigeria (Dafur et al., 2018; Okonkwo, 2011). HTS identified the *Vibrio* genus as well which indicates that some pathogenic species of *Vibrio* including *Vibrio parahemolyticus* that cause minor illnesses like diarrhoea, fever, acute gastroenteritis and nausea may be present (Oliver & Murinda, 2011). The presence of these pathogens supports the hypothesis that NFRM in Bhutan is contaminated with disease-causing pathogens.

The contamination of milk and milk products with *B. cereus, E. coli* and *S. aureus* occurs from numerous sources during milk collection, production, transportation, processing and storage (Millogo, Sjaunja, Ouédraogo, & Agenäs, 2010; Yakubu et al., 2018) as a result of unhygienic practices during these processes. In Bhutan, contamination may occur during the pre-harvesting and post-harvesting of RM while the contamination during and post-processing may be minimal as the manufacturing process of NFRM is simple. Therefore, the RM used for processing may be the key source of contaminants in NFRM like in the USA where diseases and outbreaks due to the consumption of RMP are mainly caused by the pathogens present in the RM (Boor, Wiedmann, Murphy, & Alcaine, 2017; Gould et al., 2014).

B. cereus contamination is generally from the soil (Kotiranta et al., 2000). Since its spores are resistant to heating and dehydration (MPI, 2015a), it can easily contaminate heat treated milk and milk products (Hwang & Park, 2015; Zhang, Feng, et al., 2016). It is also present in the dust, water and air (MPI, 2016; Schoeni & Wong, 2005). Therefore, *B. cereus* may also contaminate RM from the PET bottles used for fermentation. These bottles are stored in open areas and used without washing or sterilising.

In the dairy environment, since *E. coli* is naturally found in the gastrointestinal tract of the cattle, the faeces are the primary source of *E. coli* contamination (Oliver et al., 2005). Cattle are also the major reservoirs of STEC (CDC, 2014). There are two main pathways for *E. coli* contamination. RM is directly contaminated with faeces during milking (Krumperman, 1983; Oliver et al., 2005) and indirectly contaminated from the milk

handling and storage equipment, lagoons, bedding, water troughs, feed and manure which contain faeces (Oliver et al., 2005). *E. coli* has been reported with a prevalence of 33 % in RM in Bhutan (S. B. C. Rai et al., 2018). Additionally, in Bhutan, *E. coli* has been found in the rivers in Thimphu and Paro including tap water at Paro (Rinzin, 2017). Since rivers are one of the main sources for drinking water in Bhutan (National Environment Commission (Bhutan), 2016) and the majority of the RM for NFRM processing in Thimphu is produced in Paro, it is highly likely that RM may be contaminated with *E. coli* from water used in the dairy farm as well.

S. aureus is the predominant pathogen causing mastitis in cattle (Amenu et al., 2019; Chen et al., 2018) and high numbers of *S. aureus* may be found in milk if cattle have subclinical mastitis infection (Sahebekhtiari et al., 2011). Since a high prevalence of mastitis was reported in Bhutan (up to 89 %) (S. B. C. Rai et al., 2018; Tshering & Gyem, 2015), one of the main sources of enterotoxigenic *S. aureus* contamination in NFRM may be the infected cattle (Srinivasan et al., 2006). In cattle with *S. aureus* mastitis, RM is contaminated from within the udder (Zastempowska et al., 2016). *S. aureus* was also reported in the RM in Bhutan at 20 % prevalence (S. B. C. Rai et al., 2018). *S. aureus* is also naturally present as a commensal on the skin of the teat and udder of cattle (Isaac et al., 2017) and the skin, hair and nasal passages of humans (Tong et al., 2015). Humans are the chief sources of SEs producing *S. aureus* strains (FSANZ, 2018a). In Bhutan, cattle are milked by hand. Therefore, *S. aureus* may contaminate the RM and RMP during milking, handling and processing. Moreover, the high load of *E. coli* and *S. aureus* may be due to the pooling of individual farmers' RM in a single container and transporting over a long duration without refrigeration (Amenu et al., 2019).

One of the objectives of this survey was to determine the pH and acidity of NFRM. During food fermentation including NFRM, pH is one of the principal factors which preserves the food and enhances its safety (Adams & Mitchell, 2002; Campbell-Platt, 1987; Gadaga, Nyanga, & Mutukumira, 2004). The pH in fermented foods is usually less than 4 which inhibits the growth of food-borne pathogens (Kingamkono, Sjögren, Svanberg, & Kaijser, 1994). In the present survey, the overall mean pH was 4.51 ± 0.20 and there was less variation in pH of NFRM in Thimphu, Phuntsholing and NH although the fermentation temperature and period in Phuntsholing was different from the fermentation temperature and period in Thimphu and NH. LAB are mesophilic bacteria and grow rapidly above 20 ºC (König & Fröhlich, 2017; Niamsiri & Batt, 2009). Therefore, LAB

may have grown more rapidly in Phuntsholing at 20 ºC and fermented NFRM in less than a day to achieve the manufacturers' desired viscosity than Thimphu and NH at 8 – 10 ºC where LAB fermented NFRM in 3 days to achieve the manufacturers' desired viscosity.

B. cereus is unable to grow at pH 4.5 in yoghurt, and the diarrhoeal toxins are unstable between pH 4 to 11. However, the emetic toxins are stable at pH 2 (MPI, 2015a). STEC O157:H7 grows at pH 4.4 to 10. It survives in low pH foods and is more acid-tolerant in its stationary phase (MPI, 2018). The minimum pH for growth for *S. aureus* is around 4.2 (MPI, 2001). However, *S. aureus* is reported to grow and produce SEs at a minimum pH 4.0 - 4.6 (Hennekinne et al., 2012). The actual minimum pH for growth does vary slightly in different references. This supports the hypothesis that the pH of NFRM in Bhutan is not adequate to inhibit the growth of *E. coli* and *S. aureus* although it is adequate to inhibit the growth of *B. cereus*.

Other researchers report an average pH of 3.4 (Akabanda et al., 2010), 3.55 ± 0.20 (Maikai & Madaki, 2018), 3.94 ± 0.05 (Samet-Bali et al., 2016), 4.04 ± 0.04 (Okiki et al., 2018), 4.1 ± 0.40 (Gran et al., 2002) and 4.6 (Beukes et al., 2001; Okonkwo, 2011) in NFRM. The pH was different in each study due to the lack of standardised processing methods and different viscosities desired by the manufacturers.

The pH in fermented food is lowered by the lactic acid produced by LAB which inhibits the growth of pathogens (Adams & Nicolaides, 1997). Lactic acid inhibits the growth of the pathogens independent of the effect of pH as well (Charlier et al., 2008). It also produces the mild acidic taste and the characteristic tartness (König & Fröhlich, 2017; Niamsiri & Batt, 2009. Since pathogens are generally inhibited in fermented foods with pH less than 4 (Kingamkono et al., 1994), the amount of lactic acid at pH 4 may be sufficient to inhibit the growth of pathogens. The lactic acid in fermented food like yogurt is measured in terms of acidity. In the present survey, at pH 4.51 ± 0.20, the overall mean acidity was 0.60 % ± 0.09 LA which did not inhibit the growth of *E. coli* and *S. aureus*. In similar products like yoghurt, the acidity is 0.30 ± 0.03 to 0.50 ± 0.07 % LA (Olugbuyiro, 2011).

Although through fermentation, risky raw materials like RM are transformed into products with a lesser risk of causing illnesses (Adams & Mitchell, 2002), the fermentation conditions for NFRM in Bhutan did not render NFRM safe. Moreover, in

the present survey, *E. coli* and *S. aureus* were also present in high counts in NFRM which would have made fermentation less effective (Getty et al., 2000)

Although NFRM in Bhutan contains pathogens that present an immediate safety concern, no illnesses have been reported due to the consumption of NFRM in Bhutan before. This may be due to the under-reporting of such cases since these illnesses are short-lived and do not cause hospitalisations and deaths (CDC, 2006, 2018b). Moreover, there are no disease surveillance and outbreak investigation systems in Bhutan unlike the USA, Europe and New Zealand and the risk of the pathogens causing diseases in an individual also depends on numerous factors. Immunocompromised individuals may be susceptible due to age, genetic defects, diseases, and pharmacological therapy. Children and the elderly, and consumption of contaminated food for breakfast represent increased risk of food poisoning (Acheson, 2013; Adams & Mitchell, 2002; WHO, 2015).

A limitation of the present survey was that samples were frozen and stored for over 2 months before analysis. Since there were no facilities in laboratories in Bhutan to conduct the analysis, samples were transported to the Massey University laboratory. Some researchers do not recommend freezing and storing milk samples before studying the bacterial counts for risk of misdiagnosis since the bacterial counts are adversely affected after freezing. When raw milk samples were frozen at -18 ºC, *E. coli* counts significantly declined over time (Alrabadi, 2015; Hubáčková & Ryšánek, 2007). Nevertheless, in the present survey, *E. coli* were detected in more than 90 % of NFRM samples with 45 % of NFRM samples containing high *E. coli* counts of 3.04 $\log_{10}$ cfu/ml *E. coli* based on MPN estimates which is an indication that NFRM is produced under unhygienic conditions and may even contain pathogenic *E. coli* strains.

For *S. aureus*, their counts increased after 7 days of storage and decreased thereafter. However, the decrease in *S. aureus* counts were not significant after 21 days of storage (Alrabadi, 2015; Hubáčková & Ryšánek, 2007).

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

This is the first survey of NFRM in Bhutan for the prevalence of *B. cereus, E. coli* and *S. aureus*. This survey indicated the presence of *E. coli* and *S. aureus* along with other human pathogens (*Shigella* and *Vibrio* genera, and *S. agalactiae* and *S. parauberis*) using HTS and culture. Since *B. anthracis,* which is closely related to *B. cereus,* was identified by HTS, *B. cereus* was suspected in NFRM. Hence, the results indicate that NFRM in Bhutan is contaminated with pathogens and poses a major potential health hazard. Improvements in hygienic practices and control measures from the farm to the consumer are needed to reduce the contamination and the food safety risk. The guidelines on milking and animal management (DairyNZ, 2019a) and the code of hygiene practice for milk and milk products (CAC, 2004) may be adopted in Bhutan.

A key measure is to prevent faecal contamination (Baylis, 2009). Effective washing procedures for removing the faeces from the teats should be adopted. Generally, teats should be cleaned before milking (CAC, 2004) to remove faeces and dirt. In New Zealand, "strategic washing" is most commonly practiced where only the wet and visibly dirty teats are washed followed by drying using paper towels or suitable cloths. This method saves time as well. Dry and dirty teats are wiped (DairyNZ, 2019a, 2019b). Washing and sterilising the milking utensils including the PET bottles and using good quality water are important (Baylis, 2009; Murphy & Boor, 2000). The milk producers and processors should maintain their personal hygiene. Mastitis should be prevented and controlled by managing hygiene in the dairy environment and treating unhealthy cattle (Schukken, Grommers, Van De Geer, Erb, & Brand, 1990). RM storage and transportation temperatures should also be maintained to limit the growth of pathogens. The FSANZ guidelines for RM recommend cooling the milk on farm to 5 ºC within 3.5 h after the milking process and the European Codex requirements for heat treated milk products recommend cooling the milk on farm to 8 ºC within 2 h after the milking process. RM should then be transported at 5 ºC or 8 ºC. When RM is transported before cooling to 5 ºC or 8 ºC within 3.5 h or 2 hr of milking respectively, the post-milking cooling curves (Appendix E) should be used to determine the safety of the RM. The milk temperature and transportation time should fall below these curves (FSANZ, 2018b).

RM should also be fermented quickly with efficient starter cultures to achieve lower pH. The consumers and producers should also be aware of the risks of consuming the product (Verraes et al., 2015).

Nevertheless, despite the measures intended to minimise the contamination of RM and NFRM with pathogens, it is not possible to entirely eliminate them (EFSA, 2015b) and since there is no heat treatment used in Bhutan to inactivate the pathogens, the safety of NFRM can never be assured. Even in developed countries including the USA, Europe and New Zealand where better hygiene is used for RM and RMP, several disease outbreaks due to the consumption of these products have been reported (Verraes et al., 2015). Although no diseases and outbreaks have been reported in Bhutan, this is likely to be due to a lack of reporting rather than a lack of actual sickness. The product is still unsafe especially to the vulnerable population including the children, elderly and immunosuppressed people (Lund, 2015). Ideally, fermented milk products should be prepared from pasteurised milk and fermented using commercial starter cultures. Pasteurisation is the most effective method to inactivate the vegetative pathogens and assure the safety of the product (Baylis, 2009; Verraes et al., 2015). The ethnic flavour of NFRM may be preserved by developing starter cultures comprising of LAB present in NFRM as revealed by HTS. The costs of such products may be higher than the ethnic NFRM. Nevertheless, the safety of NFRM will be assured and the health risks minimised.

6.2 Recommendations for future work

The following recommendations are suggested for future work:

1. Determining the bacterial counts without freezing the samples
2. Using HTS to study the bacterial profile of the individual samples, rather than pooled samples of NFRM
3. Using strain-level analysis in combination with HTS to identify the species of *B. cereus* and pathogenic strains of *E. coli*
4. Determining the levels of *B. cereus* and *S. aureus* toxins
5. Using selective media like the Barcara agar (Tallent, Kotewicz, Strain, & Bennett, 2012) to culture *B. cereus*
6. Using methods to detect *B. cereus* spores
7. Screening NFRM for the prevalence of other human pathogens including *Salmonella* and *L. monocytogenes.*

REFERENCES

Acheson, D. (2013). Iatrogenic high-risk populations and food borne disease. *Infectious Disease Clinics of North America, 27*(3), 617-629. Retrieved from <Go to ISI>://WOS:000325191000008. doi:10.1016/j.idc.2013.05.008

Adams, M. R., & Mitchell, R. (2002). Fermentation and pathogen control: A risk assessment approach. *International Journal of Food Microbiology, 79*(1-2), 75-83.

Adams, M. R., & Nicolaides, L. (1997). Review of the sensitivity of different foodborne pathogens to fermentation. *Food Control, 8*(5-6), 227-239.

Adebesin, A. A., Amusa, N. A., & Fagade, S. O. (2001). Microbiological quality of locally fermented milk (nono) and fermented milk-cereal mixture (fura da nono) drink in Bauchi, a Nigerian city. *Bioline International, 6*, 87-89.

Agyei, D., Owusu-Kwarteng, J., Akabanda, F., & Akomea-Frempong, S. (2019). Indigenous African fermented dairy products: Processing technology, microbiology and health benefits. *Critical Reviews in Food Science and Nutrition*, 1-16. doi:10.1080/10408398.2018.1555133

Akabanda, F., Owusu-Kwarteng, J., Glover, R. L. K., & Tano-Debrah, K. (2010). Microbiological characteristics of Ghanaian traditional fermented milk product, nunu. *Nature and Science, 8*(9), 178-187. Retrieved from http://www.sciencepub.net/nature.

Alegbeleye, O. O., Guimarães, J. T., Cruz, A. G., & Sant'Ana, A. S. (2018). Hazards of a 'healthy'trend?: An appraisal of the risks of raw milk consumption and the potential of novel treatment technologies to serve as alternatives to pasteurization. *Trends in Food Science & Technology, 82*, 148-166. doi:10.1016/j.tifs.2018.10.007

Alrabadi, N. I. (2015). The effect of freezing on different bacterial counts in raw milk. *International Journal of Biology, 7*(4), 9-12. doi:10.5539/ijb.v7n4p9

Amagliani, G., Petruzzelli, A., Omiccioli, E., Tonucci, F., Magnani, M., & Brandi, G. (2012). Microbiological surveillance of a bovine raw milk farm through multiplex real-time PCR. *Foodborne Pathogens and Disease, 9*(5), 406-411. doi:10.1089/fpd.2011.1041

Amenu, K., Grace, D., Nemo, S., & Wieland, B. (2019). Bacteriological quality and safety of ready-to-consume milk and naturally fermented milk in Borana

pastoral area, southern Ethiopia. *Tropical animal health and production,* 1-6. doi:10.1007/s11250-019-01872-8

Angulo, F. J., LeJeune, J. T., & Rajala-Schultz, P. J. (2009). Unpasteurized milk: A continued public health threat. *Clinical Infectious Diseases, 48*(1), 93-100. doi:10.1086/595007

Arqués, J. L., Rodríguez, E., Langa, S., Landete, J. M., & Medina, M. (2015). Antimicrobial activity of lactic acid bacteria in dairy products and gut: Effect on pathogens. *BioMed research international, 2015*. doi:10.1155/2015/584183

Ash, C., Farrow, J. A. E., Dorsch, M., Stackebrandt, E., & Collins, M. D. (1991). Comparative analysis of Bacillus anthracis, Bacillus cereus, and related species on the basis of reverse transcriptase sequencing of 16S rRNA. *International Journal of Systematic and Evolutionary Microbiology, 41*(3), 343-346.

Axelsson, L. (2004). Lactic acid bacteria: Classification and physiology. In S. Salminen., A. V. Wright., & A. Ouwehand. (Eds.), *Lactic acid bacteria: microbiology and functional aspects* (Vol. 3, pp. 1-66). New York, USA: Marcel Dekker, Inc.

Baylis, C. L. (2009). Raw milk and raw milk cheeses as vehicles for infection by Verocytotoxin-producing *Escherichia coli*. *International Journal of Dairy Technology, 62*(3), 293-307. doi:10.1111/j.1471-0307.2009.00504.x

Begley, M., & Hill, C. (2015). Stress adaptation in foodborne pathogens. *Annual review of food science and technology, 6*, 191-210. doi:10.1146/annurev-food-030713-092350

Belfiore, C., Castellano, P., & Vignolo, G. (2007). Reduction of *Escherichia coli* population following treatment with bacteriocins from lactic acid bacteria and chelators. *Food microbiology, 24*(3), 223-229. Retrieved from <Go to ISI>://WOS:000244177700005. doi:10.1016/j.fm.2006.05.006

Bellio, A., Astegiano, S., Traversa, A., Bianchi, D. M., Gallina, S., Vitale, N., . . . Decastelli, L. (2016). Behaviour of *Listeria monocytogenes* and *Staphylococcus aureus* in sliced, vacuum-packaged raw milk cheese stored at two different temperatures and time periods. *International Dairy Journal, 57*, 15-19. doi:10.1016/j.idairyj.2016.02.003

Bennett, R. W., & Beley, N. (2001). *Bacillus cereus*. In F. P. Downes & K. Ito (Eds.), *Compendium of methods for the microbiological examinations of foods* (Vol. 4, pp. 311-316). Washington, DC, USA: American Public Health Association.

Bennett, R. W., Hait, J. M., & Tallent, S. M. (2013). *Staphylococcus aureus*. In R. G. Labbé & S. García (Eds.), *Guide to foodborne pathogens* (Vol. 2, pp. 26-44): John Wiley & Sons, Ltd.

Beukes, E. M., Bester, B. H., & Mostert, J. F. (2001). The microbiology of South African traditional fermented milks. *International Journal of Food Microbiology, 63*(3), 189-197. Retrieved from <Go to ISI>://WOS:000167207500001. doi:10.1016/s0168-1605(00)00417-7

Beuvier, E., & Buchin, S. (2004). Raw milk cheeses. In P. F. Fox, P. L. H. McSweeny, T. M. Cogan, & T. P. Guinee (Eds.), *Cheese: Chemistry, physics and microbiology* (Vol. 1, pp. 319-345). Italy: Elsevier Academic Press.

Boor, K. J., Wiedmann, M., Murphy, S., & Alcaine, S. (2017). A 100-year review: Microbiology and safety of milk handling. *Journal of Dairy Science, 100*(12), 9933-9951. doi:10.3168/jds.2017-12969

Brakstad, O. G., Aasbakk, K., & Maeland, J. A. (1992). Detection of *Staphylococcus aureus* by polymerase chain reaction amplification of the nuc gene. *Journal of clinical microbiology, 30*(7), 1654-1660.

CAC. (2004). *Code of hygienic practice for milk and milk products CAC/RCP 57-2004*. Retrieved from http://www.fao.org/fileadmin/user_upload/livestockgov/documents/CXP_057e.pdf

Campbell-Platt, G. (1987). *Fermented foods of the world: A dictionary and guide*. London, Boston: Butterworths Scientific.

Campbell-Platt, G. (1994). Fermented foods: A world perspective. *Food Research International, 27*(3), 253-257. doi:https://doi.org/10.1016/0963-9969(94)90093-0

Cancino-Padilla, N., Fellenberg, M. A., Franco, W., Ibáñez, R. A., & Vargas-Bello-Pérez, E. (2017). Foodborne bacteria in dairy products: Detection by molecular techniques. *Ciencia Investigación Agraria, 44*(3), 215-229. doi:10.7764/rcia.v44i3.1811

CDC. (2006). *Surveillance for foodborne-disease outbreaks: United States, 1998-2002*. Retrieved from U.S: https://www.cdc.gov/mmwr/preview/mmwrhtml/ss5510a1.htm

CDC. (2014). *E. coli (Escherichia coli)*: Questions and answers. Retrieved from https://www.cdc.gov/ecoli/general/index.html

CDC. (2017). Food safety: Raw milk questions and answers. Retrieved from https://www.cdc.gov/foodsafety/rawmilk/raw-milk-questions-and-answers.html

CDC. (2018a). Division of Foodborne, waterborne and environmental diseases (DFWED) : Surveillance and data systems. Retrieved from https://www.cdc.gov/nors/about.html

CDC. (2018b). National Outbreak Reporting System (NORS) Dashboard. Retrieved from https://wwwn.cdc.gov/norsdashboard/

CDC. (2018c). *Surveillance for foodborne disease outbreaks- United States, 2016 annual report*. Retrieved from Atlanta, Georgia.: https://www.cdc.gov/fdoss/pdf/2016_FoodBorneOutbreaks_508.pdf

Charlier, C., Even, S., Gautier, M., & Loir, Y. L. (2008). Acidification is not involved in the early inhibition of *Staphylococcus aureus* growth by *Lactococcus lactis* in milk. *International Dairy Journal, 18*(2), 197-203. doi:10.1016/j.idairyj.2007.03.015

Chen, J., Tang, J., Hu, K., Zhao, Y., & Tang, C. (2018). The production characteristics of volatile organic compounds and their relation to growth status of *Staphylococcus aureus* in milk environment. *Journal of Dairy Science, 101*(6), 4983-4991. Retrieved from https://ac.els-cdn.com/S0022030218302340/1-s2.0-S0022030218302340-main.pdf?_tid=4408582c-96e6-4257-8e2f-f924627914cc&acdnat=1537932042_bb86014fc9fcb455e6528d4280cb111e. doi:https://doi.org/10.3168/jds.2017-13629

Chiang, Y.-C., Tsen, H.-Y., Chen, H.-Y., Chang, Y.-H., Lin, C.-K., Chen, C.-Y., & Pai, W.-Y. (2012). Multiplex PCR and a chromogenic DNA macroarray for the detection of *Listeria monocytogens, Staphylococcus aureus, Streptococcus agalactiae, Enterobacter sakazakii, Escherichia coli* O157: H7, *Vibrio parahaemolyticus, Salmonella* spp. and *Pseudomonas fluorescens* in milk and meat samples. *Journal of microbiological methods, 88*(1), 110-116. doi:10.1016/j.mimet.2011.10.021

Chikindas, M. L., Weeks, R., Drider, D., Chistyakov, V. A., & Dicks, L. M. (2018). Functions and emerging applications of bacteriocins. *Current Opinion in Biotechnology, 49,* 23-28. doi:10.1016/j.copbio.2017.07.011

Choi, K. H., Lee, H., Lee, S., Kim, S., & Yoon, Y. (2016). Cheese microbial risk assessments: A review. *Asian-Australasian Journal of Animal Sciences, 29*(3), 307. doi:10.5713/ajas.15.0332

Christiansson, A., Naidu, A. S., Nilsson, I., Wadström, T., & Pettersson, H. (1989). Toxin production by Bacillus cereus dairy isolates in milk at low temperatures. *Applied and Environmental Microbiology, 55*(10), 2595-2600.

Claeys, W. L., Cardoen, S., Daube, G., De Block, J., Dewettinck, K., Dierick, K., . . . Herman, L. (2013). Raw or heated cow milk consumption: Review of risks and benefits. *Food Control, 31*(1), 251-262. Retrieved from <Go to ISI>://WOS:000315363600040. doi:10.1016/j.foodcont.2012.09.035

Climates to travel. (2019). Climate-Bhutan. Retrieved from https://www.climatestotravel.com/climate/bhutan

Cogan, T. M., Barbosa, M., Beuvier, E., BianchiSalvadori, B., Cocconcelli, P. S., Fernandes, I., . . . Rodriquez, E. (1997). Characterization of the lactic acid bacteria in artisanal dairy products. *Journal of Dairy Research, 64*(3), 409-421. Retrieved from <Go to ISI>://WOS:A1997XR07200008. doi:10.1017/s0022029997002185

Costard, S., Espejo, L., Groenendaal, H., & Zagmutt, F. J. (2017). Outbreak-related disease burden associated with consumption of unpasteurized cow's milk and cheese, United States, 2009–2014. *Emerging Infectious Diseases*, *23*(6), 957.

Dafur, G. S., Iheukwumere, C. C., Azua, E. T., & Dafur, B. S. (2018). Evaluation of the microbial quality of 'nono' sold in Mangu local government area of Plateau state, Nigeria. *South Asian Journal of Research in Microbiology*, 1-14. doi:10.9734/SAJRM/2018/44560

DairyNZ. (2019a). Mastitis. Retrieved from https://www.dairynz.co.nz/animal/cow-health/mastitis/

DairyNZ. (2019b). Teat washing. Retrieved from https://www.dairynz.co.nz/milking/dairy-stockmanship/teat-washing/

Davidson, P. M., Critzer, F. J., & Taylor, T. M. (2013). Naturally occurring antimicrobials for minimally processed foods. *Annual review of food science and technology, 4*, 163-190. doi:10.1146/annurev-food-030212-182535

De Jonghe, V., Coorevits, A., De Block, J., Van Coillie, E., Grijspeerdt, K., Herman, L., . . . Heyndrickx, M. (2010). Toxinogenic and spoilage potential of aerobic spore-formers isolated from raw milk. *International Journal of Food Microbiology*, *136*(3), 318-325. doi:10.1016/j.ijfoodmicro.2009.11.007

De Reu, K., Grijspeerdt, K., & Herman, L. (2004). A Belgian survey of hygiene indicator bacteria and pathogenic bacteria in raw milk and direct marketing of raw milk farm products. *Journal of Food Safety, 24*(1), 17-36.

Dewan, S., & Tamang, J. P. (2007). Dominant lactic acid bacteria and their technological properties isolated from the Himalayan ethnic fermented milk products. *Antonie Van Leeuwenhoek, 92*(3), 343-352. doi:10.1007/s10482-007-9163-5

Donkor, O. N., Henriksson, A., Vasiljevic, T., & Shah, N. P. (2007). Proteolytic activity of dairy lactic acid bacteria and probiotics as determinant of growth and in vitro angiotensin-converting enzyme inhibitory activity in fermented milk. *Le Lait, 87*(1), 21-38. doi:10.1051/lait:2006023

EFSA. (2015a). Raw drinking milk: What are the risks? Retrieved from https://www.efsa.europa.eu/en/press/news/150113

EFSA. (2015b). Scientific opinion on the development of a risk ranking toolbox for the EFSA BIOHAZ Panel. *Efsa Journal, 13*(1), 3939. doi:10.2903/j.efsa.2015.3940

EFSA. (2019). Monitoring and analysis of food-borne diseases. Retrieved from https://www.efsa.europa.eu/en/topics/topic/monitoring-and-analysis-food-borne-diseases

Egger-Danner, C., Cole, J., Pryce, J., Gengler, N., Heringstad, B., Bradley, A., & Stock, K. F. (2015). Invited review: Overview of new traits and phenotyping strategies in dairy cattle with a focus on functional traits. *Animal, 9*(2), 191-207. doi:10.1017/S1751731114002614

Eka, O., & Ohaba, J. (1977). Microbiological examination of Fulani milk (nono) and butter (manshanu). *Nigerian Journal of Science, 11*(1-2), 113.

Elliott, R. P., Clark, D. S., Lewis, K. H., Lundbeck, H., & Olson Jr, J. C. (1978). *Microorganisms in foods : Their significance and methods of enumeration* (Vol. 1). Toronto: University of Toronto Press.

FDA. (2011). Raw milk misconceptions and the danger of raw milk consumption. Retrieved from https://www.fda.gov/Food/FoodborneIllnessContaminants/BuyStoreServeSafeFood/ucm247991.htm

FDA. (2017). Grade "A" pasteurised milk ordinance. Retrieved from https://www.fda.gov/media/114169/download

French, N., Benschop, J., & Marshall, J. (2013). *Raw milk: Is it good for you?* Paper presented at the Proceedings of the food safety, animal welfare & biosecurity branch of the NZVA, New Zealand.

FSANZ. (2018a). Compendium of microbiological criteria for food. Retrieved from http://www.foodstandards.gov.au/publications/Documents/Compedium%20of%20Microbiological%20Criteria/Compendium_revised-Sep%202018.pdf

FSANZ. (2018b). Raw milk temperatures. Retrieved from http://www.agriculture.gov.au/export/controlled-goods/dairy/registered-establishment/raw-milk-temperatures

Fusco, V., Oguntoyinbo, F. A., & Franz, M. A. P. C. (2017). Fermentation to improve food security in Africa and Asia. In A. M. Grumezescu & A. M. Holban (Eds.), *Soft Chemistry and Food Fermentation* (Vol. 3, pp. 337-378): Academic Press.

Gadaga, T. H., Nyanga, L. K., & Mutukumira, A. N. (2004). The occurrence, growth and control of pathogens in African fermented foods. *African Journal of food and nutritional science, 4*(1), 5358-5374.

Geigl, E.-M. (2008). Palaeogenetics of cattle domestication: Methodological challenges for the study of fossil bones preserved in the domestication centre in Southwest Asia. *Comptes Rendus Palevol, 7*(2-3), 99-112. doi:10.1016/j.crpv.2008.02.001

Geneaid Biotech Ltd. (2019). Instruction manual: Presto mini gDNA bacteria k. Retrieved from https://www.geneaid.com/sites/default/files/GBB16.pdf

Getty, K. J. K., Phebus, R. K., Marsden, J. L., Fung, D. Y. C., & Kastner, C. L. (2000). *Escherichia coli* O157: H7 and fermented sausages: A review. *Journal of Rapid Methods & Automation in Microbiology, 8*(3), 141-170.

Gharsallaoui, A., Oulahal, N., Joly, C., & Degraeve, P. (2016). Nisin as a food preservative: Part 1: Physicochemical properties, antimicrobial activity, and main uses. *Critical Reviews in Food Science and Nutrition, 56*(8), 1262-1274. doi:10.1080/10408398.2013.763765

Ghelardi, E., Celandroni, F., Salvetti, S., Barsotti, C., Baggiani, A., & Senesi, S. (2002). Identification and characterization of toxigenic Bacillus cereus isolates responsible for two food-poisoning outbreaks. *Fems Microbiology Letters, 208*(1), 129-134.

Gopal, N., Hill, C., Ross, P. R., Beresford, T. P., Fenelon, M. A., & Cotter, P. D. (2015). The prevalence and control of *Bacillus* and related spore-forming

bacteria in the dairy industry. *Frontiers in Microbiology, 6*, 1-18. doi:10.3389/fmicb.2015.01418

Gould, L. H., Mungai, E., & Behravesh, C. B. (2014). Outbreaks attributed to cheese: Differences between outbreaks caused by unpasteurized and pasteurized dairy products, United States, 1998–2011. *Foodborne Pathogens and Disease, 11*(7), 545-551. doi:10.1089/fpd.2013.1650

Goulet, V., Hebert, M., Hedberg, C., Laurent, E., Vaillant, V., De Valk, H., & Desenclos, J.-C. (2011). Incidence of listeriosis and related mortality among groups at risk of acquiring listeriosis. *Clinical Infectious Diseases, 54*(5), 652-660. doi:10.1093/cid/cir902

Gran, H. M., Mutukumira, A. N., Wetlesen, A., & Narvhus, J. A. (2002). Smallholder dairy processing in Zimbabwe: the production of fermented milk products with particular emphasis on sanitation and microbiological quality. *Food Control, 13*(3), 161-168.

Gran, H. M., Wetlesen, A., Mutukumira, A. N., Rukure, G., & Narvhus, J. A. (2003). Occurrence of pathogenic bacteria in raw milk, cultured pasteurised milk and naturally soured milk produced at small-scale dairies in Zimbabwe. *Food Control, 14*(8), 539-544.

Granum, P. E., & Toril, L. (2013). *Bacillus cereus*. In Michael P. Doyle & R. L. Buchanan (Eds.), *Food Microbiology: Fundamental and Frontiers* (Vol. 4, pp. 491-502). Washington, DC.: ASM press.

Griffiths, M. W., & Schraft, H. (2017). *Bacillus cereus* food Poisoning. In C. E. R. Dodd., T. Aldsworth, R. A. Stein, D. O. Cliver, & H. P. Riemann (Eds.), *Foodborne Diseases* (pp. 395-405): Academic Press.

Gutiérrez, S., Martínez-Blanco, H., Rodríguez-Aparicio, L. B., & Ferrero, M. A. (2016). Effect of fermented broth from lactic acid bacteria on pathogenic bacteria proliferation. *Journal of Dairy Science, 99*(4), 2654-2665. doi:10.3168/jds.2015-10439

Gyawali, R., & Ibrahim, S. A. (2014). Natural products as antimicrobial agents. *Food Control, 46*, 412-429. doi:10.1016/j.foodcont.2014.05.047

Hamad, S. H. (2012). Factors affecting the growth of microorganisms in food. In R. Bhat, A. K. Alias, & G. Paliyath (Eds.), *Progress in food preservation* (pp. 405-426). West Sussex, UK: John Wiley & Sons Ltd.

Hennekinne, J. A., De Buyser, M. L., & Dragacci, S. (2012). *Staphylococcus aureus* and its food poisoning toxins: Characterization and outbreak investigation. *FEMS microbiology reviews, 36*(4), 815-836. doi:10.1111/j.1574-6976.2011.00311.x

Holzapfel, W. (1997). Use of starter cultures in fermentation on a household scale. *Food Control, 8*(5-6), 241-258. Retrieved from http://ezproxy.massey.ac.nz/login?url=http://search.ebscohost.com/login.aspx?direct=true&db=edselc&AN=edselc.2-52.0-0031256337&site=eds-live&scope=site. doi:10.1016/S0956-7135(97)00017-0

Horn, N., & Bhunia, A. K. (2018). Food-associated stress primes foodborne pathogens for the gastrointestinal phase of infection. *Frontiers in Microbiology, 9*(1962). Retrieved from <Go to ISI>://WOS:000442470700001. doi:10.3389/fmicb.2018.01962

Hu, D. L., & Nakane, A. (2014). Mechanisms of staphylococcal enterotoxin-induced emesis. *European journal of pharmacology, 722*, 95-107. doi:10.1016/j.ejphar.2013.08.050

Hubáčková, M., & Ryšánek, D. (2007). Effects of freezing milk samples on the recovery of alimentary pathogens and indicator microorganisms. *Acta Veterinaria Brno, 76*(2), 301-307. doi:10.2754/avb200776020301

Hwang, J. Y., & Park, J. H. (2015). Characteristics of enterotoxin distribution, hemolysis, lecithinase, and starch hydrolysis of *Bacillus cereus* isolated from infant formulas and ready-to-eat foods. *Journal of Dairy Science, 98*(3), 1652-1660. doi:10.3168/jds.2014-9042

Isaac, P., Bohl, L. P., Breser, M. L., Orellano, M. S., Conesa, A., Ferrero, M. A., & Porporatto, C. (2017). Commensal coagulase-negative *Staphylococcus* from the udder of healthy cows inhibits biofilm formation of mastitis-related pathogens. *Veterinary microbiology, 207*, 259-266. doi:10.1016/j.vetmic.2017.05.025

Ivbade, A., Ojo, O. E., & Dipeolu, M. A. (2014). Shiga toxin-producing *Escherichia coli* O157: H7 in milk and milk products in Ogun State, Nigeria. *Veterinaria Italiana, 50*(3), 185-191. doi:10.12834/VetIt.129.2187.1

Jakobsen, R. A., Heggebø, R., Sunde, E. B., & Skjervheim, M. (2011). *Staphylococcus aureus* and *Listeria monocytogenes* in Norwegian raw milk cheese production. *Food microbiology, 28*(3), 492-496. doi:10.1016/j.fm.2010.10.017

Jatmiko, Y. D., Howarth, G. S., & Barton, M. D. (2018). *Naturally fermented milk and its therapeutic potential in the treatment of inflammatory intestinal disorders.* Paper presented at the AIP Conference Proceedings, 2019.

Johler, S., Kalbhenn, E. M., Heini, N., Brodmann, P., Gautsch, S., Bağcioğlu, M., . . . Ehling-Schulz, M. (2018). Enterotoxin production of *Bacillus thuringiensis* isolates from biopesticides, foods, and outbreaks. *Frontiers in Microbiology, 9*(1915), 1-11. Retrieved from https://www.frontiersin.org/article/10.3389/fmicb.2018.01915. doi:10.3389/fmicb.2018.01915

Johler, S., Weder, D., Bridy, C., Huguenin, M.-C., Robert, L., Hummerjohann, J., & Stephan, R. (2015). Outbreak of staphylococcal food poisoning among children and staff at a Swiss boarding school due to soft cheese made from raw milk. *Journal of Dairy Science, 98*(5), 2944-2948. doi:10.3168/jds.2014-9123

Kalyoussef, S., & Feja, K. N. (2014). Foodborne Illnesses. *Advances in Pediatrics, 61*(1), 287-312. Retrieved from http://www.sciencedirect.com/science/article/pii/S0065310114000152. doi:10.1016/j.yapd.2014.04.003

Karmali, M. A., Gannon, V., & Sargeant, J. M. (2010). Verocytotoxin-producing *Escherichia coli* (VTEC). *Veterinary microbiology, 140*(3-4), 360-370. doi:10.1016/j.vetmic.2009.04.011

Kim, W., Kim, J. Y., Cho, S. L., Nam, S. W., Shin, J. W., Kim, Y. S., & Shin, H. S. (2008). Glycosyltransferase: A specific marker for the discrimination of *Bacillus anthracis* from the *Bacillus cereus* group. *Journal of medical microbiology, 57*(3), 279-286. doi:10.1099/jmm.0.47642-0

Kingamkono, R., Sjögren, E., Svanberg, U., & Kaijser, B. (1994). pH and acidity in lactic-fermenting cereal gruels: Effects on viability of enteropathogenic microorganisms. *World Journal of Microbiology and Biotechnology, 10*(6), 664-669.

König, H., & Fröhlich, J. (2017). Lactic acid bacteria. In H. Konig, G. Unden, & J. Frohlich (Eds.), *Biology of microorganisms on grapes, in must and in wine* (pp. 3-30). Heidelberg, Germany: Springer-Verlag Berlin Heidelberg.

Kornacki, J. L., & Johnson, J. L. (2001). Enterobacteriaceae, coliforms and *Escherichia coli* as quality and safety indicators. In F. P. Downes & K. Ito (Eds.),

Compendium of methods for the microbilogical examination of foods (4 ed., pp. 69-82). Washington, DC, USA: American Public Health Association.

Kotiranta, A., Lounatmaa, K., & Haapasalo, M. (2000). Epidemiology and pathogenesis of *Bacillus cereus* infections. *Microbes and infection, 2*(2), 189-198. Retrieved from http://www.sciencedirect.com/science/article/pii/S1286457900002690. doi:10.1016/S1286-4579(00)00269-0

Koutsoumanis, K. P., Lianou, A., & Gougouli, M. (2016). Latest developments in foodborne pathogens modeling. *Current Opinion in Food Science, 8*, 89-98. doi:10.1016/j.cofs.2016.04.006

Koutsoumanis, K. P., & Sofos, J. N. (2004). Comparative acid stress response of *Listeria monocytogenes, Escherichia coli* O157: H7 and *Salmonella Typhimurium* after habituation at different pH conditions. *Letters in Applied Microbiology, 38*(4), 321-326. doi:10.1111/j.1472-765X.2004.01491.x

Krumperman, P. H. (1983). Multiple antibiotic resistance indexing of *Escherichia coli* to identify high-risk sources of fecal contamination of foods. *Applied and Environmental Microbiology, 46*(1), 165-170. doi:0099-2240/83/070165-06$02.00/0

Kuang, Y., Tani, K., Synnott, A. J., Ohshima, K., Higuchi, H., Nagahata, H., & Tanji, Y. (2009). Characterization of bacterial population of raw milk from bovine mastitis by culture-independent PCR–DGGE method. *Biochemical engineering journal, 45*(1), 76-81. doi:10.1016/j.bej.2009.02.010

Lancette, G. A., & Bennett, R. W. (2001). *Staphyloccus aureus* and staphylococcal enterotoxins. In F. P. Downes & K. Ito (Eds.), *Compendium of methods for the microbiological examinations of foods* (4 ed., pp. 387-403). Washington, DC, USA: American Public Health Association.

Langer, A. J., Ayers, T., Grass, J., Lynch, M., Angulo, F. J., & Mahon, B. E. (2012). Nonpasteurized dairy products, disease outbreaks, and state laws: United States, 1993–2006. *Emerging Infectious Diseases, 18*(3), 385. doi:10.3201/eid1803.111370

Leedom, J. M. (2006). Milk of nonhuman origin and infectious diseases in humans. *Clinical Infectious Diseases, 43*(5), 610-615. doi:10.1086/507035

Liu, W., Zheng, Y., Kwok, L. Y., Sun, Z., Zhang, J., Guo, Z., . . . Zhang, H. (2015). High-throughput sequencing for the detection of the bacterial and fungal

diversity in Mongolian naturally fermented cow's milk in Russia. *BMC microbiology*, *15*(1), 45. doi:10.1186/s12866-015-0385-9

Lund, B. (2015). Microbiological food safety for vulnerable people. *International journal of environmental research and public health*, *12*(8), 10117-10132. doi:10.3390/ijerph120810117

Maikai, B. V., & Madaki, P. D. (2018). Enumeration of coliforms in fermented milk product (nono) sold in Samaru, Kaduna State, Nigeria. *Sokoto Journal of Veterinary Sciences*, *16*(4), 50. Retrieved from http://ezproxy.massey.ac.nz/login?url=http://search.ebscohost.com/login.aspx?direct=true&db=edb&AN=134100384&site=eds-live&scope=site. doi:10.4314/sokjvs.v16i4.6

Makovec, J., & Ruegg, P. (2003). Results of milk samples submitted for microbiological examination in Wisconsin from 1994 to 2001. *Journal of Dairy Science*, *86*(11), 3466-3472. doi:10.3168/jds.S0022-0302(03)73951-4

Manter, D. K., Weir, T. L., & Vivanco, J. M. (2010). Negative effects of sample pooling on PCR-based estimates of soil microbial richness and community structure. *Applied and Environmental Microbiology*, *76*(7), 2086-2090. doi:10.1128/AEM.03017-09

Marco, M. L., Heeney, D., Binda, S., Cifelli, C. J., Cotter, P. D., Foligné, B., . . . Hutkins, R. (2017). Health benefits of fermented foods: Microbiota and beyond. *Current Opinion in Biotechnology*, *44*, 94-102. Retrieved from http://www.sciencedirect.com/science/article/pii/S095816691630266X. doi:10.1016/j.copbio.2016.11.010

Marian, M., Aminah, S. S., Zuraini, M., Son, R., Maimunah, M., Lee, H., . . . Elexson, N. (2012). MPN-PCR detection and antimicrobial resistance of *Listeria monocytogenes* isolated from raw and ready-to-eat foods in Malaysia. *Food Control*, *28*(2), 309-314. doi:10.1016/j.foodcont.2012.05.030

Midura, T. F., & Bryant, R. G. (2001). Sampling plans, sample collection, shipment, and preparation for analysis. In *Compendium of methods for the microbiological examinations of foods. American Public Health Association ed., Washington, DC, USA* (Vol. 4, pp. 13-23).

Millogo, V., Sjaunja, K. S., Ouédraogo, G., & Agenäs, S. (2010). Raw milk hygiene at farms, processing units and local markets in Burkina Faso. *Food Control*, *21*(7), 1070-1074. doi:10.1016/j.foodcont.2009.12.029

Ministry of Agriculture and Forests (Bhutan). (2018). Deparment of livestock: About us. Retrieved from http://www.moaf.gov.bt/agencies/department-of-livestock/dol-about-us/

Miskimin, D., Berkowitz, K., Solberg, M., Riha Jr, W., Franke, W., Buchanan, R., & O'leary, V. (1976). Relationships between indicator organisms and specific pathogens in potentially hazardous foods. *Journal of Food Science, 41*(5), 1001-1006.

Mo, L., Yu, J., Jin, H., Hou, Q., Yao, C., Ren, D., . . . Zhang, H. (2019). Investigating the bacterial microbiota of traditional fermented dairy products using propidium monoazide with single-molecule real-time sequencing. *Journal of Dairy Science, 102*(5), 3912-3923. doi:10.3168/jds.2018-15756

Motarjemi, Y., Moy, G. G., Jooste, P. J., & Anelich, L. E. (2014). Milk and Dairy Products. In Y. Motarjemi & H. Lelieveld (Eds.), *Food Safety Management: A practical guide for the food industry* (pp. 83-117). San Diego: Academic Press.

Motato, K. E., Milani, C., Ventura, M., Valencia, F. E., Ruas-Madiedo, P., & Delgado, S. (2017). Bacterial diversity of the Colombian fermented milk "Suero Costeño" assessed by culturing and high-throughput sequencing and DGGE analysis of 16S rRNA gene amplicons. *Food microbiology*, *68*, 129-136. doi:10.1016/j.fm.2017.07.011

MPI. (2001). *Staphylococcus aureus*. Retrieved from https://www.mpi.govt.nz/dmsdocument/11051-staphylococcus-aureus-microbial-pathogen-data-sheet

MPI. (2015a). *Bacillus cereus*. Retrieved from https://www.mpi.govt.nz/dmsdocument/21545-bacillus-cereus-spore-forming

MPI. (2015b). *Escherichia coli* and STEC. Retrieved from https://www.mpi.govt.nz/dmsdocument/26024/send

MPI. (2016). Risk profile: *Bacillus cereus* in dairy products. Retrieved from https://www.mpi.govt.nz/dmsdocument/14149-risk-profile-bacillus-cereus-in-dairy-products

MPI. (2018). Shiga Toxin-producing *Escherichia coli*. Retrieved from https://www.mpi.govt.nz/dmsdocument/26030-shiga-toxin-producing-escherichia-coli-stec

MPI. (2019). Food safety: Human health surviellance. Retrieved from https://www.mpi.govt.nz/food-safety/food-safety-and-suitability-research/human-health-surveillance/

Mungai, E. A., Behravesh, C. B., & Gould, L. H. (2015). Increased outbreaks associated with nonpasteurized milk, United States, 2007–2012. *Emerging Infectious Diseases*, *21*(1), 119. doi:10.3201/eid2101.140447

Murphy, S. C., & Boor, K. J. (2000). Sources and causes of high bacteria counts in raw milk: An abbreviated review. *Dairy, Food and Environmental Sanitation, 20*(8), 1-4.

Mutukumira, A., Narvhus, J., & Abrahamsen, R. (1995). Review of traditionally-fermented milk in some sub-Saharan countries: Focusing on Zimbabwe. *Cutured Dairy Products, 30*, 6-10.

Narvhus, J. A., & Gadaga, T. H. (2003). The role of interaction between yeasts and lactic acid bacteria in African fermented milks: A review. *International Journal of Food Microbiology*, *86*(1-2), 51-60. doi:10.1016/S0168-1605(03)00247-2

National Environment Commission (Bhutan). (2016). *Bhutan drinking water quality standard, 2016*. Thimphu, Bhutan Retrieved from http://extwprlegs1.fao.org/docs/pdf/bhu181581.pdf

National Stastics Bureau of Bhutan. (2018). *2017 Population & housing census of Bhutan*. Retrieved from Thimphu, Bhutan: http://www.nsb.gov.bt/publication/files/PHCB2017_national.pdf

Niamsiri, N., & Batt, C. A. (2009). Dairy products. In M. Schaecher (Ed.), *Encyclypaedia of Microbiology*. San Diego University, San Diego.

Nyachuba, D. G. (2010). Foodborne illness: Is it on the rise? *Nutrition reviews, 68*(5), 257-269. doi:10.1111/j.1753-4887.2010.00286.x

Nzabuheraheza, F., & Nyiramugwera, A. N. (2016). Microbiological analysis of traditionally fermented milk sold in Kinigi Sector of Musanze District in Rwanda. *African Journal of food agriculture, nutrition and development, 16*(2), 10841-10852. doi:10.18697/ajfand.74.15105

NZFS. (2018). Raw milk. Retrieved from https://www.mpi.govt.nz/food-safety/food-safety-for-consumers/is-it-safe-to-eat/raw-milk/

Obande, G. A., & Azua, E. T. (2013). Extent of microbial contamination of nono, fresh cow milk and yoghurt sold in Makurdi, Benue State, Nigeria. *Journal of Microbiology and Biotechnology Research*, *3*(3), 6-14.

Obi, C. N., & Ikenebomeh, M. J. (2007). Studies on the microbiology and nutritional qualities of a Nigerian fermented milk product (nono). *International Journal of Dairy Science, 2*(1), 95-99.

Ofukwu, R., Oboegbulem, S., & Akwuobu, C. (2008). Zoonotic Mycobacterium species in fresh cow milk and fresh skimmed, unpasteurised market milk (nono) in Makurdi, Nigeria: Implications for public health. *Journal of Animal and Plant Science, 1*(1), 21-25.

Okiki, P. A., Adeniji, C. A., Oyetunji, O. A., Yusuf, O. A., & Peters, O. (2018). Assessment of the physicochemical and bacteriological qualities of nono: A fermented cow milk. *Potravinarstvo Slovak Journal of Food Sciences, 12*(1), 26-32. doi:https://doi.org/10.5219/839

Okonkwo, O. I. (2011). Microbiological analyses and safety evaluation of nono: A fermented milk product consumed in most parts of Northern Nigeria. *International Journal of Dairy Science, 6*, 181-189. doi:10.3923/ijds.2011

Oliver, S. P., Boor, K. J., Murphy, S. C., & Murinda, S. E. (2009). Food Safety Hazards Associated with Consumption of Raw Milk. *Foodborne Pathogens and Disease, 6*(7), 793-806. Retrieved from <Go to ISI>://WOS:000269633500007. doi:10.1089/fpd.2009.0302

Oliver, S. P., Jayarao, B. M., & Almeida, R. A. (2005). Foodborne pathogens in milk and the dairy farm environment: Food safety and public health implications. *Foodbourne Pathogens & Disease, 2*(2), 115-129. doi:10.1089/fpd.2005.2.115

Oliver, S. P., & Murinda, S. E. (2011). Milk and raw milk consumption as a vector for human disease. In D. O. Krause & S. Hendrick (Eds.), *Zoonotic Pathogens in the Food Chain* (pp. 99-118). UK: CAB International.

Oliwa-Stasiak, K., Kolaj-Robin, O., & Adley, C. C. (2011). Development of real-time PCR assays for detection and quantification of *Bacillus cereus* group species: differentiation of *B. weihenstephanensis* and rhizoid *B. pseudomycoides* isolates from milk. *Applied and Environmental Microbiology, 77*(1), 80-88. doi:10.1128/AEM.01581-10

Olugbuyiro, J. A. (2011). Physico-chemical and sensory evaluation of market yoghurt in Nigeria. *Pakistan Journal of Nutrition, 10*(10), 914-918.

Osvik, R. D., Sperstad, S., Breines, E. M., Godfroid, J., Zhou, Z., Ren, P., . . . Ringø, E. (2013). Bacterial diversity of amasi, a South African fermented milk product, determined by clone library and denaturing gradient gel electrophoresis analysis.

African Journal of Microbiology Research, 7(22), 4146-4158. doi:10.5897/AJMR12.2317

Panesar, P. S. (2011). Fermented dairy products: Starter cultures and potential nutritional benefits. *Food and Nutrition Sciences, 2*(1), 47-51. doi:10.4236/fns.2011.21006

Pederson, C. S. (1971). Microbiology of food fermentations. *Microbiology of food fermentations.*

Phanchung, Dorji, P., Sonam, T., & Pelden, K. (2002). Smallholder dairy farming in Bhutan: Characteristics, constraints, and development opportunities. In P. M. Tulachan, M. A. Jabbar, & M. A. M. Saleem (Eds.), *Smallholder dairy in mixed farming systems of the HKH.* (pp. 19-34). Kathmandu, Nepal: International Centre for Integrated Mountain Development.

Porcellato, D., Narvhus, J., & Skeie, S. B. (2016). Detection and quantification of *Bacillus cereus* group in milk by droplet digital PCR. *Journal of microbiological methods, 127*, 1-6. doi:10.1016/j.mimet.2016.05.012

Postollec, F., Falentin, H., Pavan, S., Combrisson, J., & Sohier, D. (2011). Recent advances in quantitative PCR (qPCR) applications in food microbiology. *Food microbiology, 28*(5), 848-861. doi:10.1016/j.fm.2011.02.008

Pouillot, R., Hoelzer, K., Jackson, K. A., Henao, O. L., & Silk, B. J. (2012). Relative risk of listeriosis in foodborne diseases active surveillance betwork (FoodNet) sites according to age, pregnancy, and ethnicity. *Clinical Infectious Diseases, 54*(suppl_5), S405-S410. Retrieved from https://doi.org/10.1093/cid/cis269. doi:10.1093/cid/cis269

Prajapati, J. B., & Nair, B. M. (2003). The history of fermented foods. In E. R. F. Farnworth (Ed.), *Handbook of fermented functional foods* (Vol. 1, pp. 1-25). Boca Raton: CRC Press.

Prudêncio, C. V., Dos Santos, M. T., & Vanetti, M. C. D. (2015). Strategies for the use of bacteriocins in Gram-negative bacteria: Relevance in food microbiology. *Journal of food science and technology, 52*(9), 5408-5417. doi:10.1007/s13197-014-1666-2

Prüß, B. M., Dietrich, R., Nibler, B., Märtlbauer, E., & Scherer, S. (1999). The hemolytic enterotoxin HBL is broadly distributed among species of the *Bacillus cereus* group. *Applied and Environmental Microbiology, 65*(12), 5436-5442.

Puniya, A. K., Kumar, S., Puniya, M., & Malik, R. (2015). Fermented milk and dairy products: An overview. In A. K. Puniya (Ed.), *Fermented milk and dairy products* (Vol. 1, pp. 32-53). Boca Raton: CRC Press.

Qiagen. (2019). QIAamp biostic bacteremia DNA kit. Retrieved from https://www.qiagen.com/us/shop/sample-technologies/dna/genomic-dna/qiaamp-biostic-bacteremia-dna-kit/#orderinginformation

Quigley, L., McCarthy, R., O'Sullivan, O., Beresford, T. P., Fitzgerald, G. F., Ross, R. P., . . . Cotter, P. D. (2013). The microbial content of raw and pasteurized cow milk as determined by molecular approaches. *Journal of Dairy Science, 96*(8), 4928-4937. Retrieved from <Go to ISI>://WOS:000322055900015. doi:10.3168/jds.2013-6688

Quigley, L., O'Sullivan, O., Beresford, T. P., Ross, R. P., Fitzgerald, G. F., & Cotter, P. D. (2011). Molecular approaches to analysing the microbial composition of raw milk and raw milk cheese. *International Journal of Food Microbiology, 150*(2-3), 81-94. doi:10.1016/j.ijfoodmicro.2011.08.001

Quigley, L., O'sullivan, O., Stanton, C., Beresford, T. P., Ross, R. P., Fitzgerald, G. F., & Cotter, P. D. (2013). The complex microbiota of raw milk. *FEMS, 37*(5), 664-698. doi:10.1111/1574-6976.12030

R Core Team. (2017). R: A language and environment for statistical com-puting. *R Foundation for Statistical Computing, Vienna, Austria. URL https://www. R-project. org*.

Rai, R., Shangpliang, H. N. J., & Tamang, J. P. (2016). Naturally fermented milk products of the Eastern Himalayas. *Journal of Ethnic Foods, 3*(4), 270-275. doi:10.1016/j.jef.2016.11.006

Rai, S. B. C., Sharma, P. M., Tenzinla., P., M., Tshomo, P., & Gurung, B. B. (2018). Microbiological quality of raw milk in Bhutan. *Bhutan journal of animal science, 2*(1), 75-81.

Rajkovic, A. (2014). Microbial toxins and low level of foodborne exposure. *Trends in Food Science & Technology, 38*(2), 149-157. doi:10.1016/j.tifs.2014.04.006

Rasko, D. A., Ravel, J., Økstad, O. A., Helgason, E., Cer, R. Z., Jiang, L., . . . Angiuoli, S. V. (2004). The genome sequence of *Bacillus cereus* ATCC 10987 reveals metabolic adaptations and a large plasmid related to *Bacillus anthracis* pXO1. *Nucleic acids research, 32*(3), 977-988. doi:10.1093/nar/gkh258

Renewable Natural Resources (Bhutan). (2018). *Statistics on dairy animal population and production trend from 2013 to 2017* (5 ed.). Thimphu, Bhutan: Renewable Natural Resources Statistics Division.

Reuben, C. R., Okolocha, E. C., Bello, M., & Tanimu, H. (2013). Occurrence and antibiogram of *Escherichia coli* O157: H7 in locally fermented milk (Nono) sold under market conditions in Nasarawa State, Nigeria. *International Journal of Science and Research, 2*(2), 591-598.

Rinzin, Y. C. (2017). *E. Coli* bacteria found in Thimphu and Paro rivers. *Kuensel*. Retrieved from http://www.kuenselonline.com/e-coli-bacteria-found-in-thimphu-and-paro-rivers/

Robinson, R. K., & Tamime, A. Y. (2006). Types of fermented milks. In A. Tamime (Ed.), *Fermented milks* (pp. 1-10). UK: Blackwell Science Ltd.

Rosengren, Å., Fabricius, A., Guss, B., Sylvén, S., & Lindqvist, R. (2010). Occurrence of foodborne pathogens and characterization of *Staphylococcus aureus* in cheese produced on farm-dairies. *International Journal of Food Microbiology, 144*(2), 263-269. doi:10.1016/j.ijfoodmicro.2010.10.004

Røssland, E., Langsrud, T., & Sørhaug, T. (2005). Influence of controlled lactic fermentation on growth and sporulation of *Bacillus cereus* in milk. *International Journal of Food Microbiology, 103*(1), 69-77. doi:10.1016/j.ijfoodmicro.2004.11.027

Sabat, G., Rose, P., Hickey, W., & Harkin, J. (2000). Selective and sensitive method for PCR amplification of *Escherichia coli* 16S rRNA genes in soil. *Applied and Environmental Microbiology, 66*(2), 844-849.

Sahebekhtiari, N., Nochi, Z., Eslampour, M., Dabiri, H., Bolfion, M., Taherikalani, M., . . . Emaneini, M. (2011). Characterization of *Staphylococcus aureus* strains isolated from raw milk of bovine subclinical mastitis in Tehran and Mashhad. *Acta microbiologica et immunologica Hungarica, 58*(2), 113-121. doi:10.1556/AMicr.58.2011.2.4

Samet-Bali, O., Felfoul, I., Lajnaf, R., Attia, H., & Ayadi, M. (2016). Hygienic quality of" Rayeb": A traditional Tunisian fermented cow's milk. *International Food Research Journal, 23*(1), 366-369.

Sarkar, S. (2008). Innovations in Indian fermented milk products: A review. *Food biotechnology, 22*(1), 78-97. doi:10.1080/08905430701864025

Schelin, J., Wallin-Carlquist, N., Cohn, T. M., Lindqvist, R., & Barker, G. C. (2011). The formation of *Staphylococcus aureus* enterotoxin in food environments and advances in risk assessment. *Virulence, 2*(6), 580-592. doi:10.4161/viru.2.6.18122

Schoeni, J. L., & Wong, L. A. C. (2005). *Bacillus cereus* food poisoning and its toxins. *Journal of Food Protection, 68*(3), 636-648.

Schukken, Y. H., Grommers, F. J., Van De Geer, D., Erb, H. N., & Brand, A. (1990). Risk factors for clinical mastitis in herds with a low bulk milk somatic cell count: Data and risk factors for all cases. *Journal of Dairy Science, 73*(12), 3463-3471. Retrieved from http://www.sciencedirect.com/science/article/pii/S0022030290790455. doi:10.3168/jds.S0022-0302(90)79045-5

Sekse, C., Holst-Jensen, A., Dobrindt, U., Johannessen, G. S., Li, W., Spilsberg, B., & Shi, J. (2017). High throughput sequencing for detection of foodborne pathogens. *Frontiers in Microbiology, 8*, 2029. doi:10.3389/fmicb.2017.02029

Shangpliang, H. N. J., Rai, R., Keisam, S., Jeyaram, K., & Tamang, J. P. (2018). Bacterial community in naturally fermented milk products of Arunachal Pradesh and Sikkim of India analysed by high-throughput amplicon sequencing. *Scientific reports, 8*(1532). doi:10.1038/s41598-018-19524-6

Shangpliang, H. N. J., Sharma, S., Rai, R., & Tamang, J. P. (2017). Some technological properties of lactic acid bacteria isolated from Dahi and Datshi, naturally fermented milk products of Bhutan. *Frontiers in Microbiology, 8*, 116. doi:10.3389/fmicb.2017.00116

Siebert, P. D., & Larrick, J. W. (1992). Competitive PCR. *Nature, 359*, 557-558.

Silva, W. P. D., Destro, M. T., Landgraf, M., & Franco, B. D. (2000). Biochemical characteristics of typical and atypical *Staphylococcus aureus* in mastitic milk and environmental samples of Brazilian dairy farms. *Brazilian Journal of microbiology, 31*(2), 103-106.

Simatende, P., Gadaga, T. H., Nkambule, S. J., & Siwela, M. (2015). Methods of preparation of Swazi traditional fermented foods. *Journal of Ethnic Foods, 2*(3), 119-125. doi:10.1016/j.jef.2015.08.008

Srinivasan, V., Sawant, A. A., Gillespie, B. E., Headrick, S. J., Ceasaris, L., & Oliver, S. P. (2006). Prevalence of enterotoxin and toxic shock syndrome toxin genes in

Staphylococcus aureus isolated from milk of cows with mastitis. *Foodbourne Pathogens and Disease, 3*(3), 274-283.

Steinkraus, K. H. (1997). Classification of fermented foods: Worldwide review of household fermentation techniques. *Food Control, 8*(5-6), 311-317. Retrieved from <Go to ISI>://WOS:000071279400010. doi:10.1016/s0956-7135(97)00050-9

Steinkraus, K. H. (2004). Origin and history of food fermentations. In Y. H. Hui, L. Meunier- Goddik, A. S. Hansen, J. Josephsen, W. K. Nip, P. S. Stanfield, & F. Toldra (Eds.), *Handbook of food and beverage fermentation technology* (pp. 1-8). USA: Marcel Dekker,Inc.

Steinkraus, K. H. (2018). Introduction to indigenous fermented foods. In K. H. Steinkraus (Ed.), *Handbook of indigenous fermented foods, second edition, revised and expanded* (Second edition ed., Vol. 2, pp. 1-6). Boca Raton: CRC Press.

Stevens, K. A., Sheldon, B. W., Klapes, N. A., & Klaenhammer, T. R. (1991). Nisin treatment for inactivation of *Salmonella* species and other gram-negative bacteria. *Applied and Environmental Microbiology, 57*(12), 3613-3615. Retrieved from <Go to ISI>://WOS:A1991GT94200035.

Sun, Z., Liu, W., Bao, Q., Zhang, J., Hou, Q., Kwok, L., . . . Zhang, H. (2014). Investigation of bacterial and fungal diversity in tarag using high-throughput sequencing. *Journal of Dairy Science, 97*(10), 6085-6096. doi:10.3168/jds.2014-8360

Sun, Z., Liu, W., Gao, W., Yang, M., Zhang, J., Wu, L., . . . Zhang, H. (2010). Identification and characterization of the dominant lactic acid bacteria from Kurut: The naturally fermented yak milk in Qinghai, China. *The Journal of general and applied microbiology, 56*(1), 1-10.

Swanson, K., Petran, R., & Hanlin, J. (2001). Culture methods for enumeration of microorganisms. In *Compendium of methods for the microbiological examination of foods* (Vol. 4, pp. 53-62).

Syed, Q. A., Buffa, M., Guamis, B., & Saldo, J. (2016). Factors affecting bacterial inactivation during high hydrostatic pressure processing of foods: A review. *Critical Reviews in Food Science and Nutrition, 56*(3), 474-483. doi:10.1080/10408398.2013.779570

Tallent, S. M., Kotewicz, K. M., Strain, E. A., & Bennett, R. W. (2012). Efficient isolation and identification of *Bacillus cereus* group. *Journal of AOAC International, 95*(2), 446-451. doi:10.5740/jaoacint.11-251

Tamang, J. P. (2010). Fermented milk. In J. P. Tamang (Ed.), *Himalayan fermented foods: Microbiology, nutrition, and ethnic values* (pp. 95-116). Boca Raton: CRC Press.

Tamang, J. P., & Kailasapathy, K. (2010). *Fermented foods and beverages of the world*. Boca Raton: CRC press.

Tamba, Z., Bello, M., & Raji, M. (2016). Occurence and antibiogram of *Salmonella* spp. In raw and fermented milk in zaria and environs. *Bangladesh Journal of Veterinary Medicine, 14*(1), 103-107.

Tenzin, T. (2018). *Status of notifiable animal diseases in Bhutan for 2017*. Retrieved from Serbithang, Thimphu: http://www.ncah.gov.bt/Downloads/File_218.pdf

Tirloni, E., Ghelardi, E., Celandroni, F., Bernardi, C., & Stella, S. (2017). Effect of dairy product environment on the growth of *Bacillus cereus*. *Journal of Dairy Science, 100*(9), 7026-7034. doi:10.3168/jds.2017-12978

Tong, S. Y. C., Davis, J. S., Eichenberger, E., Holland, T. L., & Fowler, V. G. (2015). *Staphylococcus aureus* infections: Epidemiology, pathophysiology, clinical manifestations, and management. *Clinical microbiology reviews, 28*(3), 603-661. doi:10.1128/CMR.00134-14

Troller, J., & Scott, V. (1992). Measurement of water activity (aw) and acidity. *Compendium of Methods for the Microbiological Examination of Foods. American Public Health Association, Washington, DC AquaLab, 14*, 135-151.

Tshering, D., & Gyem, K. (2015). Prevalence of clinical and sub-clinical mastitis in lactating dairy cows at the National Jersey Breeding Centre, Samtse, Bhutan. *Bhutan Journal of Natural Resources & Development, 2*(1), 33-39. doi:10.17102/cnr.2015.05

van Netten, P., & Kramer, J. M. (1992). Media for the detection and enumeration of *Bacillus cereus* in foods: A review. *International Journal of Food Microbiology, 17*(2), 85-99. Retrieved from http://www.sciencedirect.com/science/article/pii/016816059290108F. doi:https://doi.org/10.1016/0168-1605(92)90108-F

Venema, K., Venema, G., & Kok, J. (1995). Lactococcal bacteriocins: Mode of action and immunity. *Trends in microbiology, 3*(8), 299-304.

Verraes, C., Vlaemynck, G., Van Weyenberg, S., De Zutter, L., Daube, G., Sindic, M., . . . Herman, L. (2015). A review of the microbiological hazards of dairy products made from raw milk. *International Dairy Journal, 50*, 32-44. Retrieved from <Go to ISI>://WOS:000361187200005. doi:10.1016/j.idairyj.2015.05.011

Walsh, A. M., Crispie, F., Daari, K., O'Sullivan, O., Martin, J. C., Arthur, C. T., . . . Cotter, P. D. (2017). Strain-level metagenomic analysis of the fermented dairy beverage nunu highlights potential food safety risks. *Applied and Environmental Microbiology, 83*(16), e01144-01117. doi:10.1128/AEM.01144-17.

Wangdi, J., Dema, T., Karma, M., & Bhujel, P. (2014). The compositional quality of cow's milk in Bhutan. *Issues in Biological Sciences and Pharmaceutical Research, 2*(7), 62-68.

Wanjala, W., Nduko, J., & Mwende, M. (2018). Coliforms contamination and hygienic status of milk chain in emerging economies. *Journal of food quality and hazards control, 5*(1), 3-10.

Watanabe, K., Fujimoto, J., Sasamoto, M., Dugersuren, J., Tumursuh, T., & Demberel, S. (2008). Diversity of lactic acid bacteria and yeasts in Airag and Tarag, traditional fermented milk products of Mongolia. *World Journal of Microbiology and Biotechnology*, *24*(8), 1313-1325.

West, H. G. (2008). Food fears and raw-milk cheese. *Appetite, 51*(1), 25-29. Retrieved from <Go to ISI>://WOS:000256227700008. doi:10.1016/j.appet.2008.02.004

WHO. (2015). *WHO estimates of the global burden of foodborne diseases: Foodborne disease burden epidemiology reference group 2007-2015*. Retrieved from

Wilson, D. J., Gonzalez, R. N., & Das, H. H. (1997). Bovine mastitis pathogens in New York and Pennsylvania: Prevalence and effects on somatic cell count and milk production. *Journal of Dairy Science, 80*(10), 2592-2598.

Wouters, J. T., Ayad, E. H., Hugenholtz, J., & Smit, G. (2002). Microbes from raw milk for fermented dairy products. *International Dairy Journal, 12*(2-3), 91-109.

Yakubu, Y., Shuaibu, A. B., Ibrahim, A. M., Hassan, U. L., & Nwachukwu, R. J. (2018). Risk of Shiga Toxigenic *Escherichia coli* O157: H7 infection from raw and fermented milk in Sokoto Metropolis, Nigeria. *Journal of pathogens, 2018*, 1-5.

Yousef, A. E., & Courtney, P. D. (2003). Basics of stress adaptation and implications in new-generation foods. *Microbial stress adaptation and food safety, 1*, 1-30.

Yucel, N., & Ulusoy, H. (2006). A Turkey survey of hygiene indicator bacteria and *Yersinia enterocolitica* in raw milk and cheese samples. *Food Control, 17*(5), 383-388.

Zadoks, R., Gonzalez, R., Boor, K., & Schukken, Y. (2004). Mastitis-causing *Streptococci* are important contributors to bacterial counts in raw bulk tank milk. *Journal of Food Protection, 67*(12), 2644-2650.

Zakary, E. M., Nassif, M. Z., & Mohammed, G. (2011). Detection of *Staphylococcus aureus* in bovine milk and its product by real time PCR assay. *Global Journal of Biotechnology and Biochemistry, 6*(4), 171-177.

Zastempowska, E., Grajewski, J., & Twarużek, M. (2016). Food-borne pathogens and contaminants in raw milk: A review. *Annals of Animal Science, 16*(3), 623-639. doi:https://www.degruyter.com/downloadpdf/j/aoas.2016.16.issue-3/aoas-2015-0089/aoas-2015-0089.pdf

Zeder, M. A., & Hesse, B. (2000). The initial domestication of goats (Capra hircus) in the Zagros mountains 10,000 years ago. *Science, 287*(5461), 2254-2257.

Zhang, Z., Feng, L., Xu, H., Liu, C., Shah, N. P., & Wei, H. (2016). Detection of viable enterotoxin-producing *Bacillus cereus* and analysis of toxigenicity from ready-to-eat foods and infant formula milk powder by multiplex PCR. *Journal of Dairy Science, 99*(2), 1047-1055.

Zhang, Z., Tao, X., Shah, N. P., & Wei, H. (2016). Antagonistics against pathogenic *Bacillus cereus* in milk fermentation by *Lactobacillus plantarum* ZDY2013 and its anti-adhesion effect on Caco-2 cells against pathogens. *Journal of Dairy Science, 99*(4), 2666-2674.

Zhao, X., Lin, C.-W., Wang, J., & Oh, D. H. (2014). Advances in rapid detection methods for foodborne pathogens. *Journal of Microbiology and Biotechnology, 24*(3), 297-312. doi:10.4014/jmb.1310.10013

APPENDICES

Appendix A. Microbiological criteria of FSANZ

Interpretation of results

The tables below provide guidance on interpreting results for the microbiological examination of RTE foods for pathogenic microorganisms and for indicator microorganisms. The limits apply to foods sampled in the retail chain (i.e. food for sale at retail, food service wholesale and distribution) up to and including end of shelf life.

There are four categories of microbiological assessment defined based on the detection or level of microorganism found:

- Satisfactory: results are within expected microbiological levels (lower range) and present no food safety concern. No action required.
- Marginal: results are within expected microbiological levels but are at the upper range. Some action may be required to ensure food handling controls continue to be effective.
- Unsatisfactory: results are outside expected microbiological levels and indicate poor food handling practices. Further actions are required to re-establish effective food handling controls.
- Potentially hazardous: results exceed expected microbiological levels to a level that presents an immediate food safety concern. Further action is required to:
  - prevent affected product still available from being distributed or sold
  - determine the likely source/cause of the problem and ensure corrective actions are implemented.

Interpretation of results should also be based on knowledge of the food product and the production process. Care must be taken when interpreting results obtained in the absence of this information.

Appendix A1. Interpreting results for testing of pathogenic microorganisms in RTE food

Hazard	Result (cfu/g)	Interpretation	Likely cause	Actions
Bacillus cereus and other pathogenic *Bacillus* spp.	$>10^5$	Potentially hazardous	Inadequate time and temperature control during cooling and subsequent storage allowing spores to germinate and multiply. The use of poor quality highly contaminated raw ingredients, such as plant-based powders and spices, may also be a contributing factor. Inadequate acidification of foods using pH to control growth (e.g. acidified rice for sushi).	• Product disposition action required to assess safety and determine if disposal or product recall is required. Reprocessing of product not an option due to potential for toxin formation. • Investigate and review temperature and time profiles used for the cooling and storage of cooked foods. • Identify high-risk raw ingredients and consider limits for *B. cereus.* • Investigate pH and acidification process (as applicable).
	10^3– $\leq 10^5$	Unsatisfactory	As above.	• Investigate and review temperature and time profiles used for the cooling and storage of cooked foods. • Identify high-risk raw ingredients and consider limits for *B. cereus.*
	10^2 – $<10^3$	Marginal	Process controls not fully achieved or possible raw material contamination.	• Proactive investigation to ensure temperature and time profiles used for cooling and storage of cooked foods are being implemented. • Assess quality of high-risk raw ingredients.
	$<10^2$	Satisfactory		
Staphylococcus aureus and other coagulase-positive staphylococci	$>10^4$	Potentially hazardous	Inadequate temperature control and poor hygienic practices.	• Product disposition action required to assess safety and determine if disposal or product recall is required. Reprocessing of product not an option due to potential for toxin formation. • Food handling practices should be investigated to: ➢ ensure all practicable measures are being undertaken by food handlers to prevent unnecessary contact with RTE food ➢ ensure good levels of personal hygiene ➢ review temperature and time controls. • Testing for enterotoxin should be considered where cases of foodborne illness are suspected.

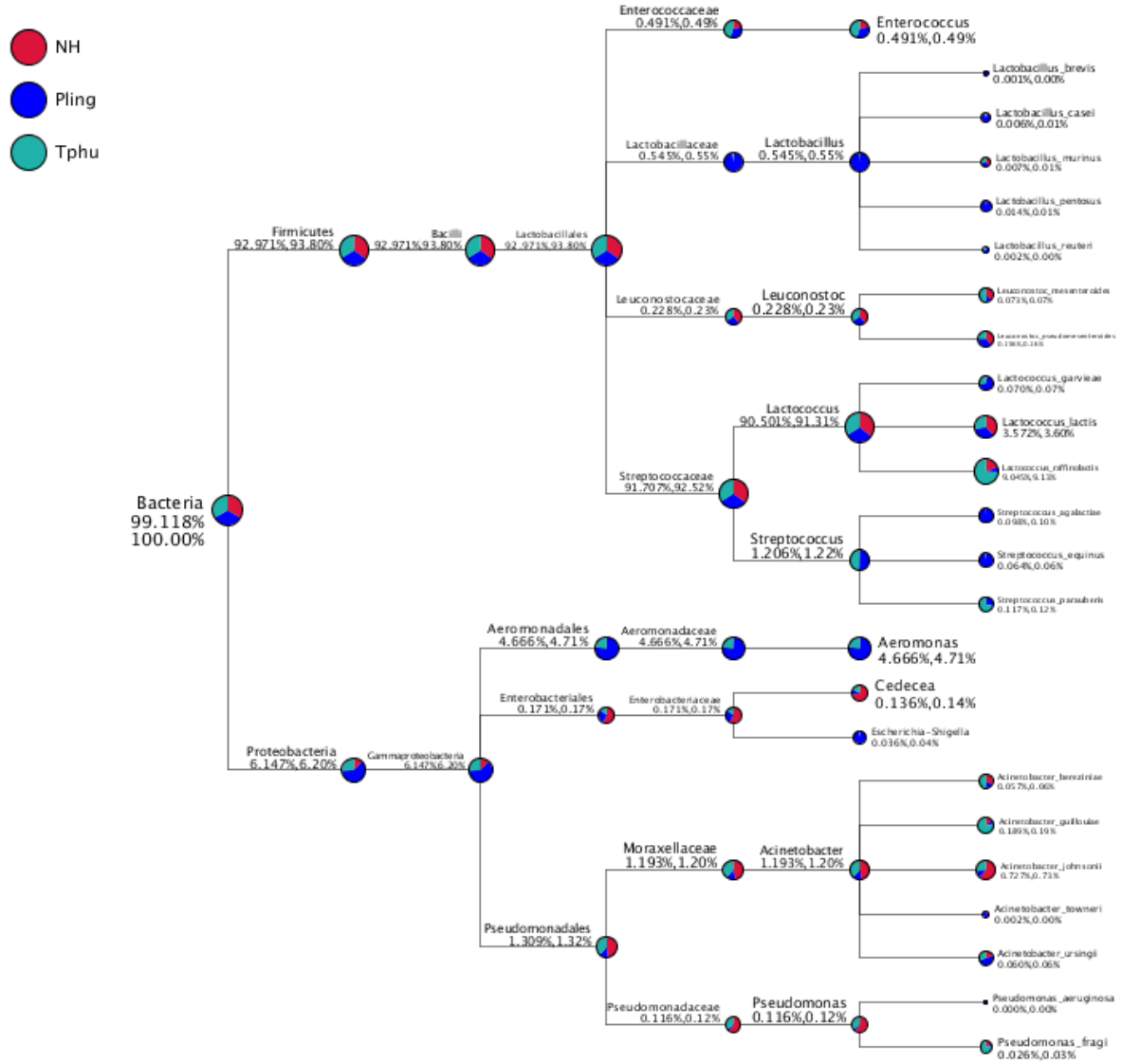
	$10^3 – \leq 10^4$	Unsatisfactory	As above.	• Food handling practices should be investigated as above. • The level of *S. aureus* determined at the time of analysis may not be the highest level that occurred in the food. If cases of foodborne illness are suspected, testing for enterotoxin should be considered. • Proactive investigation to ensure hygiene practices and temperature controls are effectively implemented.
	10^2 - $<10^3$	Marginal	Hygiene and handling controls not fully achieved	
	$<10^2$	Satisfactory		
Shiga toxin-producing *Escherichia coli* (STEC)	Detected in 25g	Potentially hazardous	Inadequate processing of raw products or cross contamination of raw materials and prepared foods. Poor time and temperature control is a contributing factor for multiplication.	• Product disposition action required to assess safety and determine if disposal or product recall is required. • An investigation should be undertaken to assess: ➢ raw material suitability ➢ the adequacy of processing used (e.g. adequate cooking, pH, water activity) ➢ the adequacy of measures implemented to prevent the likelihood of cross contamination ➢ the adequacy of time and temperature controls used. • Additional sampling of foods and environmental samples may be required. • Confirmation of toxigenic strains and serotyping required where cases of foodborne illness suspected.
	Not detected in 25g	Satisfactory		

Appendix A2. Interpreting results for testing of indicator organisms in RTE foods

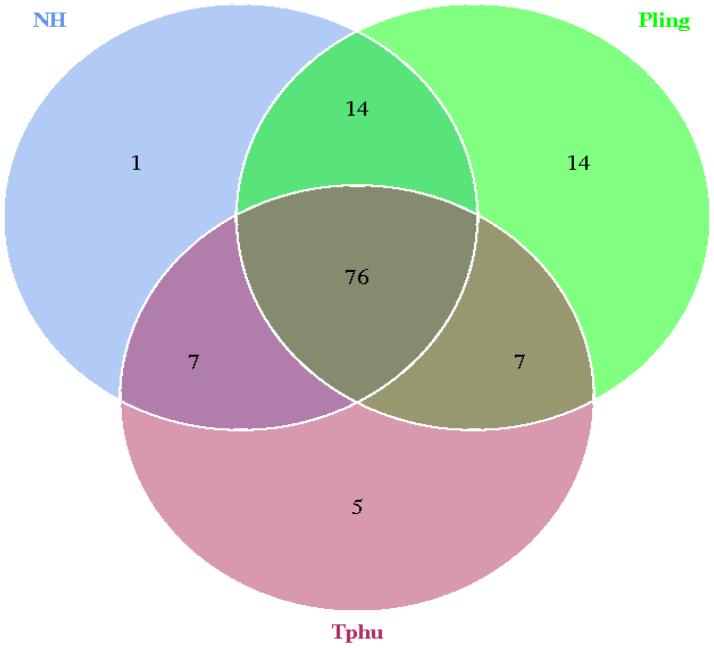
Indicator	Result (cfu/g)	Interpretation	Likely cause	Actions
Escherichia coli	$>10^2$	Unsatisfactory	For raw and processed foods indicates potential for there to have been contamination of faecal origin from poor hygienic practices (cross contamination from food contact surfaces, raw foods or food handlers) or there has been inadequate processing. For RTE foods that have not been processed (e.g. fresh produce), contamination from the primary production environment should be considered	• Review: ➢ processing controls used (such as cooking temperatures) ➢ cleaning and sanitising practices for premises and equipment ➢ food handler hygiene ➢ time and temperature control ➢ primary production controls (e.g. harvest practices, water quality, fertilizers, other inputs as appropriate). Additional food or environmental samples may be required for investigation and testing for enteric pathogens considered if appropriate.
	3 - $<10^2$	Marginal	While low levels may occasionally be found in RTE food, widespread detection in several foods or areas of the food production environment suggests poor hygienic practices.	Proactive investigation to ensure processing and hygiene controls are being implemented.
	<3	Satisfactory		

From "Compendium of microbiological criteria for food" by FSANZ (2018a) (http://www.foodstandards.gov.au/publications/Documents/Compedium%20of%20Microbiological%20Criteria/Compendium_revised-Sep%202018.pdf). p. 9-16. CC BY 3.0

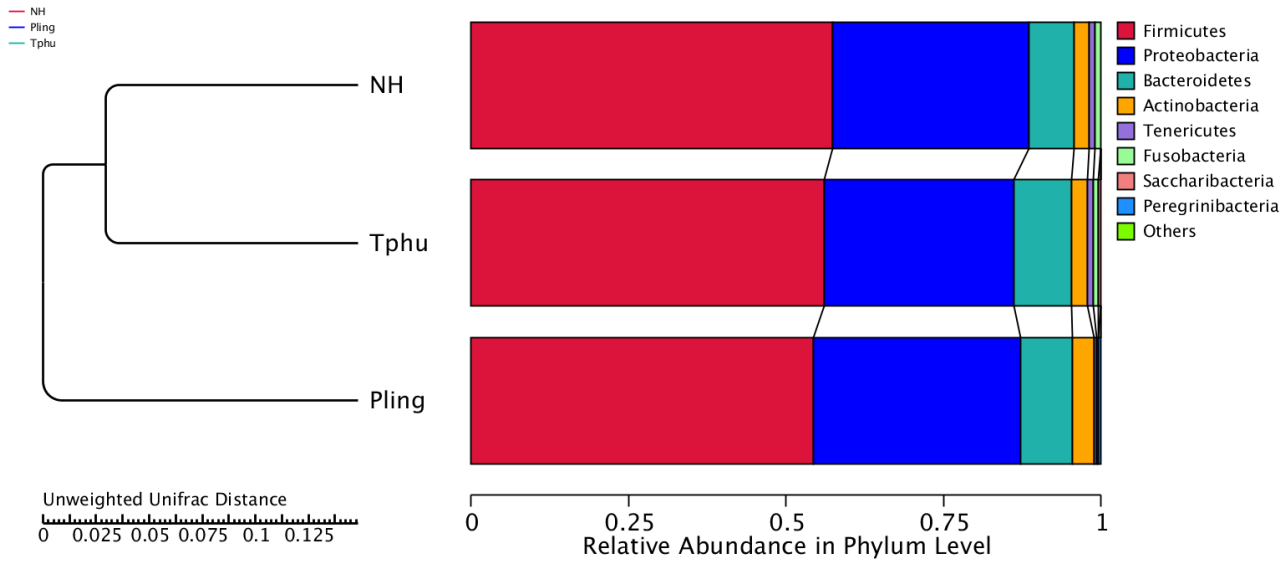
Appendix B. Taxonomy tree of NFRM in Bhutan



Appendix C. Venn diagram showing common and unique OTUS in NFRM in Thimphu, Phuntsholing and NH

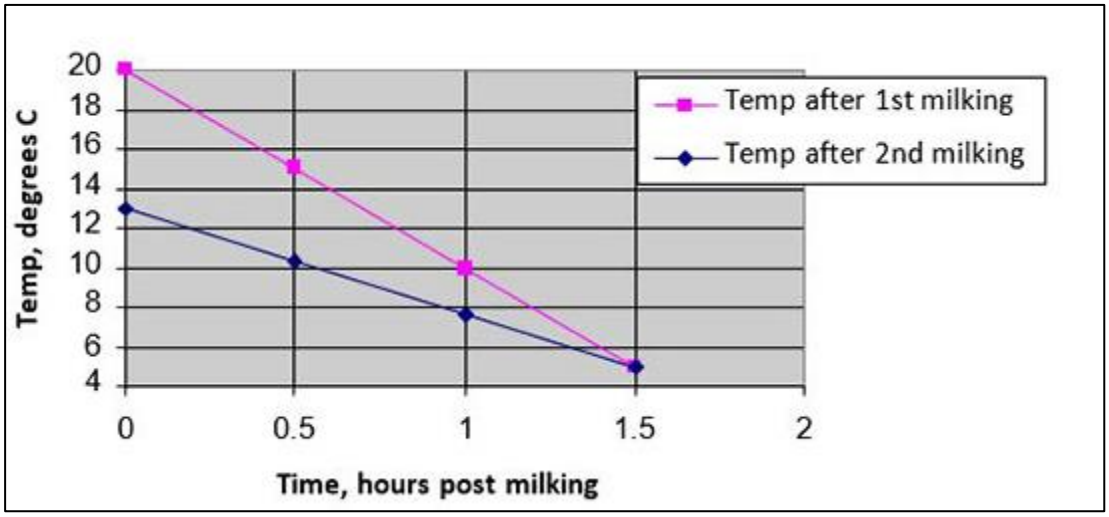


Appendix D. UPGMA tree



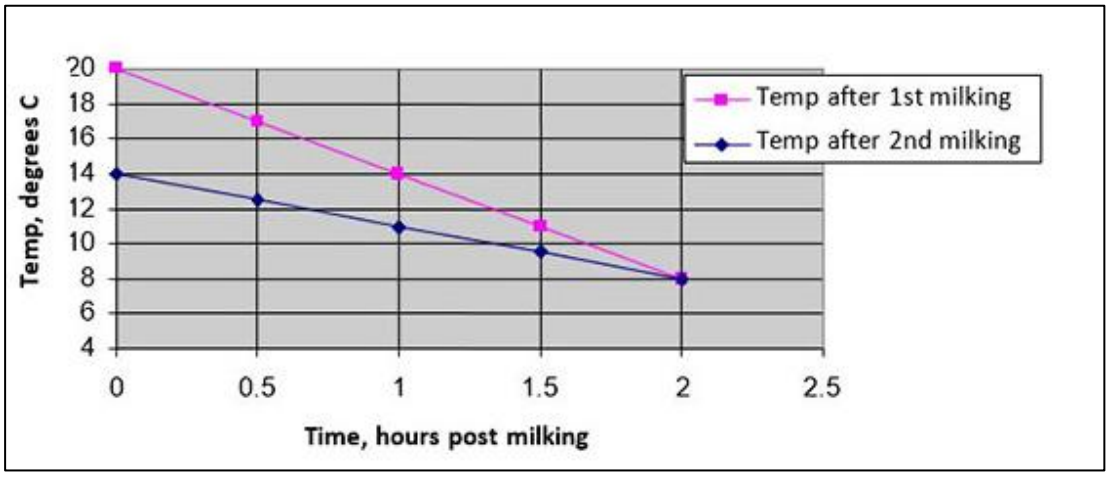
Appendix E. Post-milking cooling curves

Appendix E1. Post- milking cooling curve to meet FSANZ guidelines for RM



From "Raw milk temperatures" by FSANZ (2018b) (http://www.agriculture.gov.au/export/controlled-goods/dairy/registered-establishment/raw-milk-temperatures) CC BY 3.0.

Appendix E2. Post- milking curve to meet EC requirements for heat treated milk products



From "Raw milk temperatures" by FSANZ (2018b) (http://www.agriculture.gov.au/export/controlled-goods/dairy/registered-establishment/raw-milk-temperatures) CC BY 3.0.