INVESTIGATION INTO THE MECHANISMS BEHIND THE DIFFERENTIAL INHIBITION OF *LISTERIA MONOCYTOGENES* IN SOFT-RIPENED CHEESES

by

Justin Falardeau

B.Sc., Carleton University, 2014

M.Sc. University of British Columbia, 2017

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The following individuals certify that they have read, and recommend to the Faculty of Graduate and Postdoctoral Studies for acceptance, the dissertation entitled:

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Examining Committee:

Siyun Wang, Associate Professor, Food, Nutrition and Health, UBC
Supervisor

Christine Scaman, Associate Professor, Food, Nutrition and Health, UBC
Supervisory Committee Member

Steven Hallam, Professor, Microbiology & Immunology, UBC
Supervisory Committee Member

Vivien Measday, Associate Professor, Wine Research Centre, UBC
University Examiner

Richard Barichello, Professor, Food and Resource Economics, UBC
University Examiner

Abstract

Soft-ripened cheeses (*e.g.*, Camembert; SRC) are at risk for the growth of the *Listeria monocytogenes* due to favourable moisture and pH compared to other cheeses; however, the *L. monocytogenes* growth is not uniform across all SRC. The cheese microbiome, which is influenced by the farm and processing environments, may affect *L. monocytogenes* growth. Therefore, the purpose of this thesis was to investigate how the taxonomic and functional profiles of SRC microbiomes may affect the growth of *L. monocytogenes*.

Forty-three SRC were inoculated with *L. monocytogenes*, and pathogen growth was monitored over 12 days at 8°C by select plating. Cheeses were produced from both raw (n=12) and pasteurized (n=31) milk. The taxonomic and functional profiles of the cheeses microbiomes was investigated using 16S rRNA targeted amplicon sequencing, shotgun metagenomic sequencing of 39 and 15 cheeses, respectively.

L. monocytogenes growth differed significantly between cheeses (ANOVA; $p < 0.001$), with increases ranging from 0 to 5.4 log CFU (mean of 2.5 ± 1.2 log CFU) over the incubation period. Raw milk cheeses showed significantly lower *L. monocytogenes* growth than pasteurized milk cheeses (t-test; $p = 0.008$), possibly due to an increase in microbial richness. *L. monocytogenes* growth in cheeses was positively correlated with the relative abundance of *Streptococcus thermophilus* (Spearman correlation; $p < 0.0001$), and negatively correlated with the relative abundances of *Brevibacterium aurantiacucm* (Spearman correlation; $p = 0.0002$) and two *Lactococcus* spp. (Spearman correlation; $p < 0.01$). The inclusion of *S. thermophilus* as a starter

culture is more common in industrialize SRC production, suggesting that industrial production of SRC may increase the risk of *L. monocytogenes* growth.

L. monocytogenes growth was negatively correlated with the relative abundance of two metabolic pathways related to formaldehyde oxidation and β-D-glucuronosides degradation. No mechanism was identified for the antilisterial effects of these pathways, but it may involve the depletion of micronutrients. The presence of bacteriocin-encoding genes did not affect the growth of *L. monocytogenes* in the cheeses.

These results further our understanding of the cheese microbiome's affect on *L. monocytogenes* growth, hopefully leading to the development of SRC starter/ripening cultures that can prevent *L. monocytogenes* growth.

Lay Summary

Soft ripened cheeses, like Camembert, are at risk for the growth of the foodborne pathogen, *Listeria monocytogenes*, but the growth of this pathogen is not uniform across all soft ripened cheeses and may be inhibited by the naturally occurring microbiome in these cheeses. Importantly, the microbiome in these cheeses is affected the farm and production environments, and so differs between producers. In this study I investigated the relationship between the microbiome and the growth of *L. monocytogenes* across 39 soft ripened cheeses. The relative abundances of four species of bacteria in the cheeses were correlated with the growth of this foodborne pathogen; one positively correlated, and three negatively correlated. Two metabolic pathways in the cheese metagenome were also negatively correlated with *L. monocytogenes* growth. This work sheds light on the attributes of the cheese microbiome that affect *L. monocytogenes* growth and will help develop methods of producing safer cheeses.

Preface

A version of Chapter 1 has been published in Comprehensive Reviews in Food Science and Food Safety (Falardeau J, Trmčić A, Wang S. 2021. The occurrence, growth, and biocontrol of *Listeria monocytogenes* in fresh and surface-ripened soft and semisoft cheeses. *Compr. Rev. Food Sci. Food Saf.* 20:4019-4048). I conceived the topic of the review, collected and reviewed all cited literature, and drafted and formatted the manuscript. A. Trmčić provided critical feedback and personal expertise towards analysis of the literature. S. Wang provided supervision, feedback, and valuable suggestions throughout the production of the manuscript. All three authors reviewed and approved the final manuscript. S. Wang was the supervisory author.

A version of Chapter 2 has been published in Food Microbiology (Falardeau J, Keeney K, Trmčić A, Kitts D, Wang S. 2019. Farm-to-fork profiling of bacterial communities associated with an artisan cheese production facility. *Food Microbiol*. 83:48-58). K. Keeney conceived the study, collected the samples, and performed the DNA extractions. DNA sequencing was conducted by Genome Quebec, and sequence assembly and filtering were conducted by Dr. Yusanne Ma and Mr. Greg Taylor at the British Columbia Cancer Agency. Data analysis and interpretation was conducted by me with some guidance from A. Trmčić. I also drafted and formatted the original manuscript. All authors helped with reviewing and editing the manuscript. S. Wang was the supervisory author.

I performed the majority of the research described in Chapters 3, 4, and 5 with a few exceptions. Mr. Erkan Yildiz, a visiting scholar, performed many of the *L. monocytogenes* growth assays under my supervision. The 16S rRNA targeted amplicon sequencing of the cheese microbiomes was

conducted at the Biofactorial High-Throughput Biology Facility at the Life Sciences Institute of the University of British Columbia. The shotgun metagenomic sequencing of the cheese microbiomes was conducted by the University of Sequencing and Bioinformatics Consortium at the University of British Columbia. Feedback on experimental design and data interpretation was provided by Drs. Siyun Wang, Steven Hallam, and Christine Scaman.

All experiments involving animals were approved by the UBC Animal Care Committee (ID# A14-0279).

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List of Symbols

a_w	Water activity
bp	base pairs
cm	Centimeters
g	Grams
IU	International units
Km	Kilometers
L	Litres
M	Moles/litre
mg	Milligrams
ml	Millilitres
n	Number of samples/replicates analyzed
OD_{600}	Optical density at 600nm wavelength
p	*p*-value: probability of a type I statistical error
q-value	*p*-value corrected for multiple comparisons using the false-discovery rate method
r	Pearson's correlation coefficient
ρ	Spearman's rank correlation coefficient
R^2	Proportion of variance explained by a regression model
R^2_{adj}	Adjustment of R^2, accounting for the number of variables in the regression model

List of Abbreviations

ANI	Average nucleotide identity
ANOVA	Analysis of variance
AUC	Area under the curve
CFU	Colony forming units
CPM	Copies per million
DNA	Deoxyribonucleic acid
EC	Enzyme Commission
EU	European Union
FAO	Food and Agriculture Organization of the United Nations
FDR	False discovery rate
HQMAG	High quality metagenomically assembled genome
HTS	High throughput sequencing
LAB	Lactic acid bacteria
MAG	Metagenomically assembled genome
MFFB	Moisture content on a fat free basis
NMDS	Non-metric multidimensional scaling
NSLAB	Non-starter lactic acid bacteria
OTU	Operational taxonomic unit
PBS	Phosphate buffered saline
PC	Principal component
PCA	Principal component analysis
PCoA	Principal coordinates analysis

PE	Paired-ends
PFU	Plaque forming units
PERMANOVA	Permutational multivariate analysis of variance
rRNA	Ribosomal ribonucleic acid
RNA-Seq	RNA sequencing
RTE	Ready-to-eat
RT-PCR	Reverse transcriptase polymerase chain reaction
SRC	Soft ripened cheeses
TAMC	Total aerobic microbial count
TLAB	Total lactic acid bacteria
TSA	Tryptic soy agar
TSB-YE	Tryptic soy broth with 0.6% yeast extract
UBC	University of British Columbia
WHO	World Health Organization

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To my amazing wife, **Vicki**: I could never have accomplished this without you by my side. It has been a long road for both of us, but your consistent love and support has made this all possible. You stood with me when I started down this path (planning to only pursue a bachelor's degree!), and you are still standing with me now. You have always been my biggest cheerleader. I can't thank you enough for everything you have sacrificed and done for me. I love you more than words can say.

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To my supervisory committee members, **Drs. Christine Scaman and Steven Hallam**: Your feedback and assistance throughout this journey has been insightful. Thank you for imparting your wisdom and dedication to the proper scientific method. I have learned so much from both of you.

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For my son, Atticus:

May this be a reminder of what you can accomplish

wiht hard work, dedication, and

a loving and supporting family.

Chapter 1: Introduction and Literature Review

1.1 Introduction

Listeria monocytogenes is a Gram-positive, intracellular foodborne pathogen, and a common cause of foodborne related deaths in the developed world with a mortality rate estimated to be between 20% and 40% (Public Health Agency of Canada, 2011). The species was first described in 1926, but only came to prominence as a foodborne pathogen of importance in the 1980s, due to it's identification as a causative agent in a series of foodborne outbreaks (Farber & Peterkin, 1991). *L. monocytogenes* infection most commonly affects immunocompromised individuals, such as the elderly, as well as pregnant women and newborns, and can result in severe symptoms such as meningitis, septicemia, and miscarriage/stillbirth. In healthy individuals, listeriosis infection is less severe, showing as mild influenza and gastroenteritis (Thakur et al., 2018). Pathogenicity in *L. monocytogenes* involves a variety of virulence factors located on a pathogenicity island. These virulence factors allow the pathogen to invade the epithelial cells of the intestine, travel between cells through actin polymerization within the cytoplasm, and survive inside macrophage. Through these mechanisms, *L. monocytogenes* cells can travel through the bloodstream to various organs, including across the blood brain barrier and the placental barrier (Radoshevich & Cossart, 2018). The infectious dose is suggested to be high, ranging between 7 and 9 log CFU (Public Health Agency of Canada, 2011).

Ubiquitous in the environment, *L. monocytogenes* can easily enter food processing facilities where it can establish itself and become persistent (V. Ferreira et al., 2014). Further, *L. monocytogenes* is able to overcome many hurdles associated with food preservation, including up to 20% salinity, high acidity, and has the ability to grow at temperatures ranging from 1°C to 45°C (Wiedmann &

Sauders, 2007). These survival characteristics make *L. monocytogenes* an important risk in ready-to-eat (RTE) products since no heating step is included to eliminate the pathogen before consumption.

Soft ripened cheeses (*e.g.*, Camembert; SRC) are common RTE foods that support survival and growth of *L. monocytogenes*. Soft cheeses are defined as cheeses with a moisture on a fat free basis of greater than 67% (Canadian Dairy Information Centre, 2020; Codex Alimentarius Commission, 1978). Soft ripened cheeses are enzymatically coagulated before ripening and can be classified as either bloomy rind or washed rind cheeses. Bloomy rind SRC, characterized by the presence of white mold (*i.e.*, *Penicillium camemberti*) which grows on the cheese surface (Spinnler, 2017). In washed rind cheeses, sometime referred to as smear-ripened or red-smear cheeses, a mixture of bacteria and yeast dominate and ripen the surface of the cheese due to periodic washing of the rind with a brine solution (Jérôme Mounier et al., 2017). In both mold- and smear-ripened cheeses, acids are consumed by yeasts and molds during surface ripening which results in an increased pH of the cheese.

Due to a favourable water activity (a_w) and pH in SRC, contaminating *L. monocytogenes* can grow to dangerous levels, both during the ripening period (S. Liu et al., 2007; Lobacz et al., 2013), or at retail (Lahou & Uyttendaele, 2017). As such, several listeriosis outbreaks have been linked to soft cheeses, including SRC. In the United States, soft cheeses were responsible for 17 outbreaks of listeriosis between 2000 and 2014, resulting in 180 illnesses, 17 deaths, and 14 fetal losses. Further, 88% of patients were hospitalized, emphasizing the severity of this foodborne pathogen (Jackson et al., 2018). Similarly, multiple outbreaks of listeriosis associated with soft cheeses have

been reported in Canada (Gaulin et al., 2012; Lorraine McIntyre et al., 2015), and Europe (Fretz et al., 2010; Koch et al., 2010; Lundén et al., 2004). Therefore, new or additional methods are required to prevent and control the growth of *L. monocytogenes* in these cheeses.

Biological control, or biocontrol/biopreservation, is the use of antagonistic organisms and/or their metabolites to control or prevent the growth of another, undesirable organism (Ananou et al., 2007; Lynn McIntyre et al., 2012). As many cheeses are a product of microbiological fermentation, and already possess a large and diverse microbiome (Afshari et al., 2020; Falardeau et al., 2019), they are a good candidate for the use of microbiological control methods against *L. monocytogenes*. Methods of biocontrol that have been tested experimentally in soft cheeses include the addition of bacteriocins or bacteriocin-producing strains of bacteria, and the use of bacteriophage. One other possible source of biocontrol in SRC may be the cheese microbiome itself. Previous research has suggested that the cheese microbiome can affect the growth of *L. monocytogenes* in the cheese; due to the presence of a competing microbiota (Marielle Gay & Amgar, 2005), but also due to specific members (*i.e.*, species or strains) of the microbial community (Callon et al., 2014; Gérard et al., 2021; Retureau et al., 2010). Therefore, a deeper understanding of how the microbiome in SRC affects the growth of *L. monocytogenes* can help how to develop cheeses that are naturally inhibitory to this foodborne pathogen.

1.2 Research overview

The overall objective of this study was to investigate how variations in the natural microbiome of SRC affect the growth of *L. monocytogenes* in the cheese, particularly if the pathogen

contamination happens at the retail level, after cheese ripening. This research project was conducted in four parts based on the following four hypthotheses:

Hypothesis 1: The structure of the cheese microbiome is affected by environmental contamination throughout the cheese production continuum.

Hypothesis 1 Workflow/objective: A variety of environmental milk, and cheese samples were collected from dairy farm to final cheese, along an artisanal cheese production continuum. 16S rRNA gene targeted amplicon sequencing, was then used to determine the composition and diversity of the bacteria throughout this continuum. The objective was to investigate the microbiota from dairy farm to final cheese along an artisanal cheese-making continuum.

Hypothesis 2: The growth of *L. monocytogenes* at refrigerated temperatures is variable across SRC from different origins.

Hypothesis 2 Workflow/objective: A variety of SRC were purchased from retail, and the growth of *L. monocytogenes* at 8°C was compared across the cheeses. The growth of *L. monocytogenes* was correlated to various categorical, physicochemical, and viable microbial measures in the cheeses to determine how much variation in *L. monocytogenes* growth can be explained by these variables. The objective was to compare the growth of *L. monocytogenes* at refrigerated temperatures across a variety of SRC.

Hypothesis 3: The taxonomic profile of the SRC microbiome affects the growth of *L. monocytogenes* in the respective cheese.

Hypothesis 3 Workflow/objective: A combination of 16S rRNA and shotgun metagenomic sequencing was used to taxonomically profile the microbiome of a subset of the cheeses. A

combination of alpha-diversity, beta-diversity, and differential abundance analysis was used to identify microbiome characteristics and bacterial taxa that are significantly correlated with the growth of *L. monocytogenes* in the cheeses. The objective was to identify characteristics of the cheese microbiome that correlated with the growth of *L. monocytogenes* in SRC.

Hypothesis 4: The functional profile of the SRC metagenome affects the growth of *L. monocytogenes* in the respective cheese.

Hypothesis 4 Workflow/objective: Functional profiling of the cheese metagenomes was used to identify the presence enzymes, metabolic pathways, or bacteriocin-encoding genes in the cheeses. The construction of metagenomically assembled genomes was then used to identify species of bacteria in the cheeses that potentially carry these functions in their genomes. The objective was to identify functional characteristics of the cheese microbiome that correlated with the growth of *L. monocytogenes* in SRC

1.3 Literature review

1.3.1 Occurrence and risk of *L. monocytogenes* in soft and semi-soft cheeses

A range of estimates on the occurrence of *L. monocytogenes* in soft and semi-soft cheeses have been put forward (Table 1.1). As reviewed previously (Gérard et al., 2018), the occurrence of *L. monocytogenes* in fresh, soft, and semi-soft cheeses is highly variable, and ranged globally from 0 – 37.5% in fresh Latin-style cheeses, and from 0 – 14% in soft and semi-soft ripened cheeses. A more recent systematic review and meta-analysis of the occurrence of *L. monocytogenes* in RTE foods world-wide estimated the occurrence of *L. monocytogenes* in soft cheeses to be in the order of 2.4% (95% CI, 1.6-3.6%) (Churchill et al., 2019). In the European union, a systematic review

of European cheeses observed a mean prevalence of *L. monocytogenes* of 0.9% (CI: 0.4 - 1.9%) and 1.0% (CI: 0.4 – 2.2%) for soft/semi-soft cheeses produced from pasteurized or unpasteurized milk, respectively. In the same study, concentrations of > 100 CFU/g *L. monocytogenes* were observed in 0.2% (CI: 0.1 – 0.6%) and 0.4% (CI: 0.2 – 1.2%) of the pasteurized and unpasteurized soft/semi-soft cheeses, respectively (Martinez-Rios & Dalgaard, 2018). At the same time, a second EU study observed a prevalence of 0.2-1.8% in soft and semi-soft cheeses between 2008 and 2015, with higher occurrence observed at the processing stage than at the retail stage of sampling (Ricci et al., 2018). In a Canadian survey, *L. monocytogenes* was detected in 0.5% of 2,955 tested cheeses. Unfortunately, no information was provided on the styles of the contaminated cheeses, other than that the affected cheeses in the study were all able to support the growth of the pathogen (Ganz et al., 2020). Similarly, a joint risk assessment by Health Canada and the United States Food & Drug Administration found that the probability of *L. monocytogenes* occurrence per serving of soft-ripened cheese to be 0.6% and 0.7% for cheeses made with pasteurized milk in Canada and the United States, respectively. For raw milk soft-ripened cheeses, these number rose dramatically to 3.2% and 4.7% for Canada and the United States, respectively (Health Canada & U.S. Food and Drug Administration, 2015). This regular occurrence of *L. monocytogenes* in soft and semi-soft cheeses presents a clear risk of listeriosis in susceptible populations.

The risk of listeriosis from soft and semi-soft cheese has also been investigated (Table 1.2). Health Canada and the United States Food & Drug Administration jointly investigated the risk of listeriosis from soft-ripened cheeses. In the general population, pasteurized soft-ripened cheeses were estimated to result in 1.4×10^{-4} and 1.2×10^{-4} cases per 1 million servings, respectively in Canada and the United States. For high-risk populations, such as pregnant women, the risk is

increased to as high as 1.2 x 10^{-2} cases per 1 million servings in both countries. In soft-ripened cheeses made from raw milk, a 50- to 160-fold increase in risk relative to pasteurized milk cheeses was estimated (Health Canada & U.S. Food and Drug Administration, 2015). In the European Union, a European Food Safety Authority risk assessment of soft and semi-soft cheeses estimated the risk of listeriosis to be 1.1 x 10^{-5} cases per 1 million servings in the general population, and as high as 1.1 x 10^{-3} cases per 1 million servings in pregnant women (Pérez‐Rodríguez et al., 2017).

Table 1.1 Estimates of the *L. monocytogenes* prevalence in soft and semi-soft cheeses across various regions.

Region	Cheese type	Raw or Pasteurized	Prevalence (CI)	Reference
Global	Fresh cheese	Both	0-37.5%[a]	(Gérard et al., 2018)
Global	Ripened soft/semi-soft cheese	Both	0-14%[a]	(Gérard et al., 2018)
Global	Soft cheese	Both	2.4% (1.6-3.6%)	(Churchill et al., 2019)
European Union	Soft/semi-soft	Raw	1.0% (0.4-2.2%)	(Martinez-Rios & Dalgaard, 2018)
European Union	Soft/semi-soft	Pasteurized	0.9% (0.4-1.9%)	(Martinez-Rios & Dalgaard, 2018)
European Union	Soft/semi-soft	Both	0.2-1.8%	(Ricci et al., 2018)
Canada	Unspecified	Both	0.5%	(Ganz et al., 2020)
Canada	Soft-ripened	Raw	3.2%	(Health Canada & U.S. Food and Drug Administration, 2015)
Canada	Soft-ripened	Pasteurized	0.6%	(Health Canada & U.S. Food and Drug Administration, 2015)
United States	Soft-ripened	Raw	0.7%	(Health Canada & U.S. Food and Drug Administration, 2015)
United States	Soft-ripened	Pasteurized	4.7%	(Health Canada & U.S. Food and Drug Administration, 2015)

[a] Range of estimates from a review of multiple studies.

Contamination of *L. monocytogenes* in cheeses can come from a variety of sources, such as the milk itself, or from the processing environment. It has been estimated that the occurrence of *L. monocytogenes* in raw milk is in the order of 3% to 4% (Kozak et al., 1996). When the pathogen

is present, however, the levels are usually quite low, often less that 1 CFU/ml (D'Amico & Donnelly, 2017). Raw milk is likely not the only, or even the dominant source of contamination in cheese. For instance, in the EU *L. monocytogenes* was more commonly isolated from pasteurized milk cheeses (5.8%) than raw milk cheeses (0.1%) (Todd & Notermans, 2011). The increased occurrence in pasteurized milk cheeses is consistent with the opinion of Sauders and D'amico (2016) who emphasized the high risk of cross-contamination throughout the production and retail stages. This contamination throughout production is of special concern since the finished cheeses are regularly consumed without any further heating or processing to eliminate the pathogen.

Table 1.2 Estimated risk of invasive listeriosis due to consumption of soft and semi-soft cheeses made with raw or pasteurized milk.

Country	**Cheese**	**General Population[a]**		**High Risk Population[a b]**		**Reference**
		Raw	*Pasteurized*	*Raw*	*Pasteurized*	
United States	Soft-ripened	1.8×10^{-2}	1.2×10^{-4}	1.8	1.8×10^{-2}	(Health Canada & U.S. Food and Drug Administration, 2015)
Canada	Soft-ripened	9.5×10^{-3}	1.4×10^{-4}	9.1×10^{-1}	1.8×10^{-2}	(Health Canada & U.S. Food and Drug Administration, 2015)
European Union[c]	Soft/semi-soft	1.1×10^{-5}		1.1×10^{-3}		(Pérez-Rodríguez et al., 2017)

[a] Number of cases of invasive listeriosis per 1 million servings of soft and semi-soft cheeses made with raw or pasteurized milk.
[b] Values taken from the highest risk subgroup, which was pregnant women in all cases.
[c] European Union did not differentiate between cheeses made with raw or pasteurized milk.

The regular occurrence of *L. monocytogenes* in soft and semi-soft cheeses around the globe emphasizes the need for adequate control measures throughout the production and supply chains. Further, the common presence of the pathogen in cheeses made with pasteurized milk reminds us that pasteurization is not fail-safe, and that additional strategies should still be employed to maintain the safety of theses cheeses.

1.3.2 Minimum growth requirements of *L. monocytogenes* in soft cheeses.

While the minimum growth requirements (*i.e.*, pH and a_w) have been well studied for *L. monocytogenes* in broth media, these requirements can differ in food matrices such as soft cheeses (Jeanson et al., 2015). Therefore, a variety of studies have sought to determine the physicochemical factors affecting the growth/no-growth boundary of *L. monocytogenes* in soft cheeses, which involve an interplay between a_w, pH, and storage temperature.

The two most important factors affecting the growth/no-growth boundary in soft cheeses are pH, a measure of the acidity; and a_w, the proportion of water that is unbound and available for biological activities. In acid coagulated cheeses, growth of *L. monocytogenes* has been observed at pH levels as low as 4.9-5.0 (Genigeorgis et al., 1991; Lobacz et al., 2016), and in soft-ripened cheeses, growth of *L. monocytogenes* was observed to occur once the pH increased above 5.5 (Back et al., 1993). The type of organic acid may also affect the growth/no-growth limits since inhibition of *L. monocytogenes* is the result of the undissociated acid fraction and therefore differs between organic acids at particular pH (Wemmenhove et al., 2016). Further, the concentration of osmolytes may also affect minimum growth limits for other physico-chemical parameters, as increasing concentrations of NaCl resulted in an increased minimum pH for *L. monocytogenes* growth in Mexican cheeses (Bolton & Frank, 1999), suggesting a a_w-dependent interaction. Within a pH range of 5.6 to 6.5, low inoculums of *L. monocytogenes* (~10 CFU/ml) showed stochastic growth depending on water activity, with growth occurring 100% of the time in model cheeses at $a_w > 0.98$, but never at $a_w < 0.96$. At water activities in between those two values, the probability of growth initiation increased with the increasing a_w (Schvartzman et al., 2010). Mathematical

modelling later suggested that minimum a_w in model cheeses could range from 0.938 to 0.955, depending on temperature and pH (Schvartzman, Belessi, et al., 2011). Therefore, it is important to account for both pH and a_w when estimating the growth of *L. monocytogenes* in soft cheeses.

The storage temperature of the cheeses is also an important consideration; for the obvious reason of its effect on lag phase (Lobacz et al., 2013) and growth rate (Back et al., 1993), but also indirectly as it affects the rate of pH increase in ripened cheeses. In a comparison of cheeses stored at 3°C, 6°C, 10°C, and 15°C over 40 days after ripening, only cheeses stored at 15°C where able to show sufficient pH increase in the core to allow for *L. monocytogenes* growth. It should be noted, however, that the surface pH had already increased above the *L. monocytogenes* growth threshold before the end of ripening (Back et al., 1993). In a separate experiment where cheeses were stored at 2°C after ripening, it took until day 30 before growth of *L. monocytogenes* occurred, even at the surface, which matched the relatively slow rate of pH increase (Ramsaran et al., 1998). Therefore, appropriate cold storage conditions are an important tool in preventing or reducing the growth of *L. monocytogenes* in soft cheeses where other intrinsic factors (*e.g.*, low a_w) are not limiting the growth.

Finally, the probability that *L. monocytogenes* will survive and grow in the cheese matrix is affected by the initial level of contamination. Evidence from previous research shows that lower levels of inoculation (~10 CFU/g or CFU/cm^2) lowered the likelihood of growth occurring, although, they had no effect on growth rate when growth did occur (D'Amico et al., 2008; Schvartzman, Belessi, et al., 2011). Additionally, lower contamination levels are more affected by less favourable a_w and/or pH conditions. Schvartzman et al. (2010) found that the minimum

water activity for a 100% growth initiation in a model cheese differed from 0.98 to 0.97 for an inoculum of 1-10 log CFU/ml and 500 CFU/ml in the starting milk, respectively. Similarly, at any particular a_w, the probability of growth is affected by the pH (Schvartzman, Belessi, et al., 2011). These results emphasize the stochastic nature of growth initiation when *L. monocytogenes* is at the low concentrations expected from post-production contamination.

The complex interactions between a variety of intrinsic and extrinsic factors that influence the probability of *L. monocytogenes* growth emphasize a heterogeneous risk across cheese styles; possibly even between producers and batches. Further, this risk will likely also vary differently throughout the cheese production continuum. An understanding of where the greatest risks occur is essential to efficiently focusing control measures against the introduction and growth of *L. monocytogenes* across the cheese production process.

1.3.3 Growth of *L. monocytogenes* during the different production stages of soft ripened cheeses

Cheesemaking involves several steps designed to physically and chemically modify the milk components in a controlled way, with the particular steps differing across cheese varieties (Figure 1.1). These modifications affect the pH, a_w, salt content, and nutrient composition, and in turn, also affect the growth of *L. monocytogenes*. Therefore, the survival and growth of *L. monocytogenes* is not uniform along the different stages of the cheese production continuum, including the initial stages of cheesemaking, ripening, storage, and retail (Figure 1.2).

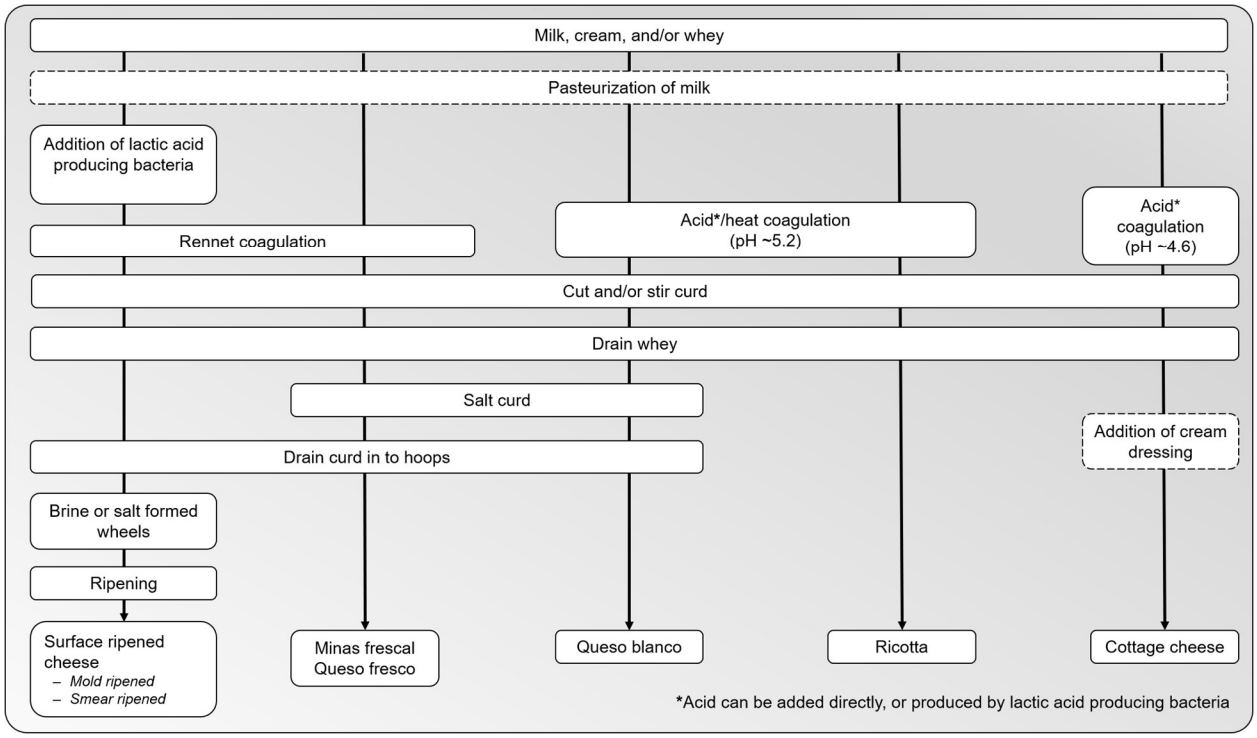

Figure 1.1 Flow diagram of the typical methods used to produce common soft cheeses based on the cheese production methods described by the research groups referenced in this review. Production procedures of cheese present on the global market can deviate from the flow diagram and may vary between different producers. Dash-outlined steps are considered optional.

1.3.3.1 Initial stages of cheesemaking

During initial stages of cheesemaking, raw or pasteurized milk is warmed to 32°C (90°F) and incubated at this temperature until either the natural population of lactic acid bacteria or added starter culture (*e.g.*, *Lactococcus lactis*, *Streptococcus thermophilus*, and/or *Lactobacillus delbrueckii*) adjust to the new conditions and start fermenting the milk sugars into acids. Alternatively, acid (*e.g.*, lactic, citric, acetic) can be added directly to the milk. Some fresh cheeses can partially or fully depend on acid, with or without heat, for coagulation, while most cheeses are made by enzyme coagulation. A typical enzyme coagulation takes between 30 to 60 minutes. Coagulation is followed by cutting and draining of the coagulum to obtain a curd that can mat

together into continuous matrix to form the final cheese wheels. Wheels of soft cheeses are formed without the use of additional mechanical pressing, and after initial drying are ready for the ripening process.

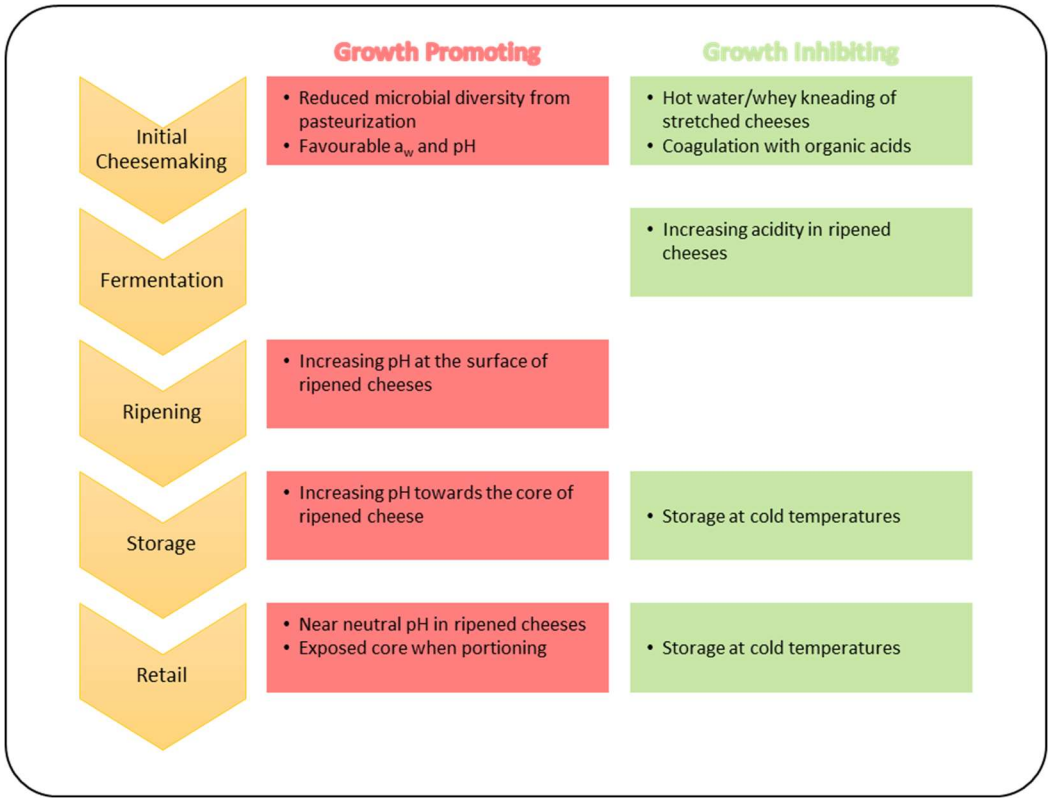

Figure 1.2 Summary of growth promoting and growth inhibiting factors encountered by *Listeria monocytogenes* during the production of soft cheeses.

Increases in *L. monocytogenes* levels during this initial cheesemaking stage can result from physical concentration during the curd formation. During coagulation, the initial microbial population of the milk is trapped in the coagulum, and this population, together with any potential *L. monocytogenes* contamination, is concentrated in the curd as the whey is filtered out. One study found that, during the manufacture of Camembert style cheese, the concentration of *L.*

monocytogenes could be increased by as much as 2 log units in the curd (S. Liu et al., 2007). Another study observed a slightly lower increase of 0.8 log units in the drained curd during the production of soft goat cheese (Morgan et al., 2001). To emphasize that this increase in *L. monocytogenes* is not solely due to growth, Schvartzman, Maffre, et al., (2011) observed that the same concentration of *L. monocytogenes* was present in the curd as in the starting milk when both were measured on a dry-weight basis. Additionally, a different study found that after 18 hours of draining, the concentration of *L. monocytogenes* in the whey was lower than in the starting milk, whereas a greater than 2 log increase was observed in the drained curd (Linton et al., 2008). Regardless of the mechanism, whether by growth, preferential partitioning into the curd, or a mixture of both, the clear result is an increased concentration of *L. monocytogenes* in the drained curd.

During the early stages of cheesemaking, conditions are highly favourable for the growth of *L. monocytogenes*, particularly in pasteurized milk cheeses. The pH has not yet decreased to inhibitory levels, moisture levels are still high since the curd has not yet fully drained off the whey, and the process is conducted close to the optimum growth temperature for *L. monocytogenes*. Due to the concentrating of *L. monocytogenes* in the curd as described above, however, it is difficult to objectively measure the growth of the pathogen during this early period. As a potential solution to this problem, Schvartzman, et al., (2011) monitored the growth of *L. monocytogenes* from milk to curd on a dry weight basis. During the first five hours of cheese making, they observed a 2-log increase in inoculated *L. monocytogenes* in cheese made from pasteurized milk, without the presence of a lag phase. Interestingly, in the same study no growth of *L. monocytogenes* was observed over the same period in cheese made with raw milk. The study's authors suggested that

the inhibition of *L. monocytogenes* growth in the raw milk may be associated with the presence of the background microbiota, but no further evidence was provided. Raw milk also possess a variety of other antimicrobial components (*e.g.*, lactoperoxidase and lysozyme) which can inhibit the growth of *L. monocytogenes* (Griffiths, 2010).

Certain processing steps might also have an antimicrobial effect on *L. monocytogenes*. For instance, the production process for some fresh kneaded or stretched cheeses includes a step where the curds are stretched in hot whey or water. This processing step has been shown to reduce the concentration of viable *L. monocytogenes* in the curd by over 5 Log CFU/g (Murru et al., 2018).

1.3.3.2 Ripening and storage

During the near constant temperature conditions encountered during the ripening and storage of cheeses, growth potential of *L. monocytogenes* is governed mostly by pH and a_w, and therefore is dependent on the style of cheese (Trmčić et al., 2017). In soft cheeses where a_w is expected to be favourable to *L. monocytogenes* growth, pH is the main factor affecting bacterium growth.

In surface-ripened cheeses, fluctuations in pH throughout the ripening process lead to variations in *L. monocytogenes* growth and survival. During the initial fermentation, a decrease in pH to below 5.0 results in a decrease or stagnation in *L. monocytogenes* populations (Back et al., 1993; S. Liu et al., 2007; Lobacz et al., 2013; Ramsaran et al., 1998; E. T. Ryser & Marth, 1987). This period of high acidity can result in as much as a 10- to 100-fold decrease in *L. monocytogenes* cells (Back et al., 1993; E. T. Ryser & Marth, 1987). These cheeses are ripened for 10-14 days at a temperature of 12°C to 15°C before being transferred to cold storage. During the end of ripening

and cold storage, the pH on the surface of the cheese begins to increase as the surface molds consume acids and degrade proteins, resulting in a pH gradient from the surface to the core. Growth of *L. monocytogenes* will begin after the pH has risen above 5.0 (S. Liu et al., 2007; Ramsaran et al., 1998), typically after >10 days of ripening (Back et al., 1993; Lobacz et al., 2013; E. T. Ryser & Marth, 1987), and occurring predominantly on the surface of the cheese rather than in the core due to the more favourable pH conditions (Back et al., 1993; E. T. Ryser & Marth, 1987). In smear-ripened (washed rind) cheeses, the pH increase on the surface was observed to be slower than in mold-ripened cheeses (Schvartzman et al., 2014), but similar growth profiles of *L. monocytogenes* on the surface were still observed (L. O'Sullivan et al., 2006; Schvartzman et al., 2014).

In fresh cheeses, where the pH is expected to remain constant, survival and growth of *L. monocytogenes* is limited by the final pH of the cheese. In acid-coagulated fresh cheeses, *L. monocytogenes* populations have been observed to grow at pH levels as low as 4.9 (Genigeorgis et al., 1991; Lobacz et al., 2016), but in cheeses with a pH below 4.9, these populations are either stagnant or may even decrease (Genigeorgis et al., 1991; Kagkli et al., 2009). In fresh cheeses coagulated with rennet rather than acidification (e.g., queso fresco), the pH remains between 6.0 and 6.7 throughout the storage period (Olarte et al., 2002; Thomas et al., 2019), and is therefore of special risk for the growth of *L. monocytogenes* throughout the entire shelf-life of the cheese.

When the pH is favourable for *L. monocytogenes* growth, the growth rate is mostly dependent on the temperature. For instance, during post-ripening cold storage of a mold-ripened cheese, *L. monocytogenes* populations were observed to increase from a starting concentration of ~3 log

CFU/g to an average of 7.5 log CFU/g, but this increase took 35 days or 6 days for storage temperatures of 3°C and 15°C, respectively (Lobacz et al., 2013). Additionally, in mold-ripened cheese stored at 2°C, an increase of only 2 log CFU/g was observed over a period of 65 days (Ramsaran et al., 1998). Temperature-dependent growth of *L. monocytogenes* has also been observed in fresh cheeses. In queso fresco, inoculated *L. monocytogenes* consistently grew up to a maximum concentration of 6 log CFU/g, but growth rate varied by storage temperature, ranging from 0.013 log CFU/h to 0.090 log CFU/h for 4°C and 30°C, respectively (Thomas et al., 2019), with similar results also observed in paneer (Sarkar et al., 2021). Temperature-dependence on growth rate can even be observed near the minimum pH required for growth. In a study by Lobacz et al., (2016), growth rates of 0.003 log CFU/h and 0.009 log CFU/hr at 3°C and 9°C storage temperatures, respectively, were observed for *L. monocytogenes* in acid coagulated fresh cheeses at pH 4.8-4.9. Therefore, proper temperature control during cheese storage is an important strategy for reducing the risk of *L. monocytogenes* in soft cheeses.

A final consideration for *L. monocytogenes* growth in acid-coagulated cheeses is the acid used for the coagulation. In the production of queso blanco, an acid coagulated Hispanic cheese, coagulation with acetic acid resulted in a reduced amount of *L. monocytogenes* growth compared to coagulation with citric or malic acids (Glass et al., 1995). Similarly, in a model system mimicking fresh soft cheese, variation in antilisterial activity was observed between organic acids: at any of the tested pH levels, propionic and acetic acids were found to have the greatest antilisterial activity followed by lactic acid then citric acid (Engstrom et al., 2020). Since antimicrobial activity depends strongly on membrane permeation by the undissociated form of organic acids, it

is likely that, at any given pH, acidulants with higher pK_a will exhibit increased antilisterial activity (George et al., 1996).

1.3.3.3 Retail

Cross contamination of cheeses with *L. monocytogenes* may occur throughout the entire distribution chain including at retail. Sub-portioning and general handling of finished cheeses can introduce *L. monocytogenes* through the interaction with utensils, cutting boards, or other surfaces of the retail environment, as outlined by Sauders and D'Amico (2016). This effect is highlighted by an outbreak of *L. monocytogenes* in Quebec, Canada where the outbreak strain was isolated from 25 cheese varieties not acquired from the implicated producer (Gaulin et al., 2012).

In whole cheese wheels, sub-portioning can contaminate the core of the cheese, which after ripening has returned to a near neutral pH and can offer more favourable growth conditions for *L. monocytogenes* than the surface rind. Indeed, a 3.22 log and 1.28 log increase in *L. monocytogenes* was observed on the cut surface of a soft and semi-soft ripened cheese, respectively, over 14 days at 7°C. By comparison, under the same conditions, the surface rinds of these cheeses only showed a 1.47 and 0.2 log increase, respectively (Lahou & Uyttendaele, 2017). The study's authors hypothesized that the increased growth potential in the core resulted from reduced biodiversity compared to the rind, but no evidence was provided to support this statement. Another possibility might be higher water activity in the core of the cheese. Regardless of the mechanism, retailers should be cautious of cross-contamination during portioning and handling of these cheeses.

1.3.4 Biocontrol of *L. monocytogenes* in soft cheese

1.3.4.1 Bacteriocins and bacteriocin-producing strains of bacteria

Bacteriocins are ribosomally synthesized peptides or proteins produced by bacteria which most commonly show antimicrobial activity against closely related bacteria; however some bacteriocins like nisin can also have a much broader spectrum of activity (Cleveland et al., 2001). Of particular interest to the dairy industry, are bacteriocins produced by lactic acid bacteria (LAB) as LAB are commonly used in food fermentations and are consequently granted Generally Regarded as Safe (GRAS) status by global food safety authorities (C. C. G. Silva et al., 2018).

Bacteriocins produced by LAB can be loosely grouped into three classes as outlined by Cotter, Hill, & Ross (2005): Class I bacteriocins, commonly referred to as lantibiotics, are heat-stable, small peptides (<5 kDa) characterized by post-translational modifications and the presence of the nonproteogenic amino acid lanthionine. Similarly, Class II bacteriocins are heat-stable, small peptides (<10 kDa), but lack lanthionine and do not undergo post-translational modifications. Finally, large (>30 kDa), heat-labile antimicrobial peptides can be grouped in to a third class known as bacteriolysins. While the specific modes of action differ between the different bacteriocins, most target the cellular envelope of their targets, inhibiting essential processes or leading to pore formation (C. C. G. Silva et al., 2018).

Of particular relevance to soft cheese production, many LAB-produced bacteriocins are known to be active against Gram-positive pathogens, including *L. monocytogenes* (Mills et al., 2011). While there is a broad spectrum of bacteriocins produced by LAB (Heng et al., 2007) and a variety of bacteriocinogenic strains were associated with cheese (Trmčić et al., 2008), here I have reviewed

those which have been investigated against *L. monocytogenes* in the soft and semi-soft cheese matrix.

1.3.4.1.1 Nisin

Nisin was first identified in 1928, and characterized in 1947 as the first bacteriocin associated with gram-positive bacteria, and therefore the most well studied (Heng et al., 2007). Nisins are class-Ia lantibiotics produced primarily by *Lactococcus lactis*, and occasionally *Streptococcus* strains (C. C. G. Silva et al., 2018). Their antimicrobial activity is the result of binding to the lipid II precursor molecule and inserting themselves in to the cellular lipid membrane, resulting in pore formation and cell death (Alvarez-Sieiro et al., 2016).

Nisin has been approved for use by the United States Food and Drug Administration (Santos et al., 2018), and recognized as safe by the Joint Food and Agriculture Organization/World Health Organization (FAO/WHO) Expert Committee on Food Additives (Favaro et al., 2015). It has also been commercialized as the product Nisaplin® (C. C. G. Silva et al., 2018). Approved concentrations of purified nisin in cheese differ by country, ranging from as low as 100 IU/g (2.5 μg/g) in Belgium compared to no upper limits in the United Kingdom (Cleveland et al., 2001). In a recent opinion by the European Food Safety Authority (EFSA) it was suggested that the use of nisin in unripened cheese at levels below 12 μg/g would not present a safety concern (Younes et al., 2017).

In fresh cheeses, purified nisin, has shown a direct bactericidal effect against *L. monocytogenes*, but this antimicrobial effect may be temporary if conditions favourable to *L. monocytogenes*

growth are present. As an example, in acidic cottage cheese (pH 4.6-4.7), the addition of 2,000 IU/g (a relatively high concentration) resulted in a 3 log decrease from the initial inoculum (4 log CFU/g) of *L. monocytogenes* over 7 days at 20°C, compared to less than a 1 log reduction in the control over the same time period (M. A. S. S. Ferreira & Lund, 1996). When even higher concentrations of nisin (20,000 IU/g) were added to queso fresco (pH 6.0) an immediate 3 log decrease in inoculated *L. monocytogenes* (4 log CFU/g) was observed, but growth of the surviving cells occurred throughout storage at 4°C (Lourenço et al., 2017) suggesting the low pH of the cottage cheese was an important to maintaining the antilisterial effect. At lower inoculation levels of *L. monocytogenes* (3 log CFU/g), a reduction of *L. monocytogenes* to below the limit of detection (1 log CFU/g) in minas-style goat cheese was observed with the addition of 500 IU/ml of nisin to the starting milk before production, and no further presence of the pathogen was detected over 10 days of storage at 8-10°C (Furtado et al., 2015). Even the addition of 50 IU/ml of nisin to the milk before the production of ricotta cheese was enough to decrease the inoculated *L. monocytogenes* in the cheese from 3 log CFU/g to below the limit of detection (2 log CFU/g) in the finished cheese. Despite more than 68% of the initially added nisin remaining in the ricotta cheese at the end of 10 weeks of storage, however, regrowth of surviving *L. monocytogenes* began after 11 and 55 days of refrigerated storage (6-8°C) when nisin was added at a concentration of 50 IU/ml and 100 IU/ml of milk, respectively (Davies et al., 1997). It is not clear what mechanism(s) led to the latent regrowth of the pathogen, but these results suggest a possible dose-dependent relationship in the long-term inhibition ability of nisin in fresh cheese. Overall, the addition of purified nisin appears to provide a maximum 3 log reduction of *L. monocytogenes*, but regrowth of surviving cells appears to be likely unless inhibited by some additional hurdle (*e.g.*, organic acids).

An increase in bactericidal activity has been suggested when combining nisin with a second bacteriocin. Complete eradication of *L. monocytogenes* was observed in Minas frescal cheese using a combination of nisin and bovicin HC5, a bacteriocin extracted from *Streptococcus bovis*. After nine days of storage at 4°C, *L. monocytogenes* decreased from 4 log CFU/g to being undetectable in 25 g samples, even after enrichment; a level maintained throughout the 30 days of the experiment (Pimentel-Filho et al., 2014). Unfortunately, the authors did not compare the inhibitory action of each bacteriocin separately, making it difficult to determine how much contribution each had towards the antilisterial effect or how much of a synergistic effect the two bacteriocins had.

Researchers have also investigated the addition nisin-producing starter cultures during the production of soft cheeses. In Camembert cheese, the addition of a nisin-producing strain of *Lc. lactis* subsp. *lactis* to the milk before cheese production produced up to 700 IU/g of nisin, and resulted in a 2 log reduction of *L. monocytogenes* relative to the control cheese within 6-9 hours of production; although, regrowth of the surviving *L. monocytogenes* began within one week of production, with the pathogen population returning to initial levels (5 log CFU/g) by six weeks of storage (Maisnier-Patin et al., 1992). The growth of the surviving *L. monocytogenes* only initiated once the pH of the ripening cheeses had risen to favourable levels, however, suggesting that it may have been the low pH of the young cheese, rather than the nisin, which prevented the growth of the pathogen after the initial antilisterial effect. In fresh cheeses, nisin-producing cultures have been tested with varying results. Despite promising results in broth media, a nisin-producing strain showed little effect on *L. monocytogenes* in cottage cheese, resulting in less than a 0.5 log decrease

from the 3 log CFU/g initially present. In comparison, without the inclusion of the bacteriocinogenic strain, the *L. monocytogenes* only increased by 0.2 log after 7 days of storage (Dal Bello et al., 2012). The study's authors suggested that low bacteriocin production *in situ* may be to blame for the reduced antilisterial action. Indeed, slow nisin production *in situ* has been observed previously, where an inoculum of 7 log CFU/ml of nisin producer *L. lactic* M78 was required to achieve 100 IU/g of cheese within 24 hours (Trmčić et al., 2011). Alternatively, in a fresh cheese model, three nisin Z-producing strains of *Lc. lactis* were each able to produce a 1.5-2 log CFU/g reduction of *L. monocytogenes* compared to the control cheese after seven days of storage at 4°C. Unfortunately, the authors did not compare with a control cheese inoculated with a non-bacteriocin producing *Lc. lactis* strain, making it difficult to confirm whether antilisterial effect was bacteriocin related, or the result of decreasing pH by the starter culture. In a different study on fresh cheese (*i.e.*, minas frescal), similar inhibition of *L. monocytogenes* was observed for both bacteriocin producing and non-producing strains of *Lc. lactis* (Furtado et al., 2015), emphasizing that the source of inhibition is not always clear. While the inclusion of nisin-producing bacterial strains may be an attractive option for cheese production, especially from a "natural" or "clean-label" perspective, it appears that these bacteria are not as effective in inhibiting *L. monocytogenes* as purified nisin.

A variety of factors may affect the antilisterial activity of nisin in soft cheeses. Nisin is known to function more effectively at acidic pH, with reduced solubility and stability above pH 6 (Ibarra-Sánchez et al., 2020). Unfortunately, a recent study found that *L. monocytogenes* showed increased resistance to nisin in cheeses at pH 5.5 compared to cheeses at pH 6.0 or 6.5, likely due to stress-related modifications to cell wall structure (Henderson et al., 2020). Lipid content and form in the medium may also affect the antimicrobial action of nisin. When comparing the action

of nisin in different types of milk, Bhatti et al. (2004) found that increased action of nisin occurred in skim milk over 2% and whole milks, and that unhomogenized milk showed increased nisin action over homogenized milk. The authors reasoned that the hydrophobic (*i.e.*, membrane interacting) portion of the nisin molecule can also bind with the lipids in the milk. In the case of homogenized milk, the homogenization process can lead to decreased size, and therefore increase surface area, of fat globules, resulting in greater binding affinity of the nisin molecules (Bhatti et al., 2004). Finally, the sensitivity of *L. monocytogenes* to nisin can vary across strains, ranging from <200 IU/g (< 5 mg/L) up to 2000 IU/ml (50 mg/L) (M. A. S. S. Ferreira & Lund, 1996), which is significant considering 12.5 mg/kg is the maximum allowable concentration in the European Union (Younes et al., 2017).

1.3.4.1.2 Pediocins

Pediocins are class IIa bacteriocins commonly associated with *Pediococcus* spp. The pediocin peptide is believed to bind to the mannose phosphotransferase system before inserting itself in to the cytoplasmic membrane and causing pore formation (Alvarez-Sieiro et al., 2016). Pediocins are effective against *Listeria* spp. and are stable over a wide range of pH (C. C. G. Silva et al., 2018). Furthermore, pediocin producing strains are designated as GRAS (Tiwari et al., 2009). Despite these beneficial traits, limited research has been focused on the use of pediocins against *L. monocytogenes* in soft cheeses.

In acid-coagulated cheese, pediocins may provide antilisterial protection in combination with organic acids. In cottage cheese (pH 5.1), the addition of purified pediocin PA-1 was able to immediately decrease an inoculum of 7.5 x 10^3 CFU/ml to below the limit of detection (10^2

CFU/ml) and maintain that level for the entire seven days of the experiment. On the other hand, dairy products with a more neutral pH (*i.e.*, cream or cheese sauce), showed regrowth of *L. monocytogenes* at day 7, even when a ten-times higher concentration of PA-1 was added (Pucci et al., 1988). While the prevention of regrowth could be attributed to the low pH, the type of organic acid used may also be of importance. For instance, ALTA 2341™, a commercial pediocin-based formulation, provided up to a 3-log reduction of *L. monocytogenes* over 21 days in queso blanco cheese (pH 5.2). Regrowth of the pathogen was only prevented, however, when acetic acid was used for coagulation, as opposed to citric or malic acids (Glass et al., 1995). The study's authors credited the increased concentration of undissociated organic acid at pH 5.2 for increased antilisterial activity of acetic acid over citric or malic acids. Similar to nisin, these studies show that pediocins can be effective at reducing *L. monocytogenes* in soft cheeses, but additional hurdles are still required to prevent regrowth of surviving pathogen cells.

Pediocin-producing cultures have also been investigated for their antilisterial efficacy in red smear cheeses; specifically, as additions to the smear community. In a Munster cheese model, the inclusion on a pediocin AcH producing strain of *Lb. plantarum* in the wash brine prevented the growth of *L. monocytogenes* inoculated on the cheese surface on the 7th day of ripening. The pathogen had been inoculated through the cheese brine at 2 log CFU/ml. In comparison, on control cheeses without the addition of the pediocin-producing culture, the inoculated *L. monocytogenes* was able to grow up to 5 log CFU/g over the 14 days of ripening post-contamination (Ennahar et al., 1998). Similar results were observed by Loessner et al. (2003) but a regrowth of surviving *L. monocytogenes* cells occurred within 14 days of contamination. This regrowth could have been the result of pediocin-resistance development in *L. monocytogenes*, which was observed by the

study's authors, or it could have resulted from declining pediocin concentrations on the surface of the cheese. Unfortunately, changes in pediocin concentration throughout the experiment were not measured. Overall, these results suggest that pediocin-producing cultures may not be suitable to long-term protection against *L. monocytogenes* growth in ripened soft cheeses. Alternatively, it was observed that, after separating the planktonic cells from the cultural supernatant of the pediocin AcH producing culture, the supernatant prevented growth of any *L. monocytogenes* for all 37 days of the experiment (Loessner et al., 2003), which might suggest, as with nisin, that the inclusion of the purified bacteriocin may be more suitable to inhibit contaminating pathogens than the inclusion of the culture itself.

1.3.4.1.3 Enterocins

Enterocins represent a diverse group of bacteriocins produced by enterococci, with many being active against *Listeria* spp. (C. C. G. Silva et al., 2018). Despite the presence of enterococci in raw milk and raw milk cheeses (Gelsomino et al., 2002), including those demonstrating enterocin-mediated antimicrobial activity (Trmčić et al., 2008), the association of certain species of *Enterococcus* with human disease has prevented enterococci cultures from receiving GRAS status by the FAO (C. C. G. Silva et al., 2018). Nevertheless, research into these bacteria as protective cultures in soft cheeses has been ongoing.

Bacteriostatic effects against *L. monocytogenes* have been observed when enterocin-producing bacterial strains have been included in the production of fresh and soft-ripened cheeses. For example, in fresh whey cheese, the inclusion of an enterocin-producing strain of *E. faecium* prevented the growth of contaminating *L. monocytogenes* over nine days of storage at 4°C (Aspri

et al., 2017). Similarly, a bacteriostatic effect over 12 days was also observed with fresh Minas cheese when inoculated with a bacteriocin producing strain of *E. muntdii*; however, the produced bacteriocin was not identified (Vera Pingitore et al., 2012). In Munster cheese, the inclusion of an enterocin-producing culture of *E. faecium* in the cheese wash at day 1 had a bacteriostatic effect against *L. monocytogenes* contamination from the wash brine at day seven. This bacteriostatic effect lasted the full 27 days of ripening, whereas the contaminating *L. monocytogenes* increased by 4 log CFU/g over the same period without the protective culture. Further, no negative effect on the rest of the cheese microbiota or pH was observed with the inclusion of the enterocin-producing strain (Izquierdo et al., 2009). These prolonged bacteriostatic could be valuable in preventing small contaminations of *L. monocytogenes* from growing to levels that would represent a risk.

Bacteriocidal effects of enterocins against *L. monocytogenes* have also been observed in fresh cheeses. In one study involving bacteriocin-producing enterococci in fresh cheese, the listeriostatic effect (< 1 log CFU/g decrease) was improved to a 2 log CFU/g decrease of inoculated *L. monocytogenes* over 7 days by the combining of two different strains of bacteriocin-producing *Enterococcus* (Coelho et al., 2014). Additionally, Aspri et al. (2017) isolated one enterocin-producing strain of *E. faecium* which showed bacteriocidal action against *L. monocytogenes*, resulting in the contaminating pathogen, initially inoculated at 3 log CFU/g, being undetectable in the fresh whey cheese at the end of the 9 days storage period.

Due to the lack of GRAS status for enterococci, and as a proof-of-concept, L. Liu et al. (2008) transformed a common starter culture strain of *Lc. lactis* into a heterologous producer of

enterocins, and tested its antilisterial effectiveness in cottage cheese production. Despite only being able to produce one quarter of the amount of enterocin as the original strain of *E. faecium*, the transformed isolate of *Lc. lactis* was still able to reduce the level of *L. monocytogenes* in the cheese by 1 log CFU/g compared to the control after 15 days of refrigerated storage. Unfortunately, the transformed strain of *Lc. lactis* was not as efficient in lowering the pH as its parental strain, but using it in conjunction with the parental strain resulted in regular acid production levels (L. Liu et al., 2008). While this proof-of-concept shows promise, however, lack of consumer acceptance for Genetically Modified Organisms as well as common regulatory limitations suggests this might not be a possible real-world solution.

1.3.4.1.4 Lacticins

Lacticins comprise two lantibiotics produced by strains of *Lc. lactis*. Lacticin 3147 is formed of a two-peptide system, while lacticin 481 is composed of a single peptide. Both lacticins have been shown to be inhibitory to *L. monocytogenes* (C. C. G. Silva et al., 2018).

A food grade strain of *Lc. lactis* transconjugated to produce the bacteriocin lacticin 3147 has been investigated relative to its parental strain for antilisterial effects in cottage cheese (O. McAuliffe et al., 1999), and smear-ripened cheese (L. O'Sullivan et al., 2006). In cottage cheese, the presence of the transconjugated strain reduced the level of inoculated *L. monocytogenes* from 4 log CFU/g to below the limit of detection (1 log CFU/g) by day 5 and 4 when incubated at 4°C and 18°C, respectively. However, they also observed a reduced acid production, believed to be a result of an inhibition of the native acidifying bacteria (O. McAuliffe et al., 1999). In smear-ripened cheese, the lacticin-conjugated strain provided a 3 log decrease in *L. monocytogenes* on

the cheese surface, but only when the antilisterial strain was added after contamination with *L. monocytogenes*. No inhibition was observed at all if the transconjucated strain was added before contamination. The lack of antilisterial action when added prior to contamination, and the regrowth when added after were suggested to be the result of an interaction of the bacteriocin with the cheese matrix, which led to a 50% drop in bacteriocin activity on the surface (L. O'Sullivan et al., 2006). The drawbacks described in these two studies suggest that lacticin 3147 is not a strong candidate bacteriocin for control of *L. monocytogenes* in cheeses.

Bacterial strains producing lacticin 481 have shown very little effectiveness in controlling *L. monocytogenes* in both cottage cheese (Dal Bello et al., 2012) or fresh latin-style cheese (Coelho et al., 2014). However, the addition of purified lacticin 481 to a fresh cheese model provided a 3-4 log reduction of *L. monocytogenes* over a 7-day period (Ribeiro et al., 2016), again highlighting the increased effectiveness of purified bacteriocins over bacteriocin-producing strains.

1.3.4.1.5 Other bacteriocins

A handful of studies have looked at less common bacteriocins as potential antilisterial additives in soft cheeses, with mixed results. A strain of *Brevibacterium linens* able to produce the bacteriocin linocin M18, and included in the wash brine was shown to inhibit the growth of *L. monocytogenes* on smear-ripened cheese (Eppert et al., 1997). Similarly, the addition of piscicolin 126 in the milk used for camembert production also showed an antilisterial effect in the finished cheese (Wan et al., 1997). Unfortunately, in both cases *L. monocytogenes* growth occurred after a certain amount of time: after 10 days of ripening, and 21 days of ripening and storage in the red smear and camembert cheeses, respectively. More recently, a bacteriocin-producing strain of *Staphylococcus*

equorum produced up to a 5 log reduction of *L. monocytogenes* within 24 hours of inoculation on the surface of a model cheese, but only when the model emulated a matured cheese. When the same strains were co-cultured in a model representing a green (*i.e.*, unripened) cheese, no antilisterial effect was observed, suggesting that the conditions present on the surface of ripened cheese are necessary for bacteriocin production by the *S. equorum* strain (Bockelmann et al., 2017).

Alternatively, two strains of *Lc. lactis* subsp. *lactis* producing unidentified bacteriocins have shown more promise, specifically in Jben, a Moroccan fresh cheese. One strain reduced a 4 log CFU/g inoculum of *L. monocytogenes* to below detectable limits within 24 hours, and the pathogen remained undetectable throughout the 20 days of the experiment at both refrigerated (7°C) and abuse (22°C) temperatures. By comparison, only a 1 log reduction of the pathogen was observed in the control cheese (Benkerroum et al., 2000). The other strain, likely producing a different, unidentified bacteriocin, was able to decrease bacterial cells below detectable limits even at an inoculum as high as 7 log CFU/g (Benkerroum et al., 2002).

1.3.4.1.6 Concerns and considerations

One concern regarding the use of bacteriocins in *L. monocytogenes* biocontrol is the possible development of resistance by the pathogen. In *L. monocytogenes*, the frequency of resistance development is strain-dependent, and has been observed to range from 10^{-7} to 10^{-2} for nisin (A. Gravesen et al., 2002) and from 10^{-6} to 10^{-4} for pediocin (A. Gravesen et al., 2002; Loessner et al., 2003). Mechanisms of resistance differ across the different bacteriocins. Nisin resistance is believed to involve modifications to the cell wall and/or cellular membrane to increase the net positive charge, repelling the cationic nisin peptides, or through increases in membrane rigidity to

prevent peptide insertion (Crandall & Montville, 1998). In class IIa bacteriocins (*i.e.*, pediocin), resistance is associated with a loss in expression of the mannose-specific phosphotransferase system, the bacteriocins' binding site (Anne Gravesen et al., 2002). Resistance to a particular bacteriocin may also result in cross-resistance with other bacteriocins sharing similar modes of action (Macwana & Muriana, 2012). This resistance phenotype may be stable over the shelf-life of soft cheeses. For example pediocin-resistance was shown to be stable over at least 100 generations *in vitro*, and was maintained throughout 35 days or ripening on the surface of red smear cheese (Loessner et al., 2003). The regrowth of *L. monocytogenes* after the initial die-off observed for many different bacteriocins may therefore be partially explained by the resistance development.

Another concern with the use of bacteriocins or their producing strains in cheese production is their potential effect on the native microbiota essential for proper flavour development in the cheeses. While a micrococcin P1 producing culture of *S. equorum* showed inhibition against *L. monocytogenes* on the surface of a washed rind cheese, it also dominated the surface microbiota, supressing the other gram-positive microbes (Carnio et al., 2000), which might affect the final sensory characteristics of the finished cheese. Similarly, the use of nisin at high concentrations shifted the cheese microbiota from Gram-positive to Gram-negative dominant communities, selecting for microbes associated with spoilage, such as *Pseudomonas* (Samelis et al., 2003).

The storage temperature of the cheeses may also affect the outcome and effectiveness of bacteriocins. An experiment comparing the antilisterial effectiveness of a Lactocin RN 78 producing culture with a freeze dried extract of the same bacteriocin found that, at 35°C, the culture

was most effective, but that the use of the freeze dried extract was required for efficient *L. monocytogenes* reduction at 4°C (Mojgani et al., 2010). This result was likely due to a lack of growth of the bacteriocin-producing culture at refrigerated temperatures, as suggested by Martinez et al. (2015) who observed a greater inhibition of *L. monocytogenes* at 15°C compared to 4°C when testing a bacteriocin producing strain of *Lb. sakei* subsp. *sakei* in cheese spread. On the other hand, increased nisin sensitivity in broth medium has been observed for *L. monocytogenes* at lower temperatures, and suggested to be the result of increased membrane fluidity (J. Li et al., 2002). A recent study of nisin in a model cheese showed a 1 log lower count of *L. monocytogenes* after 14 days when stored at 6°C compared to 14°C or 22°C (Henderson et al., 2020). It is unclear, however whether this difference is the result of increased nisin sensitivity, or just a slower re-growth of the *L. monocytogenes* at the reduced temperature. Overall, it appears that the use of purified bacteriocin at lower storage temperatures may be an effective combination for the control of *L. monocytogenes.*

The starting concentration of *L. monocytogenes* as well as the bacteriocin-producing culture will also impact the level of pathogen inhibition. The use of a micrococcin P1 producing culture of *S. equorum* in a soft cheese model showed only a 1 log CFU/g reduction compared to control after 35 days of ripening when challenge at 4 log CFU/ml brine , whereas the complete inhibition of the pathogen occurred and was maintained over a period of 35 days when challenged at only 2 log CFU/ml in the wash brine (Carnio et al., 2000). These data emphasize the effectiveness of bacteriocins when contamination events are at low levels. On the other side of the coin, sufficient production of bacteriocin and interaction with the pathogen is also necessary. In solid matrices such as cheese, where antimicrobial metabolites may not readily diffuse, the initial concentration

of inoculated bacteriocin producer will affect the amount of interaction it has with the possibly sparsely distributed *L. monocytogenes* (Jeanson et al., 2015). For instance, no inhibition of *L. monocytogenes* relative to the control was observed in quark cheese produced with a lactolisterin producing strain of *Lc. lactis* subsp. *lactis* added at 3 log CFU/g; however, when the bacteriocin-producing strain was added at a level of 5-6 log CFU/g, the inoculated *L. monocytogenes* was reduced by 2 log cycles over the 21-day storage period at 4°C (Mirkovic et al., 2020). Previous research has also shown that a high inoculum of around 7 log CFU/ml of bacteriocin producers is necessary to achieve adequate levels of nisin (Trmčić et al., 2011). Therefore, the addition of concentrated, purified bacteriocins is likely the most effective method to reduce any contaminating *L. monocytogenes* cells.

1.3.4.2 Bacteriophage

Bacteriophages are ubiquitous viruses which target bacterial cells, and are considered the most abundant organism in the biosphere (Clokie et al., 2011). Virulent bacteriophages, those that cause the death of their host cell, are considered to be a safe form of biocontrol and biopreservation since they have high specificity for their target bacterium, only attacking strains within a particular genus or species, and therefore have no effect on the indigenous human microbiome (L. O'Sullivan et al., 2019). Below I have reviewed the use of bacteriophages against *L. monocytogenes* in a cheese matrix.

The addition of bacteriophages has been shown effective in reducing *L. monocytogenes* on the surface of soft cheeses, but regrowth of the pathogen typically occurs after a period of time. In ripened cheeses, *L. monocytogenes* contamination on the surface was reduced by 2-3 logs within

one day with the application of 8-9 PFU/cm^2 of phage A511 at the beginning of the ripening period. However, after 8-10 days, in accordance with increasing pH, *L. monocytogenes* growth began to occur and an increase of up to 3 log CFU/cm^2 was observed at the end of 21 days of ripening/storage (Guenther & Loessner, 2011). Similar results were also observed in queso fresco when using bacteriophage P100 (E. N. G. Silva et al., 2014; Soni et al., 2012). In a case where the phage treatment was added 30 minutes prior to the challenge with *L. monocytogenes*, a phage titre of ~7 log PFU/cm^2 resulted in up to a 1.8 log reduction of pathogen cells, but again, regrowth still occurred, and an average difference from the control was less than 0.5 log after 14 days (Henderson et al., 2019). Therefore, the effectiveness of bacteriophage seems to be only during the initial treatment.

The regrowth of *L. monocytogenes* after the initial reduction is likely not a case of resistance development since no resistant isolates were isolated during cheese experiments using bacteriophage P100 (Carlton et al., 2005) or A511 (Guenther & Loessner, 2011). Inactivation of phage is also an unlikely cause since little to no loss of phage activity was observed over 28 days in queso fresco (Soni et al., 2012) or after six days in smear-ripened cheese (Carlton et al., 2005). One likely factor, however, is the solid nature of the food matrix, which prevents mixing and diffusion of the phage particles in search of their hosts. Indeed, as a contrast, an immediate 3 log reduction of *L. monocytogenes* was observed in chocolate milk and mozzarella cheese brine with the addition of 8.5 log PFU/ml bacteriophage A511, and instead of regrowth, no detectable *L. monocytogenes* were present even after 13 days at 6°C (Guenther et al., 2009). Further, in studies involving bacteriophages targeting other foodborne pathogens in cheese (*e.g.*, *Staphylococcus aureus*), higher rates of pathogen reduction were observed when the phages were added directly

to the milk, providing a more evenly distributed population than when applied to the surface of the finished cheese (as reviewed in García-Anaya et al., 2020). Unfortunately, to the best of our knowledge, no studies have been conducted to test the efficacy of antilisterial bacteriophages added to the milk prior to cheese production.

Regrowth of *L. monocytogenes* may be prevented if the eradication of viable pathogen cells to less than 1 CFU/g can be achieved with the initial phage treatment. When *L. monocytogenes* was inoculated at 3 log CFU/cm^2 on smear cheese, a 2 log reduction was observed within 24 hours after the addition of 8.5 log PFU/cm^2, but regrowth of *L. monocytogenes* began after day 6 of ripening. On the other hand, challenging with the same titre of phage resulted in *L. monocytogenes* undetectable after 1 day and 6 days for inoculums of 1 log or 2 log CFU/cm^2, respectively, and remained undetectable throughout 21 days of ripening/storage (Guenther & Loessner, 2011). The sufficient eradication of viable pathogen cells is also dependent on the initial phage titre. In food products, where microbial contamination is expected to be low (< 10^5 CFU/ml or g), the effectiveness of bacteriophages depends on the likelihood of them coming in to contact with a target host, and thus is dependent on having a sufficiently high concentration of phage to cover the food surface (Hagens & Loessner, 2010). For example, when *L. monocytogenes* was inoculated at the low level of only 20 CFU/cm^2 on washed rind cheeses, growth initiation at 10 days was observed even with repeated dosage of 6 log PFU/cm^2 of bacteriophage P100. A single dosage of 7.8 log PFU/cm^2, however, resulted in complete eradication of viable *L. monocytogenes*, with no detection even after selective enrichment at 21 days of storage (Carlton et al., 2005). Indeed, it has been suggested that, for optimal efficiency, not less than 8 log PFU per g, ml, or cm^2 should be used in foods (Guenther et al., 2009). Taken together, these data imply that it is the initial attack

from the applied phages that is most important in reducing the pathogen load on the surface of soft cheeses, and that subsequent propagation of the bacteriophages has little to no continued effect. Therefore, a "scorched earth" strategy of complete surface coverage is recommended for the use of bacteriophages in eradicating viable *L. monocytogenes* from the surface of soft cheeses.

The efficacy of phages against *L. monocytogenes* in cheeses can also be affected by environmental parameters. In a study by Henderson et al., (2019), the use of the commercial phage cocktail ListShield™ at various temperatures in a cheese model found a reduction in pathogen cells of 0.49 log, 0.86 log, and 1.8 log CFU/cm^2 when incubated at 6, 14, and 22°C, respectively for one day. As the study's authors pointed out, however, despite the higher antilisterial activity, 22°C is not an appropriate temperature for cheese storage. In the same study, the acidic pH of 5.5 was also shown to reduce the effectiveness of the phage cocktail compared to pH 6.0 and 6.5, implying that acidic conditions might not be ideal for bacteriophage biocontrol of *L. monocytogenes*. It should be noted, however, that in a study by Guenther & Loessner (2011), the phage treatment was added to the cheeses at a pH of ~5.5, and still resulted in up to a 3 log reduction in inoculated *L. monocytogenes*. The likely cause of these contradictory findings for pH are differences in the initial dosage of bacteriophages added to the cheeses, which were 7 log CFU/cm^2 and 8.5 log CFU/cm^2 for the two studies, respectively. This difference again points to the importance of the initial concentration of the bacteriophages in the efficiency of the antilisterial action.

One option to increase the efficacy of bacteriophage against *L. monocytogenes* in cheeses is the combined use with a second antimicrobial agent able to prevent regrowth of remaining viable cells. Soni et al., (2012) found that the use of bacteriophage P100 in queso fresco resulted in a 3 log

reduction of *L. monocytogenes*, and by incorporating the listeriostatic blend of potassium lactate-sodium diacetate, no growth of the remaining viable cells was observed for the entirety of the 28-day experiment. The use of bacteriophage on cheese may therefore be better used as one element of a hurdle strategy rather than the sole antilisterial treatment.

As an alternative to the direct addition of bacteriophages, lytic enzymes produced by phages (endolysins) have also shown antimicrobial potential. Endolysins target a conserved region of the cell wall, reducing the likelihood of resistance development (Rodríguez-Rubio et al., 2016). To the best of our knowledge, the only endolysin tested against *L. monocytogenes* in a dairy matrix is endolysin PlyP100 from the antilisterial bacteriophage P100. PlyP100 has shown lytic activity against 18 strains of *L. monocytogenes* from a various serotypes, and provided a bacteriostatic effect against the pathogen in queso fresco over 4 weeks of cold storage (Van Tassell et al., 2017). This antilisterial activity can remain consistent in the cheese over 28 days of storage, even when the pathogen was added at day 28 (Ibarra-Sánchez et al., 2018). A synergistic effect was also observed when PlyP100 was combined with nisin, resulting in 4 log reduction in queso fresco over 28 days of cold storage (Ibarra-Sánchez et al., 2018). The effectiveness of PlyP100 against *L. monocytogenes* growth in queso fresco suggests that further research into the use of endolysins for dairy safety may be warranted. Further, endolysins could prove a suitable complement to bacteriocins, providing the bacteriostatic effect necessary to prevent regrowth of surviving *L. monocytogenes* cells.

1.3.4.3 Competition with native microbiota

Beyond the production of antimicrobial compounds such as bacteriocins, the native microbial community as a whole can be important in preventing the growth of *L. monocytogenes* in soft cheeses. Eppert et al. (1997) found that, while an isolated bacteriocin producing strain of *Brevibacterium* was able to inhibit the growth of *L. monocytogenes* in smear-ripened cheeses, the bacteriocin-induced inhibition was less than that of the entire smear culture as a whole. This suggested that the inhibitory ability of the microbial community goes beyond the production of the bacteriocin. Since then, numerous studies have identified an antilisterial effect associated with the ripening microbial communities where no specific antimicrobial molecules could be identified (Guillier et al., 2008; Maoz et al., 2003; Retureau et al., 2010; Saubusse et al., 2007), or that the antilisterial activity of individual strains was insufficient to explain the total observed inhibition of *L. monocytogenes* of the total communities (Imran et al., 2010).

The ability of native microbial communities to inhibit *L. monocytogenes* in cheese is not homogeneous, and may be dependent on community origin. In red smear cheese, Maoz et al. (2003) observed that complete inhibition of *L. monocytogenes* growth on the rind over a period of 40 days was associated with an undefined ripening consortium from a particular cheese producer. In the same study, the control cheese, ripened with a defined commercial ripening culture allowed for a growth up to 6 log CFU/cm^2 over the same period. This antilisterial effect was not consistent across red smear cheese from different origins, however. A survey only found ten out of 34 ripening consortia showed significant (> 2 log) inhibition of growth compared to the control after 26 days of ripening/storage (Retureau et al., 2010). The amount of inhibition is also variable across ripening consortia. In a survey of ripening consortia from a variety of smear-ripened cheeses,

increases of inoculated *L. monocytogenes* ranged from 3 log CFU/g to 6 log CFU/g over a period of 21 days (Jérôme Mounier et al., 2008). Similar to the ripening consortia, a comparison of the milk from six different farms for the production of Saint-Nectaire cheese showed differences in the growth potential of *L. monocytogenes* in the core of the individual cheeses. Specifically, cheeses made from milk from three of the farms resulted in no *L. monocytogenes* growth at all, while a > 2 log CFU/g increase over an eight-day period was observed in the other three cheeses (Millet et al., 2006).

To a certain extent, the inhibition of *L. monocytogenes* is partially a result of the mere presence of competing microbiota already present in the cheese from the milk or cheese production environment. For instance, competing microorganisms were identified as the primary cause of an increased lag phase and slower growth rate in Camembert style cheeses made with raw milk compared to those made with pasteurized milk (M Gay & Amgar, 2005). Similarly, the presence of the biofilm microbiota from ripening shelves has been shown to reduce the growth of *L. monocytogenes* on model cheese systems (Guillier et al., 2008), and on wooden shelves (Mariani et al., 2011). Indeed, on wooden shelves, Mariani et al. (2011) found reductions of *L. monocytogenes* of up to 2 log on shelves where the biofilm was left intact, whereas a greater than 4 log increase occurred when the wood was sterilized to remove the resident biofilm. When a biofilm microbiota from ripening shelves was tested on a model cheese system, no effect on the lag phase or growth rate of *L. monocytogenes* was observed; however, the inoculated *L. monocytogenes* entered stationary phase at the same time as the biofilm community, suggesting that the established biofilm community provided a bacteriostatic effect against the pathogen (Guillier et al., 2008). This phenomenon is referred to the Jameson effect, and is believed to result

from a non-specific competition for nutrients in a multi-culture system (Mellefont et al., 2008). The variation in inhibitory potential across ripening communities, however, implies that more than just simple competition for nutrients is involved. Therefore, an understanding of the specific mechanisms and/or structure of antilisterial ripening communities may help in developing protective cultures for use against *L. monocytogenes* in soft and semi-soft ripened cheeses.

One suggested mechanism of *L. monocytogenes* inhibition from the cheese microbiota is acid production; specifically, the production of lactic and acetic acids. In Saint-Nectaire cheeses produced using milks from different farms, increased *L. monocytogenes* growth in the core was associated with cheeses where acid development was slower (Millet et al., 2006), whereas antilisterial effects were associated with increased lactate production (Callon et al., 2011; Millet et al., 2006). A similar study found that antilisterial effect of rind communities on Saint-Nectaire cheese were also associated with lactate production, as well as the consumption of citric acid and the production of acetic acid (Callon et al., 2014). Other studies, on the other hand, found no correlation between growth of *L. monocytogenes* and pH (Imran et al., 2010; Retureau et al., 2010); however these studies only compared the pH at particular time points, rather than measuring the rate of acid production or concentration of lactic acid. Since it is the undissociated form of the organic acid molecules which are antimicrobial, these results imply that, at any given pH, it is the type of organic acid present that affects the growth of *L. monocytogenes*, which is in agreement with previous studies (Engstrom et al., 2020; Glass et al., 1995).

The antilisterial activity of some cheese ripening consortia may also be related to interactions between as yet unidentified antimicrobial substances. Bleicher et al. (2010) observed a strong

bactericidal effect against *L. monocytogenes* resulting from a cell-free supernatant recovered from an 8-hour incubation of a ripening consortium from Munster cheese. The antilisterial activity was not affected by the addition of proteinase K, suggesting it was not related to bacteriocin production. The production of lactate or hydrogen peroxide was also ruled out. Fractionation of the supernatant resulted in attenuated antimicrobial activity in multiple fractions, implying that the initial bactericidal activity was the result of a variety of antimicrobial compounds. Therefore, community associated inhibition may be the result of synergistic interaction between multiple antimicrobial compounds produced by multiple community members. Additionally, interactions within a microbial community can also induce the production of antimicrobial compounds not produced in pure culture (Chanos & Mygind, 2016).

A variety of studies have been conducted in order to identify the microbes responsible for the inhibitory action of native microbial communities in cheeses. Lactic acid bacteria seem to be of importance. Gay & Amgar (2005) found that inhibition of *L. monocytogenes* in Camembert cheeses made with raw milk was largely associated with the presence of thermotolerant lactobacilli. Similarly, in a comparison of 34 ripening consortia from Saint-Nectaire cheeses found that the most antilisterial community was characterized by increased amounts of lactobacilli and leuconostocs (Retureau et al., 2010). Lactococci may also provide antilisterial protection, as *Lc. garvieae* and *Lc. lactis* populations were characteristic in milks leading to increased *L. monocytogenes* inhibition in finished cheeses, and maintaining this inhibition when tested individually (Saubusse et al., 2007). Other LAB which have been identified in antilisterial cheese communities include *Vagococcus* spp. (Callon et al., 2014; Monnet et al., 2010), *Carnobacterium maltaromaticum,* and *Enterococcus gilvus* (Callon et al., 2014). The antilisterial activity of these

taxa are likely strain specific, however; as a survey of LAB isolates from Minas cheeses by Campagnollo et al. (2018) found that only 48% of them showed antilisterial activity.

Yeasts have also been identified as potential inhibitors of *L. monocytogenes* in soft cheeses (M Gay & Amgar, 2005). A survey of 304 yeast strains from smear-ripened cheeses found 11 yeast strains with antilisterial characteristics. Ten strains belonged to *Candida intermedia*, as well as one additional strain of *Yarrow lipolytica* (Goerges et al., 2006). *Y. lipolytica* was also identified as a potential source of *Listeria* inhibition in a separate study (Monnet et al., 2010).

The inhibition of *L. monocytogenes* by microbial communities, however, is likely not associated with a single strain, species, or even genus. Indeed, testing of 400 isolates from an antilisterial ripening consortium found no inhibitory effect associated with any particular bacterium (Maoz et al., 2003), suggesting that multiple members of the community were functioning in tandem to inhibit the *L. monocytogenes*. This was further illustrated by Callon et al. (2011) where an assessment of antilisterial contributions of various groups of bacteria in an antilisterial ripening consortium from Saint-Nectaire cheese, found that the inhibitory potential of LAB against *L. monocytogenes* improved greatly when paired with the Gram-positive catalase positive members of the initial consortium. This was interesting since the Gram-positive catalase positive bacteria showed no inhibitory effect on their own. The study's authors hypothesized that increases in lactic and acetic acid production associated with the combined cultures was the possible mechanism.

Further research has also shown that inhibitory potential of microbial communities is more associated with member identities rather than increased microbial diversity. While the erosion of

consortium diversity of a mother smear of 89 strains initially showed reduced antilisterial action, reduction to 6 strains returned *L. monocytogenes* inhibition to similar levels as the original consortium. These six strains formed a minimal smear consisting of two yeast, two gram-positive bacteria, and two gram-negative bacteria (Imran et al., 2010). Further, by building this minimal smear one strain at a time, Imran et al. (2010) found that maximal antilisterial action was only present when all six strains were included. Interestingly, in a follow-up study, Imran et al. (2013) found that removing each member of these six-member consortia individually did not have an effect on the antilisterial effect of the overall smear. This suggested that there is redundancy in the microbial community members (*i.e.*, yeast, Gram-positive, Gram-negative), and that *L. monocytogenes* inhibition is the joint effort of these three communities. It also highlights that the identity of the members, instead of the number of strains is of main importance for antilisterial potential, which has also been observed in a subsequent study (Callon et al., 2014).

As the microbial community structure of ripened cheeses can be an important barrier against *L. monocytogenes*, further research is needed to more completely understand the community composition and interactions that lead to maximal antilisterial action. The use of high-throughput targeted amplicon (*i.e.*, 16S rRNA gene targeted amplicon sequencing) analysis, has already begun to shed light on how the environment throughout the cheese production continuum can shape the microbiome of the finished cheese (Bokulich & Mills, 2013; Falardeau et al., 2019; Frétin et al., 2018), and has been used to identify cheese community members which might show antilisterial activity (Gérard et al., 2021; Suárez et al., 2020). Moving forward, this type of research may help to develop protective starter cultures and ripening consortia that can prevent the growth of *L. monocytogenes*, and naturally improve the safety of soft cheeses.

1.4 High-throughput sequence analysis of microbial communities in cheese

The development of high-throughput sequencing technology has allowed researchers to probe the cheese microbiome to better understand the influence of the native microbiota on the quality and safety of cheese (Afshari et al., 2020). DNA-based investigations of microbial communities can be conducted using targeted amplicon sequencing of a taxonomic marker gene (*e.g.*, 16S rRNA gene) or through shotgun metagenomic sequencing (Forbes et al., 2017). These two molecular methods for investigating microbial communities are described in detail below.

1.4.1 Targeted amplicon sequencing

In targeted amplicon sequencing, polymerase chain reaction (PCR) is used to amplify a phylogenetic or functional marker gene in the total DNA purified from the microbial community of interest. These amplified copies of this marker gene (amplicons) are then sequenced (Bokulich et al., 2016). For bacteria, the 16S rRNA gene, due to its highly conserved nature, is the most common target for amplification (De Filippis et al., 2018). The 16S rRNA gene is approximately 1,550 base pairs in length, with nine variable regions. Typically, only a subset of the variable regions are targeted for sequencing, with primers nested in conserved intervals (Di Bella et al., 2013). As an example, the V4 and V4-V5 regions have been used for the study of environmental microbiomes through the Earth Microbiome project (Parada et al., 2016; Thompson et al., 2017). After sequencing, paired-end reads are assembled, and the resulting contigs are quality filtered before being clustered into operational taxonomic units (OTUs) based on similarity (Hugerth & Andersson, 2017). The use of 97% similarity for OTU clustering has become a standard to estimate species level differentiation between OTUs; however, this claim is not absolute, and care should be taken with this interpretation since variation is expected between the different variable

regions (Schloss, 2010). As an alternative to OTUs, Analysis Sequence Variants (ASVs), which distinguish between amplicon sequence reads by as little as a single nucleotide difference (Callahan et al., 2017). There is continued debate over which of the two clustering methods is more accurate (Callahan et al., 2017; Schloss, 2021). Once the reads are clustered into OTUs (or ASVs), they can then be taxonomically assigned through similarity to a chosen database, such as the SILVA (Yilmaz et al., 2014) or Greengenes (McDonald et al., 2012) databases. A variety of software currently exist for the processing, clustering, and classification of 16S rRNA targeted amplicon sequencing, with the two most popular being MOTHUR (Schloss et al., 2009), which clusters based on similarity (*i.e.*, OTUs) and QIIME (Caporaso et al., 2010), which makes use of ASVs.

In addition to taxonomic profiling, targeted amplicon sequencing allows for diversity analysis. Alpha diversity is a measure of diversity within samples and includes species richness (*e.g.*, total number of distinct OTUs), and species evenness, which measures how even the relative abundances are between OTUs in the sample (Zalewski et al., 2018). A common measure of sample evenness is Shannon's diversity index, which measures the uncertainty in correctly identifying a randomly selected member of an ecological community (Morris et al., 2014). Beta diversity is a measure of how similar (or different) the community structure is between samples (Zalewski et al., 2018). A variety of methods exist for calculating the similarity between samples, including the Jaccard and UniFrac methods, which are based on presence absence of taxa/OTUs, and the Bray-Curtis and weighted Unifrac methods which also take into account the relative abundances of taxa/OTU when calculating similarity (Knight et al., 2018). The UniFrac and weighted Unifrac methods also incorporate phylogenetic distance between taxa/OTUs when

constructing similarity matrices (C. Lozupone et al., 2011), which might help account for closely related species filling the same niche in two different samples.

When analyzing data generated through targeted amplicon sequencing, certain limitations of the data need to be acknowledged. The first is the compositional nature of the data. Since the maximum total reads generated during a single sequencing run is fixed, the read count of a specific OTU is only relative to all other OTUs in the sample. This relative abundance of OTUs can result in negative correlation bias where a true change in the abundance of one species can lead to an apparent (but not true) decrease in the relative abundance of others (Gloor et al., 2017). Other limitations involve varying results between different variable regions of the 16S rRNA gene (Schloss, 2010), and amplification biases resulting from differences in GC-content between taxa (Laursen et al., 2017). Differences in the copy number of the 16S rRNA gene across taxa can also lead to skewed relative abundances between OTUs (Větrovský & Baldrian, 2013). Targeted amplicon sequencing also suffers from a lack of reliable resolution at or below the species level (*e.g.*, strain), and limited capacity to provide accurate functional analysis of the microbiome (Breitwieser et al., 2017). For increased taxonomic and functional resolution, shotgun metagenomic sequencing is necessary.

1.4.2 Shotgun metagenomic sequencing

In shotgun metagenomic sequencing, the entire genomic DNA of the microbial community is fragmented and sequenced (Bokulich et al., 2016). After sequencing and quality filtering, taxonomic and functional analyses can be conducted on the assembled or unassembled reads. For example, Kraken uses *k*-mer analysis to classify against a database of genomes (Wood & Salzberg,

2014), while MetaPhlAn and HUMAnN of the bioBakery platform match reads to a curated database to produces taxonomic and functional profiles of the metagenome, respectively (Beghini et al., 2021).

Shotgun metagenomic sequencing also provides an opportunity to construct population bins or metagenome assembled genomes (MAGs). First the reads are assembled into contigs using software such as MetaSPAdes (Nurk et al., 2017) or Megahit (D. Li et al., 2015). These contigs are then binned with the goal of grouping contigs from closely related donor genotypes. In supervised binning, a database of known organismal genomes is used to help guide the binning process. In unsupervised binning, where no database of previously sequenced genomes is used as a reference, accurate binning relies on genomic features (*e.g.*, GC content, k-mer frequency distribution patterns), coverage profiles of the reads (*i.e.*, reads from a single species are expected to have similar coverage across the metagenome), or a combination of the two (Breitwieser et al., 2017). A variety of software exist for unsupervised metagenomic binning, including MaxBin (Wu et al., 2016) and MetaBAT (Kang et al., 2015). The completion (measured through the presence of single copy core genes) and contamination of the bins can be assessed using CheckM software (Parks et al., 2015).

Bins meeting defined completion and contamination criteria can be used for gene- or pathway-centric analysis (Setubal, 2021). Suggested completion and contamination criteria often vary between research groups. For instance, the MetaWRAP pipeline uses greater than 70% completion and less than 5% contamination as the default settings (Uritskiy et al., 2018), whereas greater than 90% completion and less than 5% contamination has been suggested to classify MAGs as being

of high quality (Bowers et al., 2017). MAGs of sufficient quality can then be taxonomically classified and annotated using software such as the Genome Taxonomy Database Toolkit (Chaumeil et al., 2020) and Prokka (Seemann, 2014), respectively. The construction of MAGs from the metagenomic sequence data allows for a detailed probe into the species that make up the respective microbiome and can also help to assign functional determinants of the microbial community to distinct species. These MAGs might also lead to the discovery of novel species within the microbiome (Setubal, 2021). It is essential to map raw fastq reads back to MAGs in order to assess the proportion of the metagenome contributing to analysis; a quality control step often overlooked in the literature.

Chapter 2: Farm to Fork Profiling of Bacterial Communities Associated with an Artisan Cheese Production Facility.

2.1 Introduction

Cheesemaking from the farm to the table involves direct (e.g., diary equipment and food contact surfaces) or indirect (e.g., feed, litter and washing water) contact with diverse environments and results in a native microbiota in milk and end cheese products that harbor distinct and complex bacterial communities, many of which remain poorly characterized (Montel et al., 2014). Bacterial species from these communities play critical roles in shaping the quality of final cheese products and/or causing potential spoilage and safety issues. The diversity of microbial communities in fermented foods, including cheese products, is influenced by the type of ecosystem in which they reside (Fierer & Jackson, 2006), resulting in the distinctive characteristics of such foods (Fuka et al., 2010; Pangallo et al., 2014). Despite evidence indicating that cheese style is the dominant predictor of rind microbiota (Wolfe et al., 2014), dairy farms and cheese-producing plants play key roles in defining the microbiota of cheese (Bokulich & Mills, 2013; Frétin et al., 2018; Goerges et al., 2008; Mallet et al., 2012; Vacheyrou et al., 2011; Verdier-Metz et al., 2012), and subsequently impact on the quality of artisanal (i.e., small-batch, handcrafted) cheeses.

The comprehensive profiling of microbial diversity during cheese making not only helps to establish standardized management practices by using microbial biomarkers that indicate products of consistently high quality (Bokulich et al., 2016), but also improves the mechanistic understanding of microbial community assembly in a model ecosystem (Wolfe et al., 2014). The advent of high-throughput sequencing technologies has allowed deeper probing of the microbiota

involved at various stages of dairy processing. Studies using 16S rRNA gene targeted amplicon sequencing have characterized microbiota in dairy farms (Frétin et al., 2018), raw and pasteurized milk (Quigley et al., 2013; Rodrigues et al., 2017), cheese processing facilities (Bokulich & Mills, 2013), and most commonly the finished cheeses (Delcenserie et al., 2014; Dugat-Bony et al., 2015; Quigley et al., 2012; Wolfe et al., 2014). However, the composition of bacterial communities across an enclosed cheese-making continuum, from the dairy farm to the final product have not been reported. Therefore, the objective of this current study was to use 16S rRNA gene targeted amplicon sequencing of environmental and food samples collected from a dairy farm, milk, a cheese processing plant, and final cheese products to determine the composition and diversity of bacteria throughout the cheese making process.

2.2 Materials and methods

2.2.1 Cheese production site

The cheese production site consisted of a cheese-making plant and a dairy farm that are approximately 25 Km apart in British Columbia, Canada (Fig. 2.1). Cows reside in a barn with open access to a clover pasture. Hay and straw bedding in the barn were sourced from a hay pasture located 30-40 Km from the farm. Milk obtained from the cows in a dedicated milking parlor was pooled into a bulk tank and stored for a maximum of 48 hours at 0-4°C before being transferred into a transport tank and transported approximately 20 minutes down the road to the cheese plant. The milk at the cheese plant was sourced solely from the dairy farm. The plant produces a range of products, including bloomy-rind, washed-rind, washed-curd and rindless cheeses. Some products are made from pasteurized milk and others from unpasteurized milk (Table 2.1). All cultures are added directly into the cheese milk prior to coagulation.

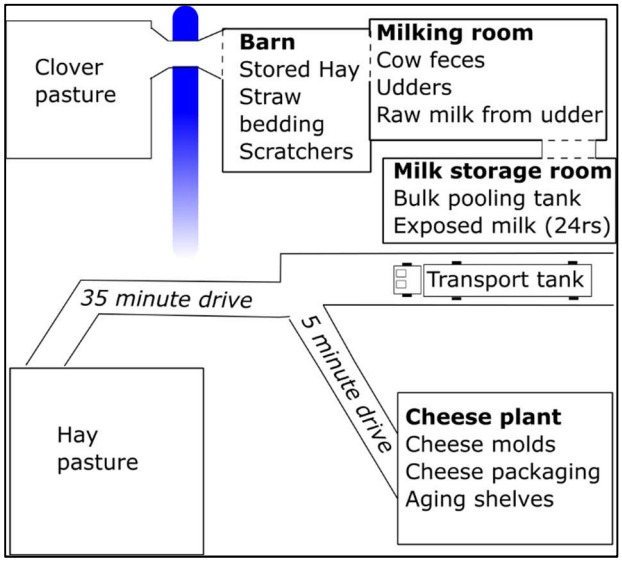

Figure 2.1 Spatial layout of sampling sites along the farm-to-cheese continuum used in this study. The cows have open access to the clover pasture, which was separated from the barn by a small stream. Hay was transported in from a pasture 30–40 Km away. The cows were led into a separate room for milking, and the milk was pooled in a separate chilled room. Milk was transported 25 Km to the cheese plant where all cheeses were produced.

2.2.2 Environmental and cheese sampling

All environmental and cheese samples were collected in two biological replicates on different days in the same season and immediately frozen (-20°C; October 21st and November 18th, 2014 respectively). Up to 20 independent (*i.e.*, separately collected) samples from 21 sample types were collected across four sample domains as outlined in Table 2.2: the dairy farm, including the hay and clover pastures, and all other samples that come into contact with this dairy farm, including the transport tank; milk, including all samples between the teat and adding of the starter cultures; the cheese plant, including any non-cheese and non-milk samples collected within the cheese plant; and samples of the four cheeses produced at the plant. Table 2.1 summarizes the characteristics of the four cheese types. All milk sample types were unpasteurized, with the cultured milk samples being from Jarlsberg cheese production. The exposed raw milk samples were taken from the bulk

tank after milking and allowed to stand, uncovered, at room temperature for 24 hours to amplify and investigate airborne contaminants that may enter the bulk tank milk while it is open during the milking process. The aging shelves only came in to contact with Gruyère cheese and the cheese packaging was clear plastic film used to wrap the cheese before sale.

Table 2.1 Production characteristics of the cheeses evaluated in this study (as reported by the cheesemaker).

Style[1]	Type	Milk Treatment	Organisms Added
Brie	Bloomy rind, soft	Pasteurized	*Lactococcus lactis* *Lactobacillus helveticus* *Lactobacillus paracasei* *Streptococcus thermophilus* *Rhodosporidium infirmominiatum* *Penicillium camembertii* *Geotrichum candidum*
Jarlsberg	Rindless, semi-hard	Unpasteurized	*Lactococcus lactis* *Lactococcus cremoris* *Lactobacillus helveticus* *Lactobacillus delbreuckii* *Steptococcus thermophiles* *Leuconostoc mesenteroides*
Gruyère	Washed rind, semi-hard, cooked[2]	Unpasteurized	*Lactococcus lactis* *Lactococcus cremoris* *Lactobacillus helveticus* *Lactobacillus delbreuckii* *Steptococcus thermophiles*
Cheddar	Rindless, hard	Unpasteurized	*Lactococcus lactis* *Lactococcus cremoris*

The Brie style cheese was aged for 10-14 days, while the other three were aged for at least 60 days.
[1]The style of the cheese was that defined by the cheesemaker and carried on the packaging label**.**
[2]The curd was cooked at 52°C for 30 minutes

Environmental samples were obtained directly by aseptic collection or swabbed as follows. The hay and clover pasture samples were collected directly (including soil and root clumps), ensuring that each sample was 100m apart. Direct samples of stored hay and straw bedding were also collected from inside the barn. Fresh, sterile gloves were used for each sampling. In addition, swab samples were collected from scratchers used by the cows in the barn area. In the milking

barn, ten cows were individually sampled on the two separate dates for a total of 20 each fecal, teat, and milk samples; total herd size was ~30 cows. Samples of cow feces (~50mg) were collected from cows in the milking parlor by aseptically scraping the inside of the rectum with a gloved finger. To swab teats, 3 cm of the distal end of each four teats and its orifice were collected at the farm by dipping swabs in sterile water and then rubbing each teat individually for 15 seconds for a total of 60 s per cow. After swabbing the teat as described prior, the teats were then disinfected using the standard farm sanitizing procedure (*i.e.*, teat dipping and wiping) and then milk samples were collected by milking with the industrial milking equipment; 5ml milk per cow was collected prior to individual cow collection tanks being pooled in the bulk tank. All experiments involving animals were approved by the UBC Animal Care Committee (ID# A14-0279). The interior surfaces of the bulk and transport tanks, and the surfaces in the cheese plant were sampled on dried equipment after routine cleaning but before production and sanitization started. Sampling after sanitation was not possible due to food safety concerns. Aged raw milk samples were collected in an open test tube and exposed to the farm air in the bulk tank room for 24 hrs before being capped and frozen; flies were aseptically removed after the 24hr period.

Swab samples were collected as follows. Cotton-tipped swabs (Puritan Medical, Guilford, ME, USA) were dipped in sterile buffer (0.15M NaCl and 0.1% Tween-20) and then rubbed vigorously in a zig zag pattern against a 17 × 17-cm surface for 60 second except for packaging materials, which were swabbed for 2 minutes instead. Because of size limitations, the entire surface of the cheese molds was sampled for 60 seconds in a zig zag pattern. In addition, the scratcher was swabbed laterally and horizontally in an approximate $5cm^3$ region for 60 seconds. Aging shelves were swabbed in distinct locations between aging rounds of Gruyère cheese.

Cheese samples were collected from the retail store at the cheese plant. These samples were all sale-ready wedges/blocks of aged cheeses, produced from milk separately collected from the same cows included in this study. The Brie cheese was aged for two weeks before being ready for sale and has a shelf life of 2.5 months. The other three cheeses were aged for 60 days before being sale-ready and can have a shelf-life of over one year. These cheese samples were transported at -20°C to the laboratory at UBC and wedges cut aseptically using a scalpel; four samples per cheese unit were collected. To focus the results on the cheese product and not confound the results with the complex environmental microbiota found upon the surface of the cheese rind, a minimum of 10mm of the outer surface of the cheese was aseptically removed and not included.

2.2.3 DNA extraction

Sample bags were opened under aseptic conditions after thawing. Approximately 5 grams of hay, clover, and pasture samples were resuspended in 25 mL of sterile HyClone DPBS +/+ in Temposacs biomerieux SA (80015) and processed in a Stomacher 400 circulator at 230rpm for 2 minutes, before being filtered and spun at 2500xg for 30 minutes to isolate microbiota. For all samples, prior to DNA extraction, cells were mechanically lysed with a 10-minute 65°C incubation prior to physical disruption using a FastPrep instrument (MP Biomedicals, Solon, OH) for two 60 sec cycles at level 5.5 m/s. DNA was extracted from cheese and milk samples using PowerFood DNA isolation kits (Mo Bio Laboratories, Carlsbad, CA, USA). DNA was extracted from pasture, clover, hay, and swabbed samples (tanks, scratchers, cheese molds and aging shelves) using PowerSoil DNA isolation kits, and from swabs of teat skin and fecal samples using PowerFecal DNA isolation kits.

Table 2.2 The sampling method and replicate number of sample types collected from four sample domains across the farm-to-cheese continuum.

Sample domain	Sample type	N[1]	Sampling method
Dairy Farm			
	Clover pasture	20	Direct
	Hay pasture	20	Direct
	Stored hay	20	Direct
	Straw bedding	19	Direct
	Scratchers	19	Swab
	Cow feces	20	Direct
	Teat skin	19	Swab
	Bulk tank[2]	20	Swab
	Transport tank[2]	20	Swab
Milk			
	Teat milk[3]	20	Direct
	Pooled pre-transport	16	Direct
	Exposed raw milk[4]	19	Direct
	Pooled post-transport	20	Direct
	Cultured milk	20	Direct
Cheese Plant			
	Cheese molds	20	Swab
	Aging shelves[5]	20	Swab
	Cheese packaging	20	Swab
Cheese[6]			
	Cheddar	11	Direct
	Gruyère	9	Direct
	Jarlsberg	8	Direct
	Brie	15	Direct

[1]Number of independently collected and sequenced samples
[2] Swabs were taken of the inside (milk contact) surfaces of the bulk and transport tanks.
[3] Teat milk was collected directly from the teat without the use of industrial milking equipment.
[4] Milk was exposed to the air in the chilled milk storage room for 24 hours.
[5] Aging shelves only came into direct contact with Gruyere cheese.
[6] Cheddar, Gruyere, and Jarlsberg cheeses were made using unpasteurized milk; Brie was produced using pasteurized milk.

2.3 Library preparation and sequencing

The V3 region of the 16S rRNA gene was amplified by PCR using TopTaq Master Mix (Qiagen, Venlo, Netherlands) and nucleotide-bar-coded primer pairs 341F: 5′-CCTACGGGAGGCAGCAG-3′and 518R: 5′-ATTACCGCGGCTGCTGG-3′ (Bartram et al., 2011). PCR products were verified on 1% agarose gel. Triplicate reactions from confirmed

amplified single-band samples (200 bp) were pooled and purified using MinElute PCR purification kits and eluted in 10 μL of nuclease-free water (Qiagen). The DNA concentration of each amplicon was determined using a Quant-iT PicoGreen dsDNA Assay Kit (ThermoFisher, Waltham, MA, USA) and Illumina HiSeq platform adaptors were added by PCR to 40 ng of pooled DNA samples. Libraries were run on a HiSeq 2500 on rapid mode (150bp PE) with a 30% PhiX spike to guarantee a diverse set of clusters (Macrogen Corp., Rockville, MD, USA), then de-multiplexed. Sequence data are available at the NCBI Sequence Read Archive under accession number PRJNA499132.

2.3.1 DNA sequence processing

The resulting sequences were processed using MOTHUR (Schloss et al., 2009) and completed as follows. Paired end sequences were assembled into contigs before screening to remove any sequences of improper length or containing ambiguous bases. The remaining sequences were then aligned and classified using the SILVA database (Yilmaz et al., 2014) and screened again to remove any sequences that were chimeric or belonging to non-prokaryotic lineages. The reads were then clustered into unique operational taxonomic units (OTUs) by phylotype (taxonomic identity). Finally, OTUs representing < 0.005% of the total reads within each sample domain were removed from the analysis as recommended (Bokulich et al., 2013).

2.3.2 Data analysis

All data were analyzed using R software (version 3.2.3; R Foundation for Statistical Computing, Vienna, Austria [https://www.R-project.org/]). Microbial communities were analyzed using the phyloseq package (version 1.22.3; McMurdie and Holmes, 2013). All graphs were produced using the ggplot2 package (version 2.2.1; Wickham, 2009) except for the Venn diagram, which was

produced using the VennDiagram package (version 1.6.20; Chen & Boutros, 2011). Dunn tests were conducted using the DescTools package (version 0.99.23; Signorell, 2017).

Alpha-diversity was analyzed using a dataset rarefied to 10,000 reads per sample. Compositional and β-diversity, was analyzed using data normalized to relative abundance. Core OTUs were defined as any OTU represented by at least 5 reads in 80% of the replicates of a sample type (*e.g.*, Cheddar cheese). Core OTUs within a sample domain (*e.g.*, Cheese) were defined as all core OTUs across all sample types within that sample domain. Core OTUs were determined and analyzed using unmodified data.

2.4 Results and discussion

2.4.1 Sequencing results

A total of 20,220,407 reads from 375 samples passed the quality filters in the MOTHUR program, with an average of 53,778 ± 28,417 reads per sample, grouped into 386 OTUs. After rarefication to 10,000 reads per sample, 343 samples remained, and 33 samples containing < 10,000 total reads were excluded. All 386 OTUs were still represented after rarefaction. It should be noted that clustering by taxa rather than by % similarity may reduce the number of individual OTUs since any reads that could not be identified to the genus level were grouped together as "unclassified" at the lowest taxonomic level at which they could be identified. *E.g.*, all reads that were identified as members of the *Lactobacillales* order but could not be identified at the family or genus level were all grouped into a single OTU.

2.4.2 Alpha-diversity

The α-diversity was measured as the richness (Observed OTUs and Chao1 richness estimate), and diversity (Shannon diversity index and inverse Simpson index) of and within sample domains (Fig. 2.2 and Table 2.3). Both richness and diversity differed significantly between sample domains across all measures (Kruskal-Wallace test; $p < 0.001$). The α-diversity was the lowest overall in cheese and highest in the dairy farm environment across all measures (Dunn tests with Holm-Bonferroni correction; $p < 0.01$ and $p < 0.05$, respectively). A decrease in diversity between cow teat skin and ripened cheeses has previously been reported (Frétin et al., 2018). This finding suggests that the cheese making process favours only a small subset of non-starter microbiota, many being crowded out by starter-related taxa.

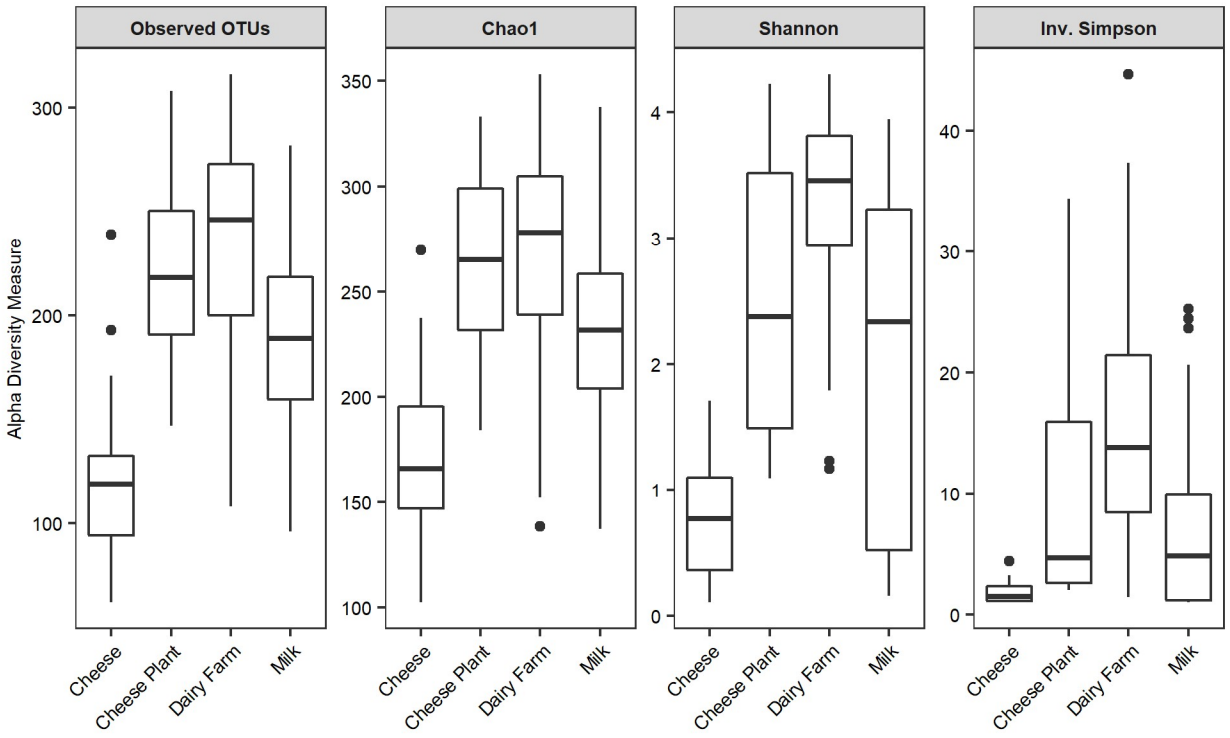

Figure 2.2 Alpha-diversity of sample domains after rarefying to an even depth of 10,000 reads per sample. Alpha-diversity is measured as the richness (i.e., Observed OTUs, Chao1) and evenness (i.e., Shannon, Inv. Simpson).

Differences in diversity were also observed within domains (Table 2.3). Within the dairy farm domain, the observed richness was lower in cow feces than in the other sample types (Dunn tests with Holm-Bonferroni correction: $p < 0.05$). This observation may be driven by the high proportion of "unclassified" OTUs in cow feces (~85%). This is because we used taxonomic-based classification of OTUs, where any sequencing reads that were labelled as "unclassified" were grouped into a single OTU. Cultured milk and raw milk exposed to air for 24 hours were significantly less rich and even compared with other raw milk samples by all measures except for Chao1 (Dunn tests with Holm-Bonferroni correction: $p < 0.05$); probably driven by the fact that cultured and raw milk samples were overwhelmingly populated by *Lactococcus* and *Pseudomonas* (relative abundance, 83% and 92%), respectively (Fig. 2.4). Additionally, richness was higher in teat milk than in pooled raw milk after transport but only significantly for Chao1 (Dunn tests with Holm-Bonferroni correction: $p < 0.05$). This difference in richness may be attributable to contamination between the teat and the bulk and transport tanks, such as from the barn environment (Vacheyrou et al., 2011) or milking equipment (Doyle et al., 2017). At the cheese plant, diversity was significantly lower on the aging shelves than on the packaging materials or the cheese mold surface (Dunn tests with Holm-Bonferroni correction; $p < 0.05$). This is not surprising as the aging shelves only have direct contact with the Gruyère cheese, likely selecting for the subset of microbes associated with the Gruyère rind. Finally, the richness of Gruyère and Jarlsberg cheeses was higher than both Cheddar and Brie cheeses by all three measures, but only significantly for Brie (Dunn tests with Holm-Bonferroni correction: $p < 0.05$). Evenness was also significantly lower in Cheddar, compared with the other three cheeses when compared by the Shannon and inverse Simpson indices (Dunn tests with Holm-Bonferroni correction: $p < 0.05$), most likely due to the

sole addition of *Lactococcus* in Cheddar production which over-represented this genus (94% relative abundance; Fig. 2.4) compared with the other three cheeses.

Table 2.3 Alpha-diversity of sample types after rarefying to an even depth of 10,000 reads per sample. Alpha-diversity is measured as the richness (*i.e.*, Observed OTUs, Chao1) and evenness (*i.e.*, Shannon, Inv. Simpson).

Sample Domain	Sample Type	N[1]	Observed OTUs[2]	Chao1	Shannon	Inv. Simpson
Dairy Farm						
	Clover pasture	19	193 ± 23^{a}	218 ± 29^{ab}	3.82 ± 0.21^{a}	24.3 ± 5.6^{a}
	Hay pasture	14	187 ± 16^{a}	209 ± 26^{a}	3.81 ± 0.31^{a}	22.7 ± 9.0^{ab}
	Hay	20	185 ± 38^{a}	223 ± 34^{ab}	2.77 ± 0.86^{bc}	9.67 ± 7.31^{cd}
	Straw bedding	19	185 ± 27^{a}	221 ± 28^{ab}	3.58 ± 0.29^{ad}	19.3 ± 6.9^{ae}
	Cow scratchers	19	207 ± 8^{a}	247 ± 23^{b}	3.39 ± 0.08^{bd}	13.1 ± 1.4^{bce}
	Cow feces	20	90 ± 11^{b}	115 ± 21^{c}	2.55 ± 0.08^{c}	7.62 ± 0.76^{d}
	Teat skin	17	178 ± 27^{a}	221 ± 35^{ab}	3.16 ± 0.35^{bcd}	11.6 ± 3.8^{cde}
	Bulk tank[3]	12	187 ± 36^{a}	214 ± 27^{ab}	3.26 ± 0.70^{abd}	14.5 ± 11.8^{bcde}
	Transport tank[3]	19	191 ± 26^{a}	218 ± 31^{ab}	3.59 ± 0.31^{ad}	18.2 ± 6.1^{abe}
Milk[4]						
	Teat milk[5]	14	139 ± 52^{a}	166 ± 54^{bc}	2.17 ± 0.92^{a}	5.37 ± 5.16^{a}
	Pooled pre-transport	16	144 ± 19^{a}	175 ± 21^{ab}	2.69 ± 0.25^{a}	6.63 ± 1.86^{ab}
	Pooled post-transport	17	201 ± 42^{a}	231 ± 35^{a}	3.34 ± 0.84^{a}	14.4 ± 5.7^{b}
	Exposed raw milk[6]	18	70 ± 16^{b}	113 ± 39^{c}	0.445 ± 0.109^{b}	1.16 ± 0.06^{c}
	Cultured milk	19	76 ± 29^{b}	118 ± 51^{c}	0.658 ± 0.396^{b}	1.57 ± 0.53^{c}
Cheese Plant						
	Cheese molds	19	165 ± 46^{a}	195 ± 40^{a}	2.82 ± 0.84^{a}	10.8 ± 8.5^{a}
	Aging shelves[7]	20	97 ± 22^{b}	143 ± 26^{b}	1.41 ± 0.25^{b}	2.64 ± 0.57^{b}
	Cheese packaging	18	199 ± 41^{a}	222 ± 39^{a}	3.29 ± 0.87^{a}	16.3 ± 11.8^{a}
Cheese[8]						
	Cheddar	11	42 ± 6^{ac}	75 ± 33^{ab}	0.272 ± 0.146^{a}	1.12 ± 0.12^{a}
	Gruyere	9	65 ± 17^{b}	95 ± 24^{b}	0.985 ± 0.244^{b}	2.11 ± 0.58^{b}
	Jarlsberg	8	57 ± 17^{ab}	98 ± 20^{b}	0.746 ± 0.329^{b}	1.69 ± 0.60^{b}
	Brie	15	38 ± 6^{c}	58 ± 25^{a}	0.920 ± 0.485^{b}	2.13 ± 0.95^{b}

[1]Number of independently collected and sequenced samples

[2]Values in the same column within of the same domain with different superscripts are significantly different (Dunn's Test; Holm-Bonferroni correction; $p < 0.05$).

[3] Swabs were taken of the inside (milk contact) surfaces of the bulk and transport tanks.

[4] All milk samples were unpasteurized

2.4.3 Taxonomic characterization

The taxonomic profile of sample domains and sample types at the phylum level are summarize in Table 2.4 and Fig. 2.3, respectively; and at lower taxonomic ranks in Fig. 2.4. Most OTUs throughout the study were attributed to the phyla *Firmicutes* (47%), *Proteobacteria* (25%), *Actinobacteria* (10%) and *Bacteroidetes* (8.9%). *Firmicutes* was the dominant phylum across all four sample domains, with an increasing trend from the dairy farm (31.1%) to the final cheese (92.1%). This trend is reasonable because cheese production is founded on the addition and selection of common starter and non-starter lactic acid bacteria (NSLAB), for example, *Lactococcus, Streptococcus,* and *Lactobacillus*, which are members of the *Firmicutes* phylum (Donnelly, 2014).

Table 2.4 Relative abundance (%) of common phyla observed across the samples domains

Phylum	Total	Dairy Farm	Milk	Cheese Plant	Cheese
Firmicutes	47.1	31.1	43.1	46.5	92.1
Proteobacteria	25.2	26.6	33.8	28.8	7.22
Actinobacteria	10.5	10.2	13.4	18.6	0.18
Bacteroides	8.94	15.5	7.38	2.97	0.21
Unclassified	7.32	14.9	1.60	2.53	0.26

2.4.3.1 Dairy farm

Firmicutes was the dominant phylum within the dairy farm environment and included the orders *Clostridiales*, *Bacillales,* and *Lactobacillales*. Among individual sample types, however, *Firmicutes* dominated only the bovine environment of straw bedding, scratchers, feces, and teat skin, and was primarily represented by the order *Clostridiales* (17% - 41% relative abundance), which was the most abundant order in the dairy farm environment (21% relative abundance), not including OTUs that were unclassified at the order level. *Actinobacteria* (for example, *Corynebacterium* and *Brevibacterium*), *Bacteroidetes* (for example, *Bacteroides* and *Alistipes*),

and *Proteobacteria* (for example, *Pseudomonas* and *Acinetobacter*) were also identified. *Firmicutes* have previously been observed to be the dominant phylum on cow teat skin, followed by *Actinobacteria* (Frétin et al., 2018; Verdier-Metz et al., 2012). Notably, the *Bacteroidetes* phylum was either undetectable (Frétin et al., 2018) or barely detectable (Verdier-Metz et al., 2012) on teat skin in these studies. Unfortunately, neither study investigated any other farm samples, making it difficult to assess the discrepancy with the current analysis. In the present study, the two genera of *Bacteroidetes* most represented on teat skin, *Bacteroides* and *Alistipes* (~1% relative abundance, each), were also represented at ≥ 1% relative abundance in cow feces, scratchers, and straw bedding. *Bacteroidetes* has been observed as the second major phylum after *Firmicutes* (~40% of reads) in cow feces (Kim & Wells, 2016; Wong et al., 2016), with *Bacteroides* as the second largest genus (Kim & Wells, 2016), emphasizing the prevalence of fecal contamination in the bovine environment.

Pasture and hay samples collected outside the bovine environment were dominated by *Proteobacteria* (41% - 76% relative abundance) and included the orders *Pseudomonadales* (5% - 11%), *Enterobacteriales* (0.5% - 51%), *Burkholderiales* (2% - 8%) and *Rhizobiales* (5% - 8%). The order *Flavobacteriales* (2% - 18% relative abundance) from the *Bacteroidetes* phylum, and *Actinomycetales* (7% - 10% relative abundance) from the *Actinomycetes* phylum were also represented, more in pasture samples that from stored hay. The microbial composition of hay and the clover pastures was similar despite a separation of several kilometers, but was also similar to forage grass grown in Europe and Asia (Estendorfer et al., 2017), and the plant phylosphere in general (Bulgarelli et al., 2013), suggesting host rather than geographical influence. The relative abundance of *Proteobacteria*, specifically the *Enterobacteriales* order was higher in stored hay

than in clover and hay pasture samples. The *Pantoea* genus in particular is associated with phytopathogenic activity (Walterson & Stavrinides, 2015), and might capitalize on reduced plant immunity after harvest, leading to an increase in growth.

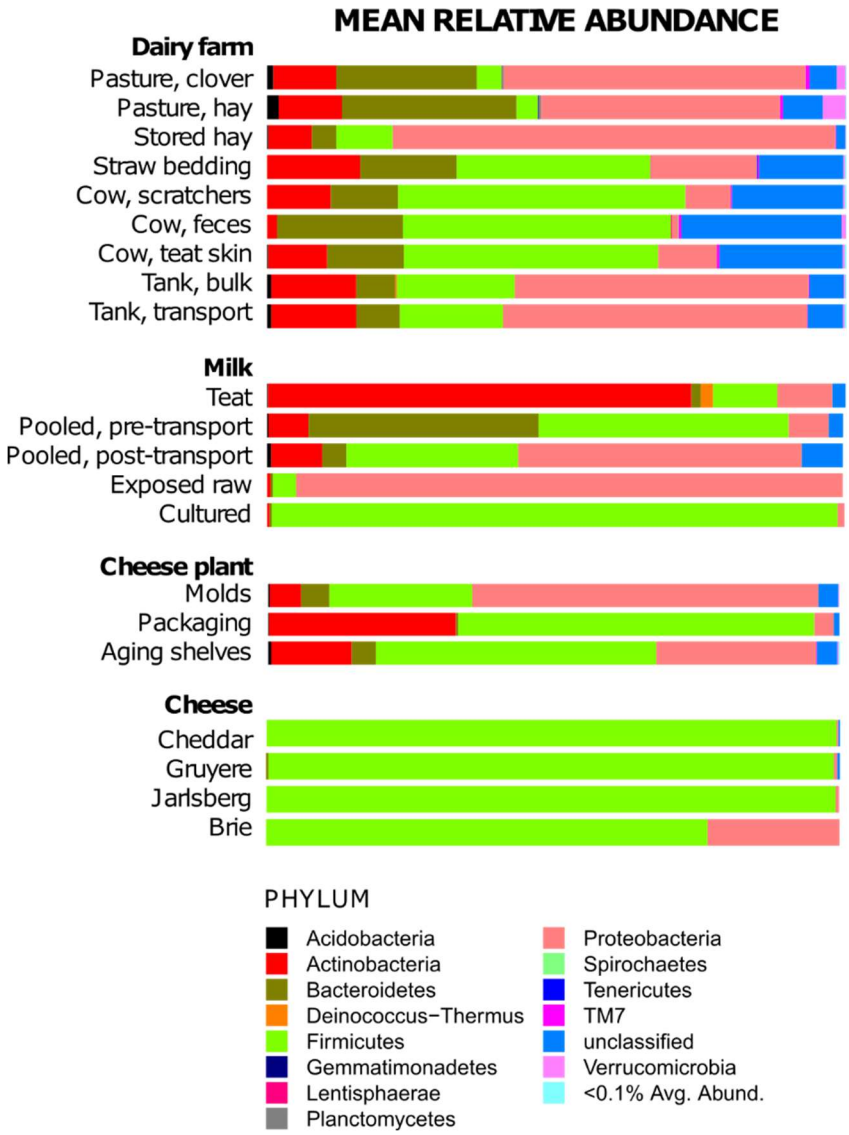

Figure 2.3 Relative abundances of the most common phyla in the dairy farm environment, milk samples, cheese plant, and finished cheeses. Common phyla were defined as any phylum representing greater than 0.1% average relative abundance across sample replicates.

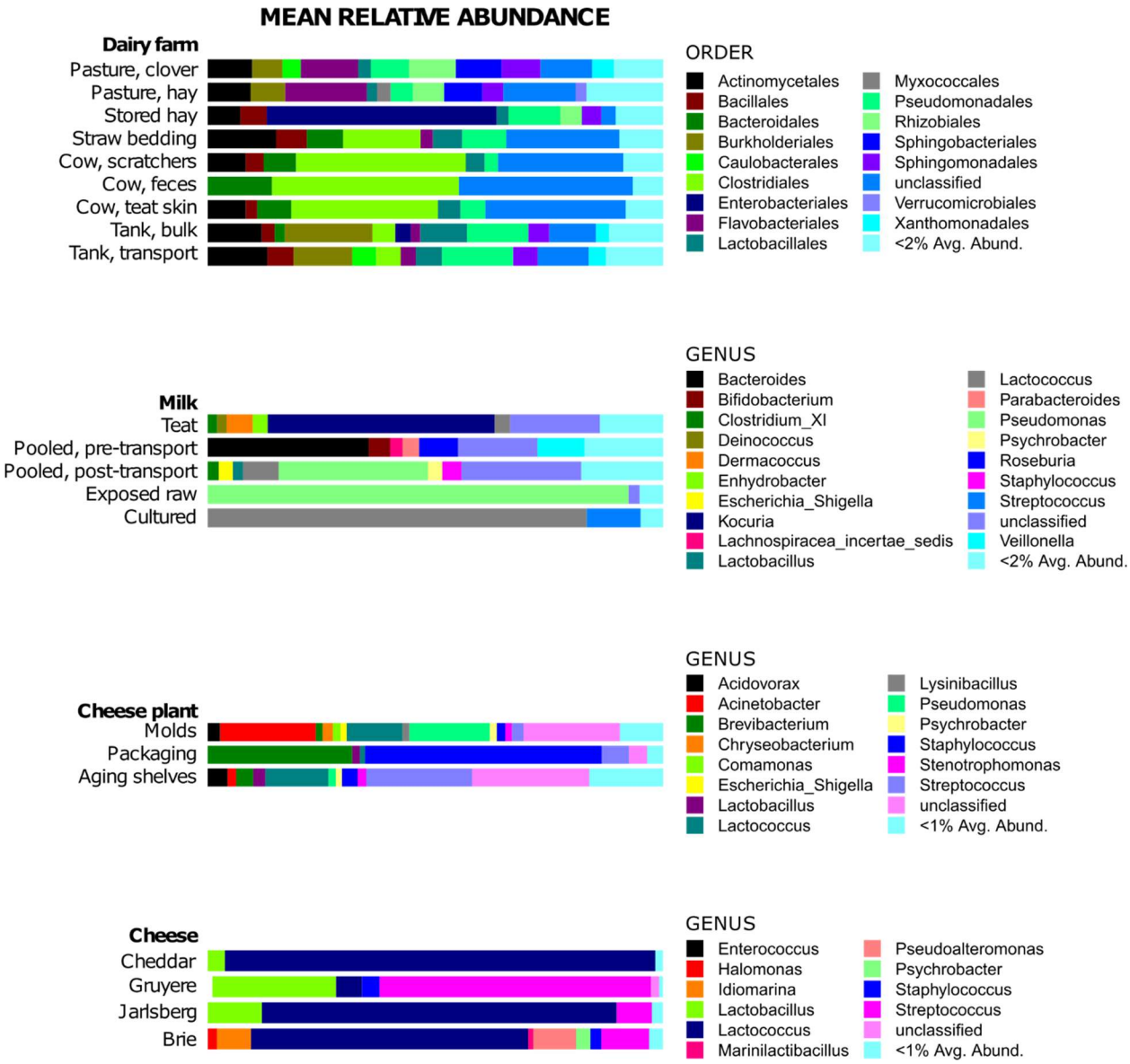

Figure 2.4 Relative abundances of the most common orders or genera in the dairy farm environment, milk samples, cheese plant, and finished cheeses.

Notably, ~60% of the reads at the genus level from the dairy farm were unclassified, emphasizing that the natural environment is still poorly characterized, and that any discussion of the present findings must rely on limited scope. Many other important, but unknown genera or species might inhabit the dairy farm environment and become apparent in the future.

Like the hay and pasture samples, the swabs of the bulk and transport tanks were dominated by *Proteobacteria* (relative abundance, 51% - 53%), specifically of the *Pseudomonadales* (*Pseudomonas*, *Psychrobacter* and *Acinetobacter*; 14% - 16% relative abundance) and *Burkholderiales* (*Acidovorax*; 13% - 19% relative abundance) orders. These two orders are associated with stored milk (Raats et al., 2011), and *Pseudomonadales* is specifically associated with storage at chilled temperatures (Kable et al., 2016; Raats et al., 2011). Unsurprisingly, as discussed below, the milk post-transport samples were similar in composition, suggesting a relationship with the storage tanks. While it is difficult to determine which direction this relationship is happening in, the sanitation step of the transport tanks might result in the added milk defining the shared microbiota.

2.4.3.2 Milk

Firmicutes was the most abundant phylum overall in the milk samples (relative abundance, 43%), followed by *Proteobacteria* (relative abundance, 33%), as previously observed (Quigley et al., 2013), but the abundance differed considerably among individual sample types. Further, not all milk samples were dominated by either of these two phyla. Specifically, the most abundant phylum in teat milk samples was *Actinobacteria* (73% relative abundance), primarily from the genus *Kocuria*, but also *Dermacoccus* and *Dietzia*. *Firmicutes* (*Lactococcus* and *Clostridium* XI) and *Proteobacteria* (*Enhydrobacter* and *Psychrobacter*), on the other hand, were only represented at low levels in the teat milk samples (relative abundance, 11% and 9.5%, respectively). Milk in the mammary gland is widely believed to be sterile (Rainard, 2017); however, the teat canal houses members of the families *Micrococcaceae* (*e.g.*, *Kocuria*), *Dietziacea* (*e.g.*, *Dietzia*),

Moraxellaceae (*e.g.*, *Enhydrobacter*, *Psychrobacter*), and *Clostrideacea* (Gill et al., 2006), which we found in the teat milk samples. We also found a very low relative abundance (~ 0.01%) of *Deinococcus* and *Dermacoccus* in hay samples, which might also be a source, although more abundant genera in hay that were not transferred, such as *Methylobacteria*, have been identified as common contaminants of raw milk (Masoud et al., 2012).

The pooled pre-transport milk samples were dominated by *Firmicutes* and *Bacteroidetes* (relative abundances of 43% and 40%, respectively), which contrasted with both the teat milk samples and the bulk storage tank swabs. Large differences between the microbiota of pooled raw milk compared to the milk directly from the teat have been observed previously, where proportions of *Micrococcaceae* (*e.g.*, *Kocuria*) were higher in the teat milk samples. These differences were attributed to contamination by the milking equipment (Doyle et al., 2017). In the present study, the most abundant genera in the pooled pre-transport milk included five anaerobic microbes (*Bacteroides*, *Parabacteroides*, *Veillonella*, *Roseburia*, and a member of the *Lachnospiraceae* family). Since these genera are not found at high relative abundance in any other milk samples, it is possible that their source was the milking equipment where lack of oxygen might provide them with a suitable niche. If the teat milk had low abundance of microbes to begin with, it would not take much contamination to give these organisms a dominant relative abundance. Longer-term storage in the refrigerated bulk tanks, however, would lead to the selection of physchrophilic bacteria (*e.g.*, *Pseudomonas* spp.), as was observed with the tank swabs. Unfortunately, no samples of the milking equipment were collected, making it impossible to confirm this idea.

After transport (equating to an extension in storage time), dominance shifted from *Bacteroidetes* to *Proteobacteria* (50% relative abundance) that were primarily *Pseudomonas*, followed by *Psychrobacter* and *Acinetobacter* (relative abundance: 33%, 3% and 1%, respectively). This shift is consistent with the community composition observed with the transport tank swabs discussed above. Previous studies have shown an increased abundance of *Gammaproteobacteria* in stored refrigerated milk (Raats et al., 2011; von Neubeck et al., 2015), with *Pseudomonas* being the main spoilage genus associated with raw milk (Ercolini et al., 2009). The microbiota in raw milk exposed to air for 24 hours was almost entirely (92% of reads) *Pseudomonas*. These spoilage bacteria, especially *Pseudomonas*, were prevalent throughout the dairy farm environment, making source attribution difficult. Previous research has also found *Pseudomonas*, along with other *Proteobacteria* to be highly abundant in aerosolized bacteria within dairy operations (Dungan, 2012). Amounts of *Lactococcus* and *Staphylococcus* also had a higher relative abundance in milk after transport, which has also been previously associated with storage/transport (Kable et al., 2016; von Neubeck et al., 2015).

Once at the cheese-making facility, the milk became dominated by the added starter bacteria (96% relative abundance; primarily *Lactococcus* followed by *Streptococcus*, *Lactobacillus* and *Leuconostoc* with a relative abundance of 83%, 12%, 1%, and 0.8%, respectively). At this point, contaminants (*Pseudomonas*, *Kocuria*, *Escherichia/Shigella*) from the farm environment remained at a low relative abundance of 0.1% - 0.4%.

2.4.3.3 Cheese plant

Swabs of cheese molds in the cheese production facility revealed a large proportion of the phylum *Proteobacteria* (60% relative abundance), specifically the genera *Acinetobacteria*, *Pseudomonas*, *Acidovorax*, *Comamonas*, *Psychrobacter*, *Stenotrophomonas* and *Escherichia/Shigella*. *Firmicutes* were also highly represented at a relative abundance of 25% and included the starter culture genera *Lactococcus* and *Streptococcus*, as well as *Staphylococcus* and *Lysinibacillus*. Finally, the relative abundance of *Actinomycetes* was 19%, which was primarily associated with the genus *Brevibacterium* (14% relative abundance). The microbial composition of packaging materials and cheese molds was similar, but the packaging materials had a greater proportion of *Firmicutes* (49% vs. 25% relative abundance), specifically *Streptococcus* (23% vs. 3%), and a correspondingly lower relative abundance of genera from the *Proteobacteria* phylum. Many of these genera were also found in the post-transport milk, suggesting that the cheese molds, and perhaps even the cheese packaging, are affected by the raw milk entering the dairy facility; although, whether the cheese packaging is affected by the milk or just as a by-product of the resulting cheese cannot be determined. A previous study of cheese-making plants (Bokulich and Mills, 2013) found the processing environment to be dominated by *Lactococcus*; however, all the investigated plants pasteurized their milk upon arrival. Alternatively, in the present study, raw milk was used to make most of the cheeses, which could explain the wider diversity of genera found on the cheese molds and packaging.

It should be acknowledged that the milk/cheese-contact surfaces from the bulk tank to the cheese molds were sanitized after sampling, but before contact with the milk or cheese. It is difficult to say what amount of effect this will have on how the tank or mold surfaces affect the milk, but it

seems probable that the observed microbiota are being carried through the system by the milk, and less by the storage/transport/production system contaminating the milk.

The aging shelves used for the Gruyère cheese were dominated by *Firmicutes* (62% relative abundance), mostly *Staphylococcus* with a minor representation of *Streptococcus*, *Lactococcus*, and *Lactobacillus,* and the Actinomycete *Brevibacterium* (31% of the reads). *Staphylococcus* and *Brevibacterium* are two of the most dominant genera on washed rind cheeses such as Gruyère (Wolfe et al., 2014), and have been associated with aging rooms for washed rind cheeses (Bokulich & Mills, 2013). Neither *Brevibacterium* nor *Staphylococcus* were intentionally added by the cheesemaker. Both were observed on the teat skin in this study, but have also been associated with the brine used in washed rind cheeses (Marino et al., 2017; J. Mounier et al., 2006), making it impossible to determine the initial source of these organisms. Either way, it seems that the Gruyère/aging shelf environment preferentially selects for these microorganisms.

2.4.3.4 Cheese

The cheeses were dominated by *Firmicutes*, representing 92% of the reads. Especially in Cheddar cheese, where 95% of total reads were attributed to *Lactococcus* with a low abundance of *Lactobacillus*. The trend in Jarlsberg cheese was similar to that of Cheddar but with a slightly higher relative abundance of *Lactobacillus* (12%), and the added presence of *Streptococcus* (8%) and *Leuconostoc* (0.9%) which were not part of the starter culture of Cheddar. *Lactobacillus* was also omitted as a starter in the Cheddar cheese, emphasizing that the starter genera cannot be attributed solely to the starter culture, with NSLAB strains influencing the final cheese (Goerges et al., 2008). Both the Cheddar and Jarlsberg cheeses were rindless, meaning they were aged while

wrapped in plastic, which might explain the overabundance of starter culture among their microbiota compared to the other two cheeses which did have rinds. The formation of the rind is responsible for raising the pH of the finished cheese (Montel et al., 2014; Jérôme Mounier et al., 2008), allowing the growth of less acidophilic genera. Therefore, the lack of rind on Cheddar and Jarlsberg cheeses may reduce the likelihood of non-starter-related genera from growing.

The Gruyère cheese was dominated by *Streptococcus* (60% relative abundance) followed by *Lactobacillus, Lactococcus* and *Staphylococcus* (27%, 6% and 4% relative abundance, respectively). Increasing cheese hardness has been associated with an increase in the relative abundance of *Lactobacillus* (Quigley et al., 2012), whereas curd cooking at 50°C decreases *Lactococcus* and increases *Streptococcus* (Masoud et al., 2012). *S. thermophilus, Lb. helveticus*, and *Lb. delbrueckii* are important starters of cooked cheeses, as they can tolerate the high temperatures used to scald the curd (Scott et al., 1998), explaining their increased relative abundance in Gruyère which is cooked at 52°C for 30 minutes.

Finally, the Brie cheese was dominated by *Lactococcus* and *Streptococcus* but was unique in having a significant proportion (23%) of *Proteobacteria*, specifically the genera *Pseudoalteromonas*, *Idiomarina*, *Psychrobacter*, *Halomonas* and notably, the marine lactic acid bacterium *Marinilactibacillus*. The presence of these non-starter bacteria was unexpected as Brie was the only cheese made with pasteurized milk. The increased presence of non-starter cultures in the Brie cheese might be attributed to the fact that this soft cheese contains more moisture than the other three firmer cheeses; however, the abundance of non-starter bacteria has been observed to be higher in hard, rather than in soft cheeses (Quigley et al., 2012). Another possibility is that

these genera are added through the dry salting process as *Idiomarina*, *Halomonas*, and *Marinilactibacillus* have been attributed to salt derived from sea water (Coton et al., 2012; Delcenserie et al., 2014; Marino et al., 2017). Physico-chemical properties of brie-style cheese (*e.g.*, higher moisture, salt, and pH) together with reduced microbial population coming from raw milk due to milk pasteurization might introduce an environment in which this halotolerant population might thrive. The *Psychrobacter* in soft cheese has been attributed to carryover from raw milk (Coton et al., 2012), but some *Psychrobacter* spp. have also been isolated from sea water (Yoon, 2005). In addition, *Pseudoalteromonas* and *Psychrobacter* are associated with rooms dedicated to the ripening of bloomy rind cheeses (Bokulich & Mills, 2013). The pH of the cheese might also be involved. Soft ripened cheeses such as Brie tend to be less acidic (Scott et al., 1998), which is associated with the growth of *Halomonas* and *Pseudoalteromonas* (Wolfe et al., 2014).

2.4.4 Shared- and co-occurrence of core OTUs across sample domains

Core OTUs were assessed to determine common genera across all sample domains. Fig. 2.5 shows a Venn diagram demonstrating how these core OTUs were shared across the sample domains. Among 229 OTUs meeting the criteria, 102 were shared by at least two domains and 32 were shared between all four domains. Of 211 core OTUs observed in the farm environment, less than half of these (*i.e.,* 99 OTUs) were shared with other domains, emphasizing the higher diversity observed in the farm environment compared to the downstream sample domains.

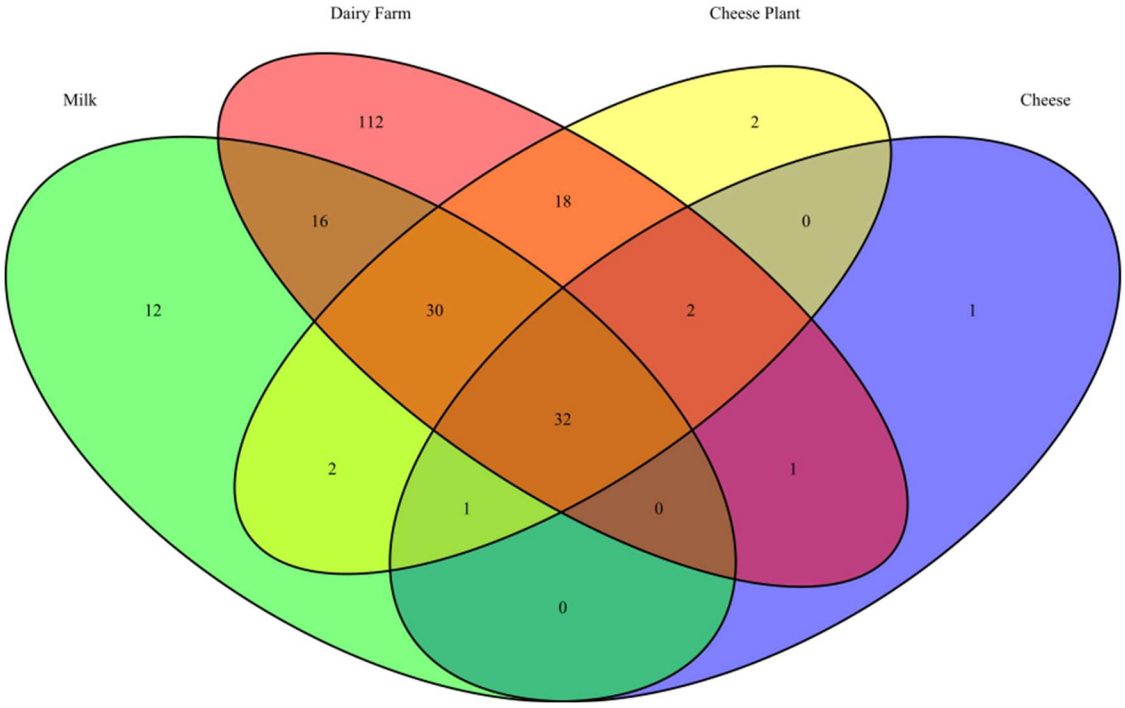

Figure 2.5 Venn diagram of core OTUs within each sample domain. Core OTUs were defined as any OTU that showed at least 5 reads in>80% of replicates of at least on sample type within that domain.

Of the 102 shared core OTUs, 59 of them were identifiable at the genus level and are shown in Table 2.5. A total of 15 identified OTUs were present across all four sample domains and included common starter genera *Lactococcus*, *Lactobacillus*, and *Streptococcus*, and other genera associated with cheese such as *Brevibacterium*, *Corynebacterium* and *Staphylococcus*. This finding emphasizes that cheese-related microbes are present at all stages of the processing continuum, suggesting the possibility that the microbiota in cheeses may be, at least partially, attributed to as far back as the dairy farm environment, as previously suggested (Frétin et al., 2018; Verdier-Metz et al., 2012). One exception to this was *Leuconostoc*, which was only observed as a core OTU in the cheese and milk domains. As the *Leuconostoc* genus was unique to the Jarlsberg

cheese, it implies that "specialist" microbes for individual cheese varieties may not be readily present in all dairy farm environments, and therefore are required to be added by the cheesemaker.

2.4.5 Beta-diversity

Principal coordinates analysis (PCoA) was conducted using both the Bray-Curtis and Jaccard methods in order to analyze β-diversity across the various sample types and domains. Since the Jaccard distance only accounts for the presence/absence of OTUs, whereas the Bray-Curtis difference takes abundance into account, the similar results of these analyses suggest that the microbial composition, and not individual microbial abundance drives the distance between samples (Fig. 2.6). Normal confidence ellipses around each sample domain showed considerable overlap, accept for the dairy farm and cheese domains. This suggests that each sample domain is similar in composition to the one preceding it in the continuum and emphasizes the movement of bacteria through the processing chain. The cheese, however, is different in composition than that of the dairy farm, emphasizing the affect of the cheese making process in selecting for a specific subset of microbes, and echoing the reduction in α-diversity observed in Table 2.3.

To further investigate the composition relationships between sample types, a dendrogram based on the Jaccard distance was generated (Fig. 2.7A). The sample types were grouped into three main clusters. The first contained Brie, Cheddar and Jarlsberg cheeses, and cultured milk. The second contained pooled raw milk in the bulk tank and all samples from the bovine environment (*i.e.*, bedding, scrachers, feces, and utters). The third main cluster contained samples from hay and pasture, teat milk, raw milk after exposure to air for 24 hours, bulk and transport tanks, the cheese plant samples, and Gruyère cheese.

Table 2.5 Taxonomic classification of core OTUs[1] shared between at least two domains and identified at the genus level.

Phylum	Family	Genus	DF[2]	M	CP	Ch
Firmicutes	*Aerococcaceae*	*Facklamia*	x	x	x	
Firmicutes	*Bacillaceae_1*	*Bacillus*	x	x	x	
Firmicutes	*Bacillales_Incertae_Sedis_XII*	*Exiguobacterium*	x		x	
Firmicutes	*Carnobacteriaceae*	*Atopostipes*	x	x	x	x
Firmicutes	*Clostridiaceae_1*	*Clostridium_sensu_stricto*	x	x		
Firmicutes	*Enterococcaceae*	*Enterococcus*	x	x	x	
Firmicutes	*Erysipelotrichaceae*	*Erysipelotrichaceae_incertae_sedis*	x	x		
Firmicutes	*Erysipelotrichaceae*	*Turicibacter*	x	x	x	
Firmicutes	*Lachnospiraceae*	*Blautia*	x	x		
Firmicutes	*Lachnospiraceae*	*Clostridium_XlVa*	x	x		
Firmicutes	*Lachnospiraceae*	*Lachnospiracea_incertae_sedis*	x	x	x	
Firmicutes	*Lachnospiraceae*	*Roseburia*		x	x	
Firmicutes	*Lactobacillaceae*	*Lactobacillus*	x	x	x	x
Firmicutes	*Leuconostocaceae*	*Leuconostoc*		x	x	x
Firmicutes	*Peptostreptococcaceae*	*Clostridium_XI*	x	x	x	x
Firmicutes	*Planococcaceae*	*Planococcus*	x		x	
Firmicutes	*Ruminococcaceae*	*Oscillibacter*	x	x		
Firmicutes	*Staphylococcaceae*	*Jeotgalicoccus*	x	x		
Firmicutes	*Staphylococcaceae*	*Staphylococcus*	x	x	x	x
Firmicutes	*Streptococcaceae*	*Lactococcus*	x	x	x	x
Firmicutes	*Streptococcaceae*	*Streptococcus*	x	x	x	x
Firmicutes	*Veillonellaceae*	*Veillonella*	x	x	x	
Proteobacteria	*Acetobacteraceae*	*Roseomonas*	x		x	
Proteobacteria	*Bradyrhizobiaceae*	*Bradyrhizobium*	x	x		
Proteobacteria	*Caulobacteraceae*	*Brevundimonas*	x	x	x	
Proteobacteria	*Comamonadaceae*	*Acidovorax*	x	x	x	
Proteobacteria	*Comamonadaceae*	*Comamonas*	x		x	
Proteobacteria	*Enterobacteriaceae*	*Buttiauxella*	x	x		
Proteobacteria	*Enterobacteriaceae*	*Escherichia_Shigella*	x	x	x	
Proteobacteria	*Enterobacteriaceae*	*Pantoea*	x			x
Proteobacteria	*Halomonadaceae*	*Halomonas*	x	x	x	x
Proteobacteria	*Moraxellaceae*	*Acinetobacter*	x	x	x	x
Proteobacteria	*Moraxellaceae*	*Enhydrobacter*	x	x	x	
Proteobacteria	*Moraxellaceae*	*Psychrobacter*	x	x	x	x
Proteobacteria	*Pseudoalteromonadaceae*	*Pseudoalteromonas*		x	x	
Proteobacteria	*Pseudomonadaceae*	*Pseudomonas*	x	x	x	x
Proteobacteria	*Rhodobacteraceae*	*Paracoccus*	x	x	x	
Proteobacteria	*Xanthomonadaceae*	*Pseudoxanthomonas*	x		x	
Proteobacteria	*Xanthomonadaceae*	*Stenotrophomonas*	x	x	x	
Acidobacteria	*Acidobacteria_Gp4_family_incertae_sedis*	*Gp4*	x	x		
Acidobacteria	*Acidobacteria_Gp6_family_incertae_sedis*	*Gp6*	x	x	x	
Actinobacteria	*Bifidobacteriaceae*	*Bifidobacterium*	x	x	x	
Actinobacteria	*Bifidobacteriaceae*	*Gardnerella*	x		x	
Actinobacteria	*Brevibacteriaceae*	*Brevibacterium*	x	x	x	x
Actinobacteria	*Corynebacteriaceae*	*Corynebacterium*	x	x	x	x
Actinobacteria	*Dietziaceae*	*Dietzia*	x	x	x	
Actinobacteria	*Microbacteriaceae*	*Microbacterium*	x		x	
Actinobacteria	*Micrococcaceae*	*Kocuria*	x	x	x	x
Actinobacteria	*Nocardioidaceae*	*Nocardioides*	x		x	
Actinobacteria	*Propionibacteriaceae*	*Propionibacterium*	x	x	x	
Bacteroidetes	*Bacteroidaceae*	*Bacteroides*	x	x	x	x
Bacteroidetes	*Flavobacteriaceae*	*Chryseobacterium*	x	x	x	
Bacteroidetes	*Flavobacteriaceae*	*Flavobacterium*	x		x	x
Bacteroidetes	*Porphyromonadaceae*	*Parabacteroides*	x	x	x	
Bacteroidetes	*Prevotellaceae*	*Prevotella*	x	x		
Bacteroidetes	*Rikenellaceae*	*Alistipes*	x	x	x	x
Bacteroidetes	*Sphingobacteriaceae*	*Pedobacter*	x		x	x
Verrucomicrobia	*Verrucomicrobiaceae*	*Akkermansia*	x	x		
TM7	*TM7_family_incertae_sedis*	*TM7_genus_incertae_sedis*	x	x	x	

[1] Core OTUs were any any with at least 5 reads in >80% of replicates of at least one sample type within that domain.
[2] DF = dairy farm, M = milk, CP = cheese plant, Ch = cheese

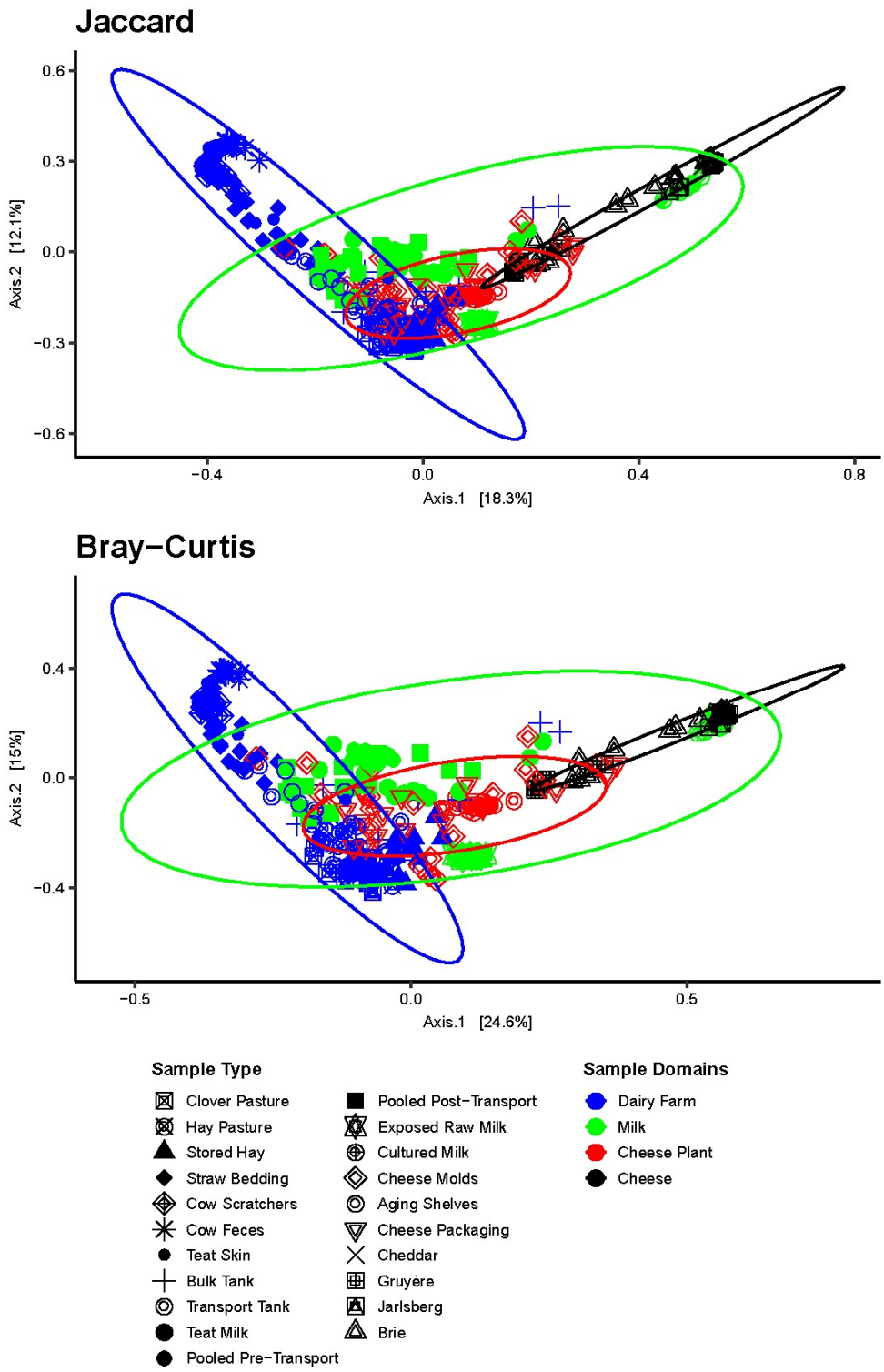

Figure 2.6 Principal co-ordinate analyis of rarefied samples, (10,000 reads each), calculated using the Jaccard and Bray-Curtis distance metrics.

The clustering of the cultured milk with three of the cheeses is likely driven by a reduced number of OTUs in these samples, rather than a unique set. These cheeses and cultured milk had lower richness compared most other samples (Table 2.3), while all but one of the core OTUs of the cheese domain are shared with other domains (Fig. 2.5). That the Gruyère cheese clustered separately from the other cheeses and was closely related to its aging shelves was notable, but not unexpected. Aging and rind formation might provide Gruyère cheese with different microbiota from the other three cheeses. As described above, aging with the rind helps to neutralize the initial acidic pH of the young cheese, allowing for less acidophilic genera to grow in the cheese. Moreover, bacteria from the rind and aging shelf may have penetrated to the cheese core, something not likely with the other three cheeses which were not aged directly on the aging shelves. Since the cultured milk was not pasteurized but still clustered separately from the milk arriving at the cheese plant, the starter culture addition might "crowd out" other OTUs by lowering their relative abundance and reducing their chances of being collected in the sample.

The clustering of samples from the bovine environment suggests a unique group of OTUs. In fact, many of the core OTUs in the dairy farm were unique to that domain (Fig. 2.5), and since the other farm samples clustered closely with other domains, many of those unique core OTUs were probably derived from these bovine environment samples. The grouping of the pooled raw milk pre-transport in this cluster indicates that the initial flora of the milk in the bulk tank may be dictated by contaminants from the bovine environment. For example, some core OTUs, such as *Blautia* and *Akkermansia*, shared between the dairy farm and milk domains were found in the bulk tank milk but were essentially absent in other raw milk samples. As noted above, feces and teat

skin are important sources of bacteria in raw milk (Doyle et al., 2017; Vacheyrou et al., 2011; Verdier-Metz et al., 2012).

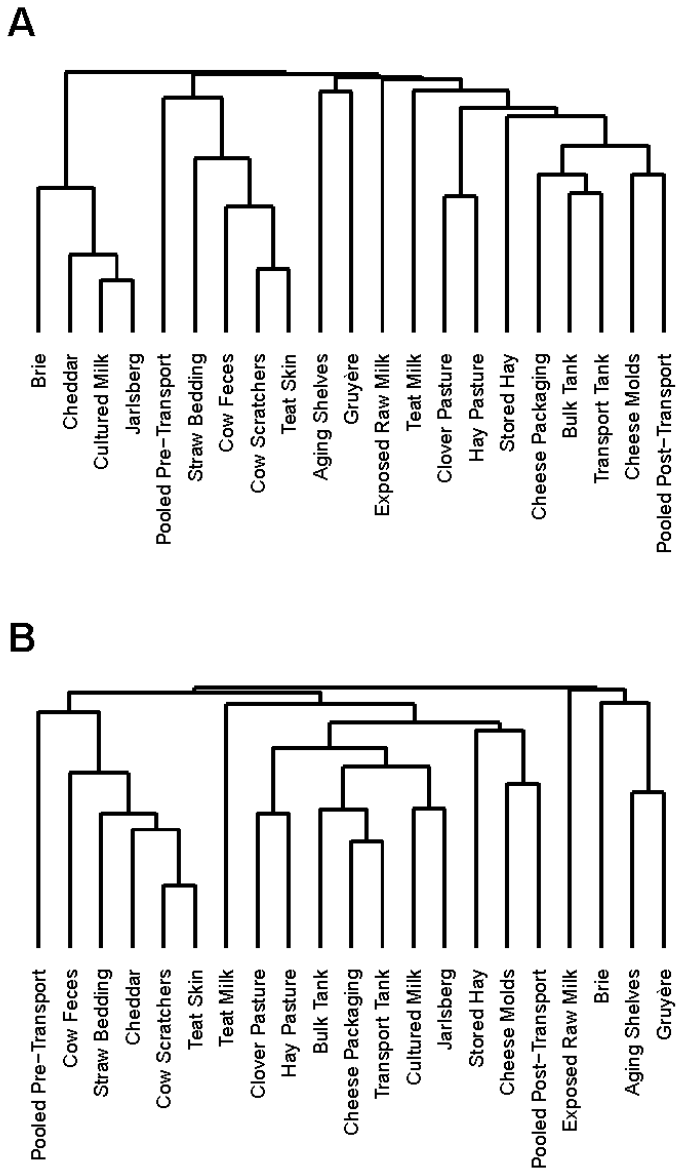

Figure 2.7 Dendrograms showing the relatedness between sample types with (A) and without (B) the presence of starter-associated genera, (*Lactococcus*, *Lactobacillus*, *Streptococcus*, and *Leuconostoc*), using the Jaccard dissimilarity method. Dendrograms were produced using data that had been rarefied to 10,000 reads each. The dataset with the starter-associated genera removed was further normalized to relative proportions to account for the varied loss of reads between sample types.

In contrast to the pooled pre-transport raw milk, the teat milk and post-transport raw milk samples clustered with samples from the cheese molds and packaging from the cheese plant, the bulk and transport tanks, and the samples collected from the hay and pasture. The separate clustering of these two raw milk samples from the bulk tank milk suggests that the unique OTUs associated with the bovine environment dominate the milk microbiota only during the milking process. Since the milk storage environment is refrigerated from this point on, the separate clustering may be a result of increased selection for psychrophiles and environmental contaminants, such as *Pseudomonas*, *Psychrobacter* and *Flavobacterium*. This is further emphasized with the close clustering with the exposed raw milk with was almost completely composed of *Pseudomonas*. Perhaps the teat milk was contaminated from the common environmental contaminants associated with the pastures and stored hay through the teat skin's contact with the straw bedding. The refrigerated storage of the milk in the bulk and transport tanks allows these bacteria to thrive, and therefore affecting the surface environment of these two storage tanks.

A sub-cluster within the milk/cheese plant/pasture cluster included the cheese mold and packaging samples along with the transport and bulk tanks, and the pooled raw milk after transport. Bokulich and Mills (2013) previously showed that house microbiota differ between cheese processing facilities. In the present study, both the cheese molds and the packaging materials share several common genera (> 1% relative abundance) with the milk after transport and/or the transport tank, such as *Acinetobacter*, *Acidovorax*, and *Brevibacterium*. While it is not possible to conclusively prove this with 16S rRNA gene targeted amplicon sequencing, it is possible that the milk coming into the cheese processing facility helps to define that facility's unique microbiota.

In order to probe the composition and abundance of environmental genera not associated with the added starter cultures, a subset of data was created where the dominant starter genera (*Lactococcus*, *Lactobacillus*, *Streptococcus*, and *Leuconostoc*) were removed. Table 2.6 shows that these starter genera comprised > 90% of reads from the finished cheeses, except for Brie cheese, where the relative proportion of the non-starter taxa was significantly higher at 31.3% ± 24.2% (Tukey's HSD; $p < 0.05$) compared with the other three cheeses. This was interesting since Brie was the only type of cheese that was made with pasteurized milk; although, as mentioned above, the presence of these bacteria may result from the salting of the cheese. The other three raw-milk cheeses did not significantly differ in their proportion of background microbiota, but the Jarlsberg and Gruyère cheeses had the lowest (1.6% ± 0.6%) and highest (8.8% ± 7.2%) abundance, respectively.

Table 2.6 Relative proportion of reads representing the background microflora of each cheese. Background microflora was considered all OTUs that are not part of the starter culture (*i.e.*, *Lactobacillus*, *Lactococcus*, *Streptococcus*, and *Leuconostoc*).

Cheese	Pasteurized?	Relative proportion of background microflora
Cheddar	No	0.0156 ± 0.0052^{a}
Gruyere	No	0.0878 ± 0.0720^{a}
Jarlsburg	No	0.0155 ± 0.0064^{a}
Mt. Ida	Yes	0.313 ± 0.242^{b}

Values with different superscripts are significantly different (Tukey's HSD; $p < 0.05$)

A reassessment of sample relatedness based only on background microbiota is shown in Fig. 2.7B. While the Gruyère and Brie cheeses still clustered with the aging shelves, the Cheddar clustered with the bovine environment, and the Jarlsberg was most similar to the cultured milk in a cluster that also included the pasture samples, the bulk and transport tanks, and the cheese packaging. This clustering suggests that the dairy farm may be an important source of non-starter related taxa

in Cheddar and Jarlsberg, but that the two cheese styles differ in which microorganism are maintained. It should be noted, however, that by removing the starter related taxa, we have likely also removed strains of NSLAB acquired from the environment, so care should be taken when interpreting these results.

The analyses of microbiota in this current study were conducted using 16S rRNA gene targeted amplicon sequencing, which presents certain limitations. Of main concern is a reduced level of resolution when compared to other, more expensive methods such as shot-gun metagenomics (Poretsky et al., 2014); specifically the inability probe beyond the genus level to confirm shared strains between samples. Biases in amplification resulting from differences in genomic GC-content (Laursen et al., 2017) or variable copy number of the 16S rRNA gene (Větrovský & Baldrian, 2013) should also be considered. Therefore, this work provides a good first insight into the different microbiota across the cheese-making continuum, but future work involving deeper sequencing technologies will be required to further investigate these relationships.

2.5 Conclusion

Throughout the cheese making process, a higher diversity was observed in the dairy farm environment relative to the finished cheese. The microbiota of the milk changes drastically between the teat and storage tank, either through selection or addition of microbes from the local environment (*e.g.*, teat skin or straw bedding), before prolonged storage appears to select for common diary-related taxa such as *Pseudomonas* and *Lactococcus*. The cheese production process then leads to a relatively higher abundance of a small subset of starter-related taxa, while a larger subset of environmental microbes (*e.g.*, *Staphylococcus* and *Pseudoalteromonas*) remain as a

minor component of finished cheeses. The microbial composition of the finished cheeses seems to differ between cheese styles, primarily through variation in the added starter cultures, but possibly also by differences in processing steps, such as cooking or aging. Future investigation at the strain level will be required before these relationships between the microbiota of cheeses, the processing environment, and diary farm can be fully elucidated.

Chapter 3: Differential Growth of *Listeria monocytogenes* in Soft Surface-Ripened Cheeses

3.1 Introduction

Listeria monocytogenes is a psychrophilic foodborne pathogen, and one of the leading causes of foodborne related deaths in the developed world (Thakur et al., 2018). Due to its ability to persist in the food processing environment (V. Ferreira et al., 2014), *L. monocytogenes* is a common contaminant of ready-to-eat foods, and has resulted in many foodborne outbreaks associated with soft cheeses (Falardeau et al., 2021).

Soft ripened cheeses (SRC) have a moisture on a fat free basis (MFFB) of greater than 67% (Canadian Dairy Information Centre, 2020; Codex Alimentarius Commission, 1978) and can be either mold-ripened (*e.g.*, Brie, Camembert) or smear-ripened (*i.e.*, washed rind cheeses). Due to a favourable a_w and pH in these cheeses, *L. monocytogenes* contamination can grow to dangerous levels, both during the ripening period (S. Liu et al., 2007; Lobacz et al., 2013), or at retail (Lahou & Uyttendaele, 2017). Various biocontrol strategies have been investigated for controlling the growth of this foodborne pathogen in these cheeses (*e.g.*, bacteriocins and bacteriophage), but with limited success in in the long run (Falardeau et al., 2021).

The potential for *L. monocytogenes* growth in SRC is not heterogeneous, with certain washed rind SRC showing inhibition against the growth this bacterium despite favourable conditions of a_w and pH (Gérard et al., 2020). Previous research has suggested an effect of the milk treatment (Marielle Gay & Amgar, 2005), ripening culture (Imran et al., 2010; Monnet et al., 2010; Retureau et al.,

2010; Saubusse et al., 2007), and/or the production/concentration of organic acids (Callon et al., 2011, 2014; Millet et al., 2006), but no clear mechanism has yet been elucidated. Therefore, the objective of this study was to 1) compare the growth of *L. monocytogenes* at refrigerated temperatures in a variety of SRC from various origins, and 2) investigate the relationship between *L. monocytogenes* growth and various physicochemical and biological characteristics of the individual cheeses.

3.2 Materials and methods

3.2.1 Inoculum preparation

All assays were conducted using *L. monocytogenes* BCCDC-A3, which had been previously isolated from a cheese sample at a dairy plant in British Columbia. *L. monocytogenes* BCCDC-A3 was identified as a member of serogroup 4b/4d/4e using a multiplex PCR assay designed to differentiate between the major serovars of *L. monocytogenes* (Doumith et al., 2004). For each biological replicate, an individual isolate of *L. monocytogenes* BCCDC-A3 was grown for 24-28 hours at 37°C in tryptic soy broth (Becton, Dickinson and Company; Franklin Lakes, NJ) with 0.6% yeast extract (Becton, Dickinson and Company; TSB-YE) while shaking, to achieve a concentration of approximately 10^9 CFU/ml. An aliquot of this culture was then diluted 100X into fresh TSB-YE and incubated at 8°C without shaking until early stationary phase (OD_{600} ~0.900; 7-10 days). These cold-adapted cultures were then washed three times with phosphate buffered saline (pH 7.4; Alfa Aesar; Haverhill, MA; PBS), and resuspended in PBS to achieve a concentration of 10^5 CFU/ml.

3.2.2 Measuring the growth of *L. monocytogenes* in soft surface-ripened cheeses

The growth of *L. monocytogenes* was monitored in 43 SRC, comprised of thirty-six bloomy rind and seven washed rind (Table 3.1). Cheeses were chosen to maximize the number of artisan-produced samples. Thirty were produced with pasteurized milk, and 12 were produced from raw milk. The remaining cheese was produced using thermized milk, which involves heating the milk at 57-68°C for 10-20 seconds (Rukke et al., 2011). Cheeses were produced in France (n = 29), Canada (n = 12), and Denmark (n = 2). Cheeses from Canada were further grouped into three regions: Quebec (n = 7), Ontario (n = 2), and British Columbia (n = 3). Each cheese was aseptically aliquoted into 3.0 g ± 0.1g sub-portions and divided into sterile 118 ml Whirl-Pak™ sample bags (Whirl-Pak®, Madison, WI). Each portion represented a cross-section of the cheese and included both rind and core. For each biological replicate (n = 3), up to 10 sub-portions were inoculated with 30 µl of cold-adapted *L. monocytogenes* culture to achieve a concentration of ~10^3 CFU/g. Negative controls were inoculated with 30 µl of sterile PBS. Inoculated cheeses and negative controls were all incubated at 8°C until enumeration. At the same time, up to three samples of approximately 25 g were reserved and stored at -20°C for microbiome analysis (Chapters 4 and 5).

The growth of *L. monocytogenes* in each cheese was measured at least every second day as follows. One inoculated sample bag for each biological replicate was diluted 10X in sterile PBS and homogenized in a stomacher for 2 minutes at 230 rpm. The homogenized samples were then diluted and spread-plated on PALCAM agar (Neogen Corp.; Lansing, MI). The plated samples were enumerated after incubation at 37°C for 24 hours. Negative controls for each cheese were

also enumerated at every second enumeration point to ensure the cheeses remained free of any countable *L. monocytogenes*.

For each biological replicate, the growth of *L. monocytogenes* was modelled over 12 days using logistic regression. For each sample, the growth was measured as both the area under the modelled growth curve (AUC) normalized to the starting concentration, and the total increase in *L. monocytogenes* cells over the course of the experiment. For categorical analysis, (*e.g.*, Principal Components Analysis), cheeses were grouped into four categories based on the total increase in *L. monocytogenes* modelled over the 12 days: no growth (<1 log CFU increase; "-"), low growth (1 log – 2 log CFU increase; "+"), medium growth (2 log – 3.5 log CFU increase; "++"), and high growth (> 3.5 log CFU increase; "+++").

3.2.3 Physicochemical and microbial analysis of cheeses

The pH of each cheese was measured in triplicate from three different areas of the cheese (*i.e.*, edge, rind, core) using an Oakton pHTestr® 50S Spear-Tip Waterproof Pocket Tester (Cole-Parmer Canada Company; Montreal, QC). The water activity (a_w) of each cheese was measured on triplicate 3.0 g ± 0.1 g aliquots using an Aqualab Series 3 water activity meter (Decagon Devices, Inc.; Pullman, WA).

Total aerobic microbial count (TAMC) and total lactic acid bacteria (TLAB) were measured for each cheese (n = 3) by spread plating. At three different time points during the *L. monocytogenes* growth assay, uninoculated, negative control portions of cheese were prepared and homogenized as described above. For TAMC, appropriate dilutions were enumerated on tryptic soy agar (TSA)

after incubation at 30°C for 48 hours. TLAB dilutions were enumerated on both M17, and de Man, Rogosa and Sharpe (pH 5.4; MRS-5.4) agars (HiMedia Laboratories; West Chester, PA). Samples on M17 agar were enumerated after aerobic incubation at 37°C for 48 hours, while samples on MRS-5.4 were enumerated after anaerobic incubation at 37°C for 72 hours.

3.2.4 Data analysis

All data were analyzed using R software (version 4.1.0; R Foundation for Statistical Computing, Vienna, Austria [https://www.Rproject.org/]) with the assistance of the tidyverse collection of packages (version 1.3.1; Wickham et al., 2019). Growth modelling was achieved using the growthcurver package (version 0.3.1; Sprouffske and Wagner, 2016). PERMANOVA analysis was conducted using the vegan package (version 2.5-7; Oksanen et al., 2020), and Fisher's LSD was calculated using the agricolae package (version 1.3-5; de Mendiburu, 2021). To correct for multiple comparisons where necessary, p-values were adjusted using the False Discovery Rate (FDR) method and are labelled as q-values.

3.3 Results

3.3.1 Differential growth of *L. monocytogenes* across surface-ripened cheeses

The growth of *L. monocytogenes* at 8°C in the cheeses was modeled over 12 days (Fig. 3.S1). The AUC of *L. monocytogenes* growth differed significantly across cheeses (Fig. 3.1A; ANOVA; $p < 0.0001$), with the total increase in pathogen ranging from no growth to as high as a 5.4 Log CFU increase over the refrigerated incubation period (mean increase = 2.5 ± 1.2 Log CFU). Out of the 43 cheeses assessed, six, seven, 20, and 10 cheeses were assigned to the no, low, medium, and high growth groups, respectively (Table 3.1).

Table 3.1 – Milk treatment, origin, growth category of the cheeses used in this study. Also shown are the cheese samples that were analyzed by 16S rRNA targeted amplicon sequencing (16S) and the sample analyzed by shotgun metagenomic sequencing (SMS).

Cheese	Milk	Origin	Rind	Growth[a]	16S	SMS
1-A	Pasteurized	Quebec	Bloomy	++	X	
1-B	Pasteurized	France	Washed	+++	X	
1-C	Raw	France	Bloomy	++	X	
1-D	Raw	Quebec	Bloomy	++	X	
2-A	Pasteurized	France	Bloomy	++	X	
2-B	Pasteurized	France	Bloomy	++	X	
2-C	Raw	France	Bloomy	-	X	
2-D	Raw	France	Bloomy	+	X	
3-A	Pasteurized	France	Bloomy	++	X	
3-B	Pasteurized	France	Washed	++	X	
3-C	Raw	France	Bloomy	-	X	
3-D	Raw	France	Bloomy	+	X	
4-A	Pasteurized	France	Bloomy	++	X	
4-B	Pasteurized	Denmark	Bloomy	+++	X	
4-C	Pasteurized	Quebec	Bloomy	+++	X	
4-D	Pasteurized	Quebec	Bloomy	+++	X	
5-A	Pasteurized	France	Bloomy	-	X	X
5-B	Pasteurized	British Columbia	Bloomy	+++	X	
5-C	Pasteurized	France	Washed	++	X	X
5-D	Pasteurized	British Columbia	Bloomy	+++	X	X
6-A	Pasteurized	France	Bloomy	-	X	X
6-B	Pasteurized	Quebec	Bloomy	++	X	
6-C	Pasteurized	Ontario	Bloomy	++	X	
6-D	Pasteurized	France	Bloomy	++	X	
7-A	Thermized	France	Bloomy	+++	X	
7-B	Pasteurized	France	Bloomy	+	X	
7-C	Pasteurized	Ontario	Bloomy	+++	X	
7-D	Pasteurized	British Columbia	Bloomy	++	X	X
7-E	Pasteurized	Denmark	Bloomy	+++	X	X
8-A	Pasteurized	France	Bloomy	++	X	X
8-B	Pasteurized	Quebec	Washed	-	X	X
8-C	Pasteurized	France	Bloomy	+++	X	X
8-D	Raw	France	Bloomy	+	X	X
8-E	Pasteurized	France	Washed	++	X	X
9-A	Raw	France	Washed	+	X	X
9-B	Raw	France	Bloomy	+	X	X
9-C	Pasteurized	Quebec	Bloomy	++	X	X
9-D	Pasteurized	France	Bloomy	++	X	
9-E	Raw	France	Washed	++	X	X
10-A	Pasteurized	France	Bloomy	+		
10-B	Pasteurized	France	Bloomy	++		
10-C	Raw	France	Bloomy	-		
10-D	Raw	France	Bloomy	++		

Growth categories were based on total increase of *L. monocytogenes* 12 days and were defined as no growth (<1 log CFU increase; "-"), low growth (1 log – 2 log CFU increase; "+"), medium growth (2 log – 3.5 log CFU increase; "++"), and high growth (> 3.5 log CFU increase; "+++").

On average, cheeses made with raw milk showed significantly lower growth of *L. monocytogenes* compared to cheeses made with pasteurized milk (Fig 3.1B; t-test; $p = 0.001$). Further, cheeses made with pasteurized milk were present in all *L. monocytogenes* growth categories, whereas raw milk cheeses were only represented in the no, low, and medium growth categories (*i.e.*, no increase greater than 3.5 Log CFU *L. monocytogenes* was observed in any of the raw milk cheeses; Table 3.1). It should be noted, however, that *L. monocytogenes* growth in some individual pasteurized milk cheeses was lower than in some raw milk cheeses.

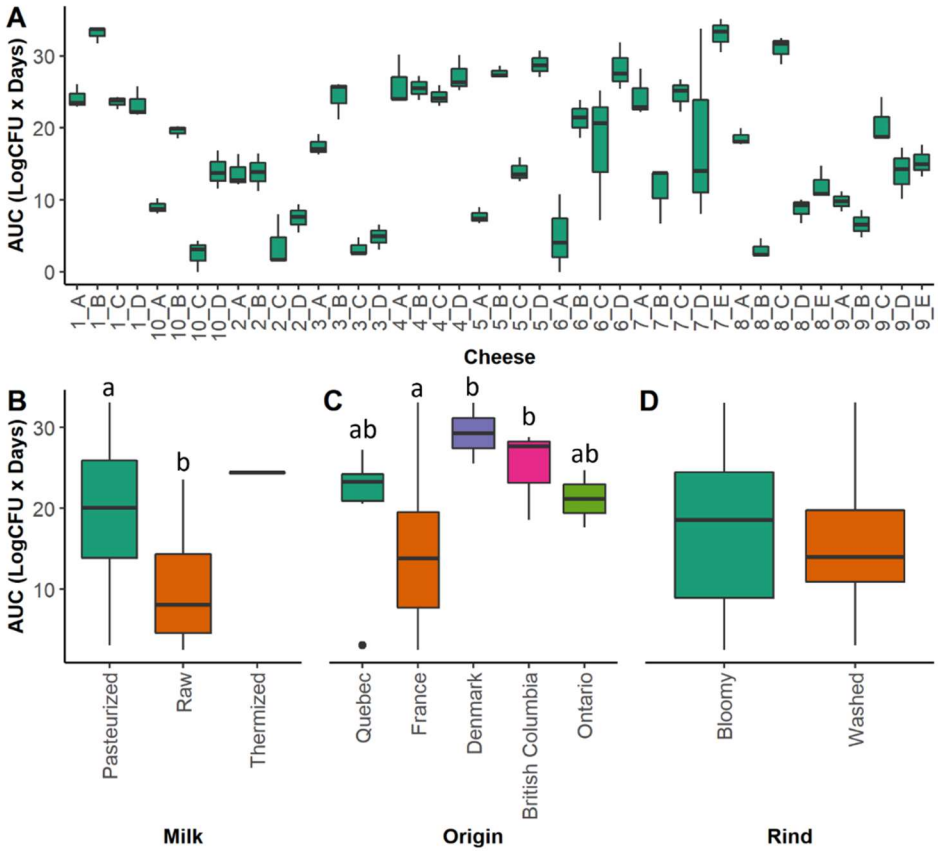

Figure 3.1 – Boxplots comparing of the growth of *Listeria monocytogenes* across the individual cheeses (A), the treatment of the milk used for cheesemaking (B), the origin of the cheeses (C), and the rind type of the cheeses (D). Boxes in boxplots B, C, and D labelled with different lowercase letters have significantly different growth of *L. monocytogenes* from each other (Fisher's LSD, $p < 0.05$). No post-hoc analysis is shown for boxplot A due to the large number of groups.

A significant difference in *L. monocytogenes* growth was also observed between cheeses of different origins (Fig. 3.1C), with cheeses from France showing significantly lower average growth of *L. monocytogenes* compared to cheeses from both British Columbia and Denmark (Fisher's LSD; $p < 0.05$). Additionally, each origin group was represented in multiple growth categories (Table 3.1), but only cheeses from France and Quebec were represented in the no and low growth categories. The medium growth category contained cheese from France, Quebec, Ontario, and British Columbia, but not the cheeses from Denmark. All origins were represented in the high growth group. No significant difference in the average growth of *L. monocytogenes* was observed between bloomy and washed rind cheeses (Fig. 3.1D); t.test; $p = 0.70$), and both rind types were represented in all four growth groups (Table 3.1).

3.3.2 Physicochemical and microbial analysis of cheeses

The average pH across the tested cheeses was 6.9 ± 0.5 and ranged from 5.5 to 7.7 (Table 3.2). Cheeses made with raw milk showed a significantly higher pH than pasteurized milk cheeses (t.test; $n = 39$; $p = 0.04$). No significant difference in pH was observed across origin, rind, or growth categories. Further, no correlation was observed between the growth of *L. monocytogenes* and cheese pH (Fig. 3.2A).

The a_w of cheeses ranged from 0.95 to 0.99, with a mean value of 0.97 ± 0.01 (Table 3.2). Raw milk cheeses showed a significantly lower average water activity than cheeses made with pasteurized milk (t.test; $n = 42$; $p = 0.02$). There was also a significant difference in a_w between growth categories (ANOVA; $n = 42$; $p = 0.02$), with high growth cheeses having a higher average a_w than cheeses from both no and low growth categories (Fisher's LSD; $p < 0.05$). As shown in

Fig. 3.2B, the water activity of the cheeses was also significantly correlated with the growth of *L. monocytogenes* (Spearman's rank correlation; $\rho = 0.417$, $p = 0.005$).

Table 3.2 – Summary of physicochemical and microbial characteristics of cheeses. Values in the same column of the same subsection (*e.g.*, Milk, Origin) are significantly different (Wilcoxon test or Dunn's test; $p < 0.05$).

				Viable Plate Count (Log CFU/g)[1]		
	n	**pH[2]**	**a_w**	**TSA**	**M17[3]**	**MRS-5.4**
Milk						
Raw	12	7.1 ± 0.3[a]	0.96 ± 0.01[a]	9.0 ± 0.5[a]	8.5 ± 0.5	7.2 ± 0.9
Pasteurized	30	6.8 ± 0.6[b]	0.97 ± 0.01[b]	8.2 ± 1.1[b]	8.0 ± 1.2	6.7 ± 1.6
Thermized	1	6.9	0.98	7.2	7.2	6.9
Origin						
Quebec	7	7.0 ± 0.4	0.97 ± 0.01	8.1 ± 1.2[a]	8.3 ± 0.7	7.4 ± 0.7[ab]
France	29	6.9 ± 0.4	0.97 ± 0.01	8.5 ± 0.7[a]	8.0 ± 0.9	6.6 ± 1.4[ac]
Denmark	2	6.5 ± 0.4	0.97 ± 0.02	5.8 ± 1.5[b]	6.6 ± 3.5	5.0 ± 2.7[c]
British Columbia	3	7.0 ± 0.9	0.97 ± 0.01	9.1 ± 0.3[a]	9.1 ± 0.2	8.5 ± 0.2[b]
Ontario	2	6.4 ± 1.2	0.96 ± 0.01	8.8 ± 0.1[a]	9.0 ± 0.3	8.1 ± 0.1[ab]
Rind						
Bloomy	36	6.9 ± 0.5	0.97 ± 0.01[a]	8.3 ± 1.1	8.2 ± 1.2	6.9 ± 1.2
Washed	7	7.1 ± 0.3	0.976 ± 0.004[b]	8.7 ± 0.4	7.9 ± 0.5	6.3 ± 2.2
Growth						
-	6	7.0 ± 0.6	0.96 ± 0.01[a]	8.6 ± 1.0[a]	7.7 ± 1.0	7.2 ± 1.0
+	7	7.1 ± 0.4	0.96 ± 0.01[a]	9.0 ± 0.5[a]	8.4 ± 0.7	7.1 ± 1.3
++	20	6.7 ± 0.5	0.97 ± 0.01[ab]	8.6 ± 0.6[a]	8.4 ± 0.7	6.9 ± 1.2
+++	10	7.1 ± 0.5	0.98 ± 0.01[b]	7.4 ± 1.4[b]	7.8 ± 1.8	6.3 ± 2.1
Total (range)	43	6.9 ± 0.5 (5.5 – 7.7)	0.97 ± 0.01 (0.95-0.99)	8.4 ± 1.0 (4.8-9.6)	8.1 ± 1.1 (4.1-9.5)	6.8 ± 1.4 (3.0[4]-8.9)

Values are listed as the mean ± standard deviation

[1]TSA and M17 plates were incubated aerobically for 48 hours at 30°C and 37°C, respectively. MRS-5.4 plates were incubated anaerobically for 72 hours at 37°C.

[2]the pH was measured in 39 of 43 cheeses.

[3]M17 plate count was only measurable in 42 of 43 cheeses.

[4]Below the reliable limit of enumeration (~3.4 Log CFU/g).

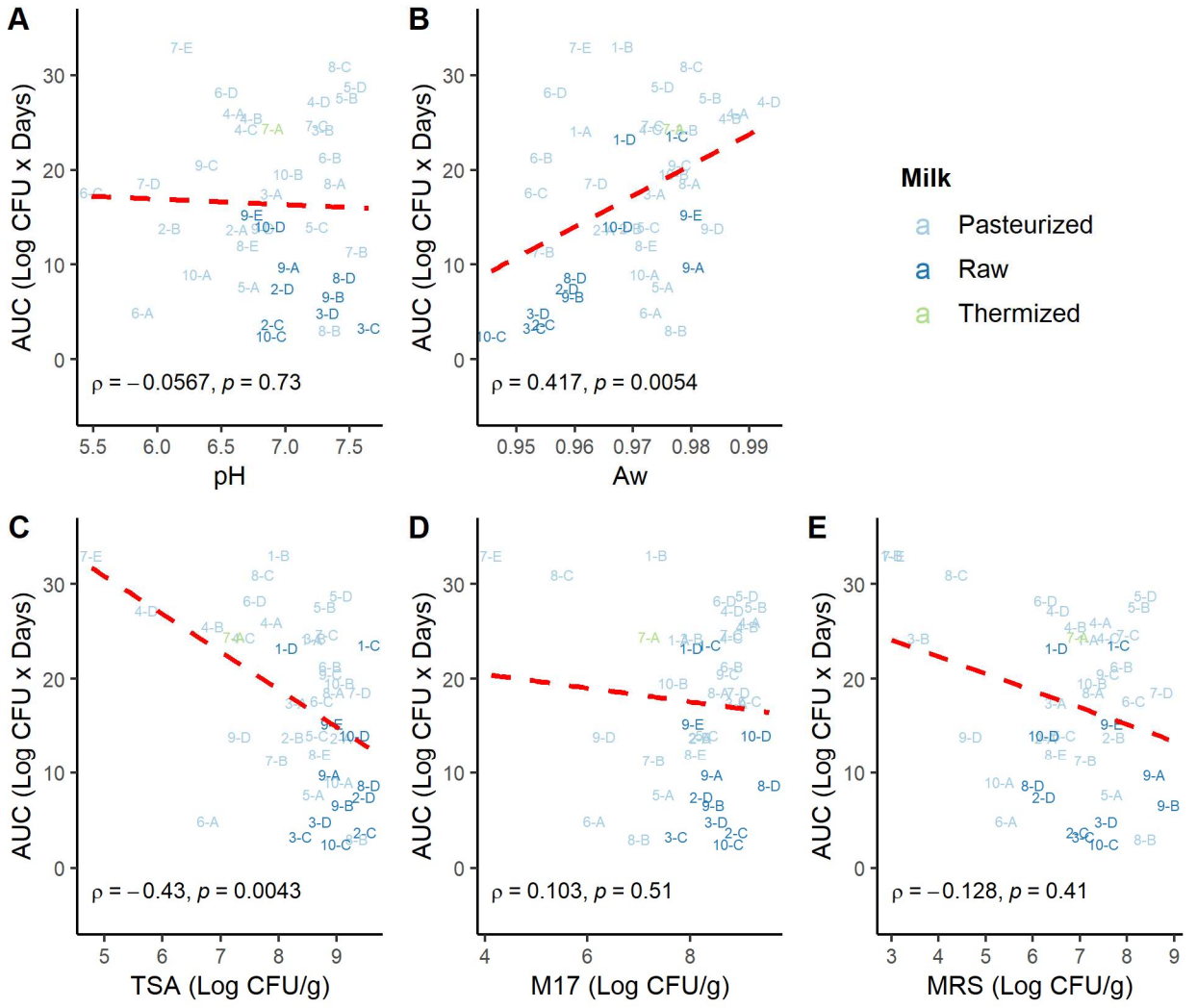

Figure 3.2 – Correlation plots between *L. monocytogenes* growth in the cheeses and their respective pH (A), water activity (B), total microbial count on tryptic soy agar (TSA) incubated aerobically at 30°C for 48 hours (C), total microbial count on M17 agar incubated aerobically at 37°C for 48 hours (D), and total microbial count on de Man, Rogosa and Sharpe agar (pH 5.4; MRS) incubated at 37°C for 72 hours (E). Cheeses are coloured based on their milk treatment, and the dashed red line represents the modelled linear regression through the data. The Spearman rank correlation (ρ) is shown at the bottom left corner of each plot.

The TAMC was enumerated for all cheeses by spread plating on TSA and incubating at 30°C (Table 3.2). The mean TAMC across all cheeses was 8.4 ± 1.0 Log CFU/g, and ranged from 4.8 Log CFU/g to 9.6 Log CFU/g. On average, TAMC was significantly higher in raw milk cheeses than in pasteurized milk cheeses, with mean TAMC of 9.0 ± 0.5 Log CFU/g and 8.2 ± 1.1 Log

CFU/g, respectively (t.test; $n = 42$; $p = 0.001$). Differences in TAMC were also observed across cheeses from different origins. Specifically, the cheeses from Denmark had an average TAMC of 5.8 ± 1.5 Log CFU/g; significantly lower (Fisher's LSD; $p < 0.05$) than the average TAMC from the other four origins which ranged from 8.1 ± 1.2 Log CFU/g to 9.1 ± 0.3 Log CFU/g. Finally, the average TAMC in the high growth category (7.4 ± 1.4 Log CFU/g) was significantly lower (Fisher's LSD; $p < 0.05$) than in the other three growth categories, which ranged from 8.6 ± 1.0 Log CFU/g to 9.0 ± 0.5 Log CFU/g. Further, a significant negative correlation was observed between the TAMC and the growth of *L. monocytogenes* in the cheeses (Spearman's rank correlation; $\rho = -0.43$, $p = 0.004$; Fig. 3.2C).

Viable plate count was also used to enumerate TLAB on both M17 (aerobic) and MRS-5.4 (anaerobic) agars (Table 3.2). The average viable plate counts on M17 agar was 8.1 ± 1.1 Log CFU/g across cheeses, with a range of 4.1 Log CFU/g to 9.5 Log CFU/g. On MRS-5.4 agar, the average viable plate count across cheeses was 6.8 ± 1.4 Log CFU/g, and ranged from below the limit of enumeration (~3.4 Log CFU/g) to 8.9 Log CFU/g. No significant difference in M17 plate counts was observed between any of the categorical grouping variables (ANOVA; $p > 0.05$), nor were M17 plate counts correlated with the growth of *L. monocytogenes* (Fig. 3.2D). Counts on MRS-5.4 agar, however, showed a significant difference between the different cheese origins (ANOVA; $p = 0.02$). The lowest MRS-5.4 counts were observed in the cheeses from Denmark (5.0 ± 2.7 Log CFU/g) and France (6.6 ± 1.4 Log CFU/g), with the highest MRS-5.4 counts observed in cheeses from British Columbia (8.5 ± 0.2 Log CFU/g). Despite these differences in MRS-5.4 counts, no correlation was observed between MRS-5.4 plate counts and the growth of *L. monocytogenes* (Fig. 3.2E).

Multivariate analysis of the cheese data was conducted using principal component analysis (PCA). When all five physicochemical and microbial metrics were included (Fig. 3.3A), 64.3% of the variation between cheese samples was explained by the first two principal components. The three microbial enumerations (*i.e.*, TSA, M17, and MRS-5.4 plate counts) appeared to be correlated with each other and showed a strong relationship to PC1. On the other hand, pH and a_w showed mutual correlation and were more strongly related to PC2. Regardless, no clear separation between the *L. monocytogenes* growth categories was observed (PERMANOVA; p = 0.12). Multiple linear regression estimated that 32% of the variation in *L. monocytogenes* growth was explained by the five physicochemical and microbial metrics.

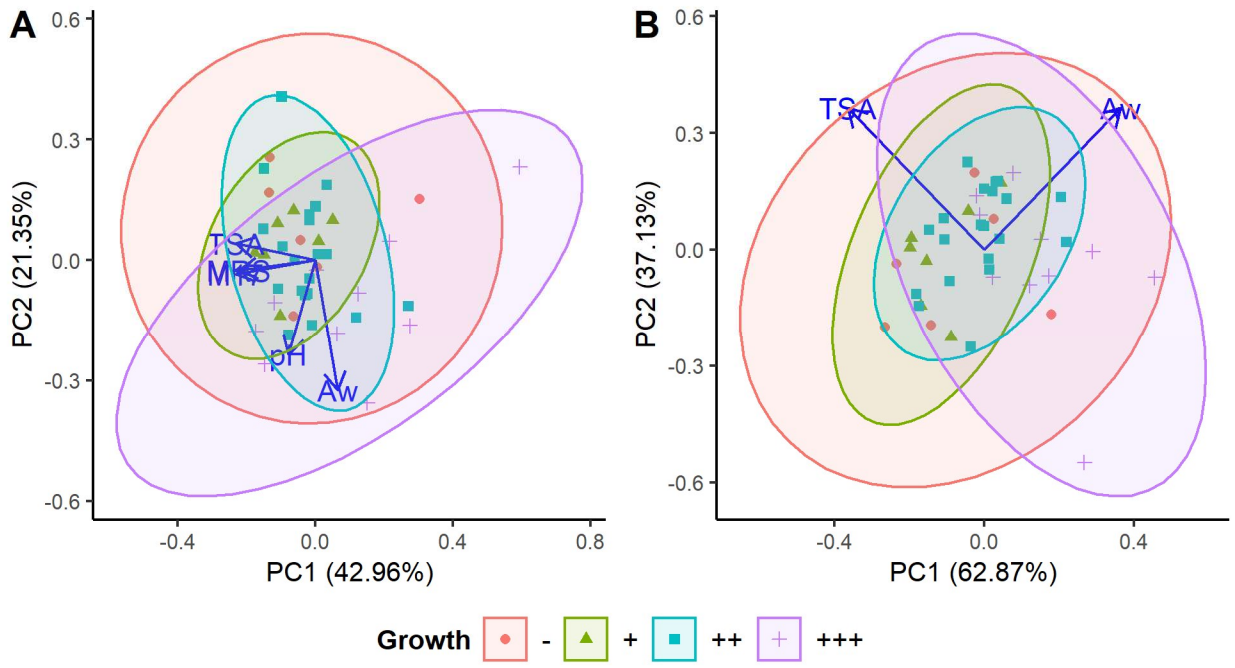

Figure 3.3 – Principal component analysis of cheeses using all physicochemical and microbial metrics (A) and only those physicochemical and microbial metrics which were significantly correlated with the growth of *L. monocytogenes* (B). The shape/colour of samples represents their respective *L. monocytogenes* growth category: no growth (-), low growth (+), medium growth (++), and high growth (+++). Coloured ellipses represent the 95% confidence interval of the growth group based on a multivariate normal distribution.

When only the metrics that showed significant correlation with *L. monocytogenes* growth were included in the analysis (*i.e.*, a_w and TSA), a significant separation was observed between the growth categories (PERMANOVA, $p = 0.005$; Fig. 3.3B). Further analysis by pairwise PERMANOVA showed that the high growth category was significantly separated from the low and medium growth category ($q < 0.05$), but, interestingly, no significant separation was present between the high and no growth categories ($q = 0.18$). Multiple linear regression analysis suggested that approximately 26% of the variation in *L. monocytogenes* growth was explained by these two variables.

3.4 Discussion

This experiment investigated the growth of *L. monocytogenes* in SRC at refrigerated temperatures and assessed whether differences in *L. monocytogenes* growth could be explained by physicochemical and/or microbial characteristics of the cheeses. As expected, *L. monocytogenes* growth was observed in most of the cheeses. Previous research has shown *L. monocytogenes* growth in SRC at refrigerated temperatures during ripening (Lobacz et al., 2013), after ripening (Gérard et al., 2020), and at retail establishments (Lahou & Uyttendaele, 2017), all with similar or greater pathogen increases when compared to the mean increase of *L. monocytogenes* observed in the present experiment (2.5 ± 1.0 Log CFU). Of interest to this current study was the variation in *L. monocytogenes* growth across the various cheeses tested, including multiple cheeses showing less than a 1 log CFU increase of *L. monocytogenes*, and two even showing less than a 0.5 log CFU increase of *L. monocytogenes* over the course of the experiment. This variation in *L. monocytogenes* growth is of note because SRC, due to their favourable moisture content and near-neutral pH, are expected to promote the growth of *L. monocytogenes* (Falardeau et al., 2021).

Previous studies have documented variation in *L. monocytogenes* growth in lab-prepared semi-soft washed rind cheeses (Callon et al., 2011, 2014; Millet et al., 2006; Retureau et al., 2010) and in lab-prepared Camembert style cheeses (Marielle Gay & Amgar, 2005), and a recent study also observed a significant variation in the growth of *L. monocytogenes* across a small sampling of washed rind SRC in Belgium (Gérard et al., 2020), but to the best of my knowledge, this is the first study documenting significant variation across a large sampling of SRC from retail. From a food safety standpoint, an understanding of factors leading to a reduction in *L. monocytogenes* growth in SRC is of value to both the dairy industry and food safety regulators.

Not unexpectedly, *L. monocytogenes* growth, on average, was significantly lower in cheeses made with raw milk compared to those produced by pasteurized or thermized milk. Previous research has shown that *L. monocytogenes* was unable to grow in raw milk during the initial stages of cheese making (Schvartzman, Maffre, et al., 2011), and that a reduced growth of the pathogen was observed in Camembert cheeses made with raw milk as compared to those made with pasteurized milk (Marielle Gay & Amgar, 2005). The inhibition of *L. monocytogenes* in these cheese studies was suggested to be an effect of increased microbial competition associated with the unpasteurized milk microbiota. Evidence for the protective effect of the native microbiota can be seen in the negative correlation between *L. monocytogenes* growth and TAMC observed in the current study. This relationship between *L. monocytogenes* growth and TAMC suggests that increasing concentrations of native microbes may be able to crowd out the contaminating pathogen, possibly through non-specific competition for nutrients (Mellefont et al., 2008). The natural microbiota of various food systems have previously been shown to inhibit the growth of *L. monocytogenes* (Al-Zeyara et al., 2011), and microbial competition against *L. monocytogenes* has also been observed

in other food systems, including apples (Leverentz et al., 2006), lettuce (Oliveira et al., 2012), and ready-to-eat meats (Amézquita & Brashears, 2002). Ironically, the antilisterial effect of raw milk during the initial stages of cheese production may result in reduced lactate production by the starter culture, and end up allowing a higher rate of *L. monocytogenes* growth in the raw milk cheeses during the ripening period (Schvartzman, Maffre, et al., 2011). It remains to be seen, however, if it is strictly the number of competing bacteria that are present, or if microorganism representing specific taxa are also important to the inhibition of the contaminating *L. monocytogenes*. Further, microbial competition associated with raw milk is not the only factor associated with *L. monocytogenes* inhibition since several pasteurized milk cheeses allowed less growth of the pathogen than many of the raw milk cheeses, including some with a relatively lower concentration of total aerobic bacteria. A variation in the average *L. monocytogenes* growth between different origins was also observed. Specifically, the lowest levels of *L. monocytogenes* were observed in cheeses made in France. However, since 11 out of 12 of the raw milk cheeses were from France, it is likely that this effect of origin is confounded by the differences in milk treatment. It should also be acknowledged that low sample sizes for some regions (*i.e.*, Denmark, Ontario, and British Columbia) make it difficult to say with confidence that these regional differences truly exist.

Despite the reduced *L. monocytogenes* growth associated with increasing TAMC, no significant correlation was observed between *L. monocytogenes* growth TLAB. This lack of correlation between pathogen growth and TLAB was surprising since the antilisterial effects associated with microbial competition in cheese has previously been tied to the presence of lactobacilli (Marielle Gay & Amgar, 2005; Retureau et al., 2010). Various strains of *Lactococcus lactis* (a commonly used starter culture in SRC production) have also shown inhibition against *L. monocytogenes* (Ho

et al., 2018). Finally, the inhibition of *L. monocytogenes* in cheese has also been associated with lactic acid production, typically associated with species of lactic acid bacteria (Callon et al., 2011; Millet et al., 2006). It is possible that strain effects of the LAB could be at play here since high levels of LAB were present in both inhibitory and non-inhibitory cheeses in the current study. Any antilisterial effect could be associated with phenotypic differences such as variations in lactate production (Ho et al., 2018) or the ability to produce bacteriocins (Mills et al., 2011). Additionally, *Lc. lactis* can enter a viable but non-culturable (VBNC) state during sugar starvation (Ganesan et al., 2007), meaning that the TLAB plate counts might not be completely accurate since the cheeses would be expected to be depleted of sugars after ripening, leading to the formation of VBNC cells.

Two important physicochemical factors affecting *L. monocytogenes* growth in soft cheeses are pH and a_w (Falardeau et al., 2021). No correlation between *L. monocytogenes* and pH was observed, but the a_w did show a significant correlation with pathogen growth, accounting for an estimated 17% of the total observed variation of *L. monocytogenes* growth across the tested cheeses. Previous modelling data has suggested a stochastic effect on whether *L. monocytogenes* growth would initiate at a_w values between 0.96 and 0.98 (Schvartzman et al., 2010), and the minimum a_w for *L. monocytogenes* growth was estimated to range from 0.938 to 0.955, depending on temperature and pH (Schvartzman, Belessi, et al., 2011). Provided a favourable pH was present, growth of *L. monocytogenes* was always observed in model cheeses with $a_w > 0.98$ (Schvartzman et al., 2010). To the best of my knowledge, however, this is the first time that a direct correlation between growth and a_w in soft cheeses was observed. It is likely that the stochastic effect of lower a_w values can be extended to what proportion of viable cells would double at any point in time, thereby reducing the total rate of growth across the entirety of the *L. monocytogenes* population.

Despite the interaction between pH and a_w associated with *L. monocytogenes* growth described by Schvartzman et al. (2010), the lack of correlation between pathogen growth and pH observed in this current study was not surprising. Since the cheeses were all purchased from retail, the ripening process had increased the pH back to near neutral levels, removing the inhibitory barrier against *L. monocytogenes* associated with the early stages of cheesemaking (Falardeau et al., 2021). Indeed, the pH of the cheeses ranged from 5.5 – 7.7, and previous research has shown that *L. monocytogenes* growth can initiate in SRC once the pH has increased above 5.5 (Back et al., 1993). Since the pH of the retail SRC is already expected to be favourable to *L. monocytogenes* growth, the a_w is therefore the primary physicochemical factor for food safety. However, like in the relationship between *L. monocytogenes* growth and TAMC, some cheeses with relatively a_w were more inhibitory to *L. monocytogenes* than those with lower a_w. Therefore, univariate analysis of cheese a_w is not sufficient to predict the probable growth rate of *L. monocytogenes*, and a multivariate approach is likely required.

Multivariate analysis showed the combined measurement of physicochemical and microbial characteristics was not sufficient to fully explain the differential growth of *L. monocytogenes* across the tested cheeses. The observed orthogonal relationship between the physicochemical and microbial characteristics, however, does suggest that a_w and total aerobic count are independent of each other. Further, multiple linear regression analysis showed a greater predictive power when the two metrics are combined, than when each metric were assessed individually. Interestingly, even though differences were observed between the growth categories in in the PCA, there was no separation between the high and no growth categories. This lack of separation implies that the cheeses in the no growth category (< 1 log CFU increase) may possess a characteristic that was

not measured in the current experiment; possibly related to the structure of the microbiome community within the individual cheeses.

3.5 Conclusion

L. monocytogenes is capable of growth in SRC at refrigerated temperature, but the amount of growth varies across cheeses. Raw milk cheeses are less likely to allow the growth of *L. monocytogenes*, possibly due to an increased concentration of native bacteria already present in the cheese. The a_w of the cheeses is also predictive of *L. monocytogenes* growth, but large amounts of variation in pathogen growth are not fully explained by the physicochemical or microbial factors measured here. Future research will investigate the structure of the cheese microbiome to determine if and how the identities of the competing bacteria can further explain the observed variation in *L. monocytogenes* growth.

Chapter 4: Taxonomic Analysis of the Microbiome of Soft Surface-Ripened Cheeses and its Relationship to the Growth of *Listeria monocytogenes*

4.1 Introduction

Despite the expected growth of *Listeria monocytogenes* in soft surface-ripened cheeses (SRC), the level of growth across these cheeses is not homogeneous (Chapter 3). Furthermore, this differential growth of *L. monocytogenes* across cheeses cannot be completely explained through the common physiochemical parameters known to affect microbial growth (*e.g.*, pH, a_w), or by common microbial parameters such as the total aerobic microbial count or total lactic acid bacteria count. Previous research has suggested that the cheese microbiome can affect the growth of *L. monocytogenes* in the cheese; due to both the presence of a competing microbiome (Marielle Gay & Amgar, 2005), but also to specific members (*i.e.*, species or strains) of the microbial community (Callon et al., 2014; Gérard et al., 2021; Retureau et al., 2010). Previous research has also highlighted microbiome and competitive exclusion effects on the occurrence and persistence of *L. monocytogenes* within food processing facilities (Fox et al., 2014; Tan et al., 2019; Zwirzitz et al., 2021).

With the advent of high-throughput sequencing (HTS) technology, researchers have been able to investigate the microbial communities of cheese using metataxonomic (*i.e.*, 16S rRNA gene targeted amplicon sequencing) and shotgun metagenomic methods (Afshari et al., 2020). Of importance are the observations that i) finished cheeses possess a non-trivial number of microbial taxa not inoculated by the cheesemaker (Dugat-Bony et al., 2016; Quigley et al., 2012; Wolfe et al., 2014), ii) the final cheese microbiota is affected by the microbiome of the cheese processing

facility (Bokulich & Mills, 2013), and iii) that many of the taxa present in the cheese processing facility and finished cheeses are also present at the dairy farm environment (Falardeau et al., 2019). Recent research has also highlighted the interaction between this cheese microbiome and *L. monocytogenes* in gouda cheese made with unpasteurized milk (Salazar et al., 2021).

A variety of studies have been conducted to determine the microbial taxa associated with *L. monocytogenes* inhibition in surface-ripened cheeses, but no clear mechanism has yet been elucidated. For example, investigations focused on Saint Nectaire style cheeses, a semi-soft washed rind cheese, have attributed observed antilisterial effects with the presence of Lactobacilli and *Leuconostoc* spp. (Retureau et al., 2010); *Lactococcus lactis* and *Lactococcus garvieae* (Saubusse et al., 2007); and species of *Vagococcus*, *Carnobacterium*, and *Enterococcus* (Callon et al., 2014). In a study investigating a variety of cheeses from Belgium, the inhibition of *L. monocytogenes* in washed rind soft cheeses was most strongly correlated with the relative concentration of *Lactococcus* spp., but also to the genera *Psychrilobacter*, *Fusobacterium*, and *Alkalibacterum* (Gérard et al., 2021), but this data was only based on 4 cheeses, and none of the mold-ripened cheeses tested showed any inhibition of *L. monocytogenes*.

The objectives of this experiment were to use a combination of 16S rRNA gene targeted amplicon sequencing and metagenomic methods to i) investigate how the microbiome differs across SRC from a large variety or origins, and ii) determine how the structure and membership of these microbiomes is associated with the growth of *L. monocytogenes* in their respective cheeses.

4.2 Materials and methods

4.2.1 Cheese samples

The 16S rRNA gene targeted amplicon sequencing and metagenomic analyses were conducted on a subset of the cheeses described in Chapter 3 and summarized in Table 3.1. For 16S rRNA gene targeted amplicon analysis, 39 of the originally 43 cheeses were selected. The remaining four cheeses were omitted because their collection and growth assays were conducted after the initial 39 cheeses were submitted for sequencing. A subset of 15 cheeses were selected for metagenomic sequencing. Cheeses subjected to metagenomic sequencing were selected to cover a large variation of factors. Ten cheeses were from France, one from Denmark, and four from Canada (2 each from British Columbia and Quebec). Twelve cheeses were made with pasteurized milk, and three cheeses were made with raw milk. Ten were bloomy rind cheeses, and five were washed rind cheeses. Finally, six cheeses were from the medium growth category, while three cheeses each were chosen from the no, low, and high growth categories.

4.2.2 DNA extractions from cheese

Total microbial DNA was extracted from up to four 3.0g ± 0.1g portions of each cheese as follows. Each cheese portion was diluted 10X in sterile PBS and homogenized as described in Chapter 3. DNA extraction was then conducted on 1.0 ml aliquots of the homogenates using the DNeasy PowerFood Microbial Kit (Qiagen Inc.; Toronto, ON).

4.2.3 Library preparation and sequencing

For 16S rRNA gene targeted amplicon sequencing, dual-indexed sequencing libraries were prepared as described previously (Comeau et al., 2017). Briefly, a one-step 10 ul PCR reaction

was performed on a LabCyte Access Workstation using Quanta 5PRIME HotMasterMix with 1 ng input DNA. Amplification was conducted using complete "fusion primers" that included Illumina Nextera adaptors, indices, and sequences targeting the V4-V5 region of the 16S rRNA gene (515F: 5'- GTGYCAGCMGCCGCGGTAA-3' and 906R: 5'- CCGYCAATTYMTTTRAGTTT-3'; Parada et al., 2016). The resulting amplicons were quantified using a picogreen assay (Quant-iT™ PicoGreen™ dsDNA Assay Kit, ThermoFisher), before pooling together 2 ng from each sample. The pooled library was then subjected to cleanup using the AmpureXP PCR cleanup protocol (Beckman) and quantified using a picogreen assay. Sequencing of the pooled library was conducted using an Illumina MiSeq sequencer with Reagent Kit v3 (600-cycle; 2 x 300bp paired-end reads) following the manufacturer's recommendations, and with the addition of 10% PhiX.

For metagenomic sequencing, library preparation and sequencing were performed by the Sequencing and Bioinformatics Consortium at the University of British Columbia. Sequencing libraries were prepared using the Illumina DNA Prep library preparation kit following the manufacturer's directions. The resulting libraries were pooled and loaded into a single NextSeq Mid output flow cell for sequencing. Paired-end, 150 bp reads were generated using the Illumina Sequencer, and raw base call data were converted to FastQ format using the bcl2fastq conversion software from Illumina.

4.2.4 Bioinformatic sequence analysis

For the 16S rRNA gene targeted amplicon sequencing, the resulting sequences were processed using mothur software (version 1.43.0; Schloss et al., 2009) following the creators' recommended

guidelines. Briefly, paired end sequences were assembled into contigs and screened to remove any contigs of improper length or containing ambiguous bases. The remaining sequences were aligned and classified using the SILVA database (version 132; Yilmaz et al., 2014) before removal of chimeric sequences or sequences from non-prokaryotic lineages. The sequences were then clustered into operational taxonomic units (OTUs) based on 97% similarity and quality filtered to remove any OTUs representing <0.005% of total reads as previously recommended (Bokulich et al., 2013). Finally, the samples were all rarefied to an even depth of 3,000 reads per sample, which allowed > 90% of samples to be retained after normalization.

For the shotgun metagenomic sequence analysis, sequence data was processed using the READ_QC module of the MetaWRAP metagenomic wrapper suite (version 1.3.2; Uritskiy et al., 2018). Cleaned, unassembled reads were then classified using Kraken 2 (version 2.1.2; Wood & Salzberg, 2014), and species level abundance was estimated using Bracken (version 2.5; Lu et al., 2017). Taxonomic analysis of the metagenomics sequence data was conducted using only representatives from the kingdom Bacteria. Strain level analysis was conducted using PanPhlAn software (version 3.0; Beghini et al., 2021) on all bacteria that were present at > 1% total relative abundance across the metagenomic sequence data, and were also present in the PanPhlAn pangenome library. PanPhlAn functions by identifying the presence/absence of genes from the metagenome in a curated pangenome of the respective species (Beghini et al., 2021). Any strains characterized by the PanPhlAn software were assumed to be the only strain present in the respective cheese.

4.2.5 Statistical analysis

Statistical analysis, including spearman's rank correlation, t-test, and ANOVA, was conducted using R software (version 4.1.0; R Foundation for Statistical Computing, Vienna, Austria [https://www.Rproject.org/]) with the assistance of the tidyverse collection of packages (version 1.3.1; Wickham et al., 2019). To correct for multiple comparisons where necessary, p-values were adjusted using the False Discovery Rate (FDR) method and are labelled as q-values. For these analyses, the significance level (α) was set at 0.05.

Taxonomic analysis was conducted using phyloseq (version 1.36.0; McMurdie & Holmes, 2013), accept for permutational multivariate analysis of variance (PERMANOVA), which was conducted using the vegan software package (version 2.5-7; Oksanen et al., 2020) using the default settings. For β-diversity analysis, including PCoA and PERMANOVA, dissimilarity between the microbial communities of cheeses was measured using the weighted UniFrac distance method (C. A. Lozupone et al., 2007).

Differential abundance was calculated using the ANCOMBC package (version 1.2.2; Lin & Peddada, 2020). Only OTUs or species with sufficient prevalence were included, to prevent taxa from a single cheese affecting the results. For 16S rRNA gene analysis, sufficient prevalence was defined as OTUs with at least 0.1% relative abundance (~ 10 reads) in at least three different cheeses. For shotgun metagenomic sequencing, species were considered sufficiently prevalent if they were represented by at least 100 reads in at least three different cheeses.

4.3 Results

4.3.1 Sequence processing

4.3.1.1 16S rRNA gene targeted amplicon sequencing

Metataxonomic analysis based on 16S rRNA gene targeted amplicon sequencing was used to survey the microbial composition of 39 of the cheeses used in this study (Table 3.1). A total of 3,896,539 reads from 150 samples were recovered after sequence assembly and quality filtering, with an average of 25,977 ± 20,552 reads per sample grouped into 223 OTUs. After rarefaction to 3,000 reads per sample, 135 samples and 222 OTUs remained.

4.3.1.2 Metagenomic sequencing

Metagenomic sequencing was used to conduct species- and strain-level taxonomic analysis on a subset of 15 cheeses used in this study (Table 3.1). A total of 137,937,980 paired end reads from 58 samples remained after quality filtering. Not including two sample replicates which failed sequencing (<100 reads), the average quality-filtered read count per sample was 2,463,175 ± 941,408. During taxonomic analysis, 102,471,448 reads were assigned to 7,201 species, with 99,170,141 of those reads being assigned to 6,841 species within the kingdom Bacteria. After rarefaction to even depth (729,719 reads per sample replicate) 7,121 species were still present across the 56 samples, with 6,783 of those being from the kingdom Bacteria. An average of 2,268 ± 1,247 species of bacteria were observed in each sample.

4.3.2 Alpha-diversity

Analysis of the alpha-diversity of the cheeses using 16S rRNA gene targeted amplicon sequencing showed differences in both the richness and evenness of microbial communities across the cheeses

(Fig. 4.1). The number of observed OTUs in each cheese ranged from 5 to 65, with an average of 34 ± 15 (Table 4.1). The species richness (*i.e.*, number of observed OTUs) was negatively correlated with the AUC of *L. monocytogenes* growth in the individual cheeses (r = -0.391; n = 39; p = 0.014; Fig. 4.2A). A similar trend was also observed between the average species richness of growth categories but was not statistically significant (ANOVA; p = 0.063; Table 4.1). The species richness was separate from the TAMC (Chapter 3), as the two were not significantly correlated (r = 0.24; p = 0.13). No significant correlation between Shannon diversity and *L. monocytogenes* growth was observed. Raw milk cheeses, on average, had a greater number of observed OTUs than cheeses made with pasteurized milk (t.test; n = 38; $p < 0.001$), but the cheese with the highest richness in the study was made with pasteurized milk (Fig. 4.1A). Similarly, the average Shannon diversity was greater in raw milk cheeses than in pasteurized milk cheeses (t.test; n = 38; $p < 0.001$), but the greatest Shannon diversity was observed in a cheese made with pasteurized milk; the same cheese which showed the greatest number of observed OTUs (Fig. 4.1B). Washed rind cheeses showed a higher level of observed OTUs than bloomy rind cheese (Table 4.1), but this difference was not significant (t.test; p = 0.073). On the other hand, the Shannon diversity was significantly different between the rind types (t.test; p = 0.005), with washed rinds having a higher Shannon diversity index (Table 4.1). No significant differences were observed between cheeses of different origin with respect to the number of observed OTUs (ANOVA; p = 0.19), or regarding the Shannon diversity index (ANOVA; p = 0.45).

To assess how well the observed richness within samples predicted the growth of *L. monocytogenes* when combined with previous correlating factors (*i.e.*, TAMC and a_w), principal component analysis was conducted (Fig. 4.3). Despite the addition of a third correlating factor,

no significant separation was observed between the growth categories (PERMANOVA; p = 0.061). Further, multiple linear regression combining the three metrics still only explained 26% of the observed variation (R^2_{adj} = 0.264; n = 39; p = 0.003); the same amount of variation in *L. monocytogenes* that was explained when only TAMC and a_w were included in the regression analysis (Section 3.3.2).

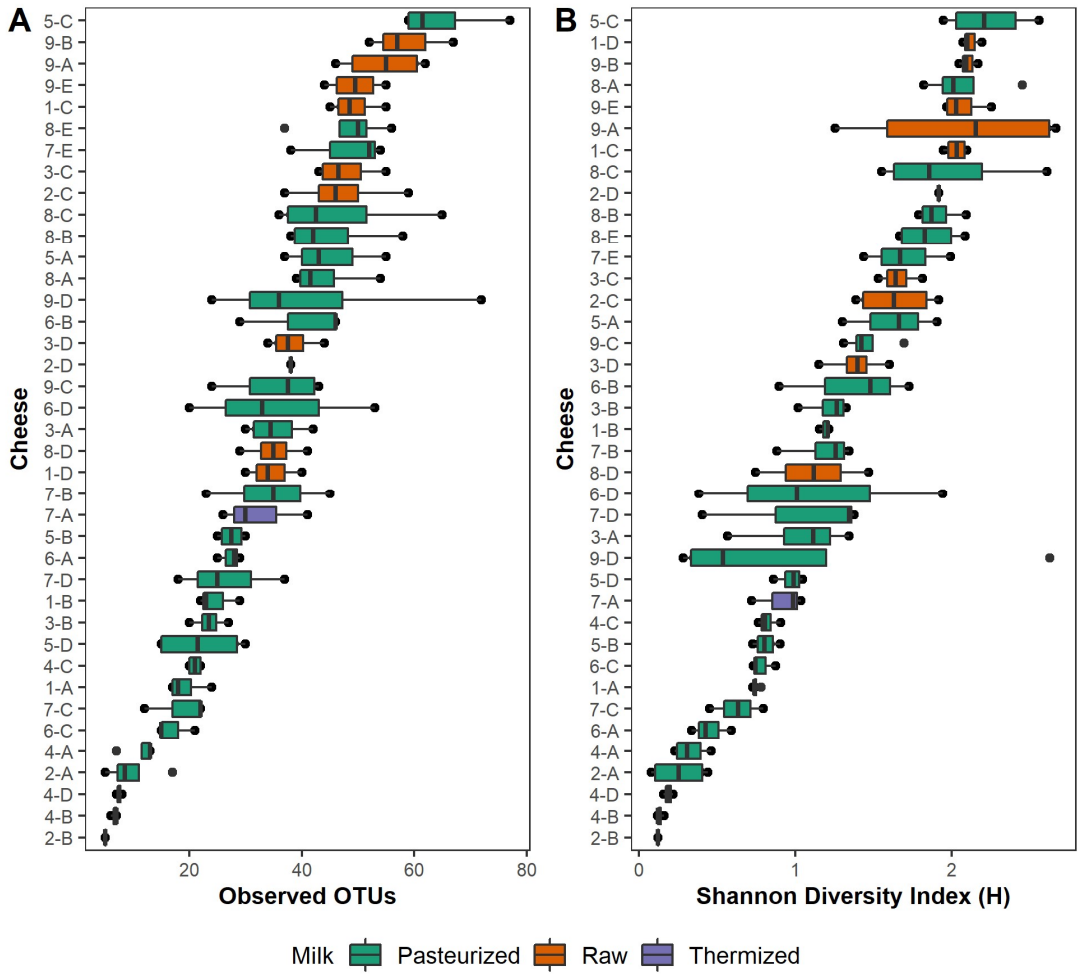

Figure 4.1 – Boxplots showing the alpha-diversity of cheeses investigated in this study based on 16S rRNA gene targeted amplicon sequencing, and measured by total observed OTUs (A) and the Shannon Diversity Index (B). Cheese samples are coloured based on milk treatment.

4.3.3 Taxonomic analysis

Taxonomic analysis at the phylum level is summarized in Fig. 4.4A and Table 4.2. Firmicutes was the dominant phylum observed, representing 78.1% ± 23.4% of the observed reads across the tested cheeses. Proteobacteria and Actinobacteria made up the bulk of the remaining taxa with 15.2% ± 18.4% and 6.6% ± 10.0% of the remaining reads in each cheese, respectively. Small amounts of the phyla Bacteroidetes Fusobacteria, and Deinococcus-Thermus were also observed. Most individual cheeses were dominated by the Firmicutes phylum, but three of the tested cheeses had Proteobacteria as their most abundant phylum.

Table 4.1 – Alpha diversity of cheeses investigated in this study measured by 16S rRNA gene targeted amplicon sequencing. Values in the same column of the same subsection (*e.g.*, Milk, Origin) are significantly different (t-test; $p < 0.05$).

Category	n	Observed OTUs	Shannon Diversity
Milk			
Raw	10	45 ± 8[a]	1.81 ± 0.35[a]
Pasteurized	28	30 ± 15[b]	1.08 ± 0.61[b]
Thermized	1	32	0.91
Origin			
Quebec	7	29 ± 13	1.23 ± 0.68
France	25	38 ± 15	1.38 ± 0.64
Denmark	2	27 ± 29	1.11 ± 2.24
British Columbia	3	25 ± 3	0.94 ± 0.12
Ontario	2	18 ± 1	0.70 ± 0.11
Rind			
Bloomy	32	31 ± 14	1.14 ± 0.62[a]
Washed	7	44 ± 15	1.79 ± 0.42[b]
Growth			
-	6	42 ± 9	1.45 ± 0.57
+	7	43 ± 11	1.63 ± 0.45
++	20	33 ± 16	1.27 ± 0.68
+++	10	25 ± 14	0.93 ± 0.58
Total (range)	39	34 ± 15 (5 – 65)	1.26 ± 0.63 (0.12 – 2.23)

Values are listed as the mean ± standard deviation

The relative abundance of the three most common phyla varied with respect to the growth of *L. monocytogenes*, as summarized in Table 4.2 and Figure 4.5. Across all growth categories, the

Firmicutes phylum was most prominent, ranging from 64.7% ± 28.4% relative abundance in the low growth category, up to 96% ± 5.3% relative abundance in the high growth category. The relative abundance of Firmicutes was significantly higher in the high growth category compared to the other three categories of *L. monocytogenes* growth (Dunn's test; $q < 0.05$). Further, there was also a significant correlation between the relative abundance of Firmicutes and growth of *L. monocytogenes* in the cheeses, as measured by AUC (Spearman rank correlation; $\rho = 0.396$; $p = 0.013$; q-value = 0.038). Proteobacteria were more prevalent in the no, low, and medium *L. monocytogenes* growth categories (19.4% ± 20.9%, 23.6% ± 25.5%, and 17.8% ± 18.3% relative abundance, respectively) compared to the high growth category (3.3% ± 4.0% relative abundance), but this difference was not statistically significant (Kruskal-Wallace; $p = 0.069$). A significant negative correlation, however, was observed between the growth of *L. monocytogenes* and the relative abundance of Proteobacteria in the cheeses (Spearman rank correlation; $\rho = -0.351$; $p = 0.028$; q-value = 0.057). Finally, Actinobacteria showed a significantly lower relative abundance in the high growth category (0.7% ± 1.5% relative abundance) compared to the no growth (6.5% ± 6.4% relative abundance), low growth (11.6% ± 14.6% relative abundance), and medium growth (8.3% ± 10.7% relative abundance) categories (Dunn's test; q-value < 0.05). The relative abundance of Actinobacteria was also significantly negatively correlated with the AUC of modelled *L. monocytogenes* growth in the cheeses (Spearman rank correlation; $\rho = -0.475$; $p = 0.0023$; q-value = 0.014).

Significant variation in the top three phyla was also observed relative to other characteristics of the cheeses (Table 4.2). Bloomy rind cheeses had a significantly higher relative abundance of Firmicutes than washed rind cheeses (Kruskal-Wallace; $p = 0.008$), while washed rind cheeses had

a significantly higher relative abundance of Proteobacteria than bloomy rind cheeses (Kruskal-Wallace; $p = 0.004$). Additionally, Actinobacteria had a higher relative abundance in cheeses made with raw milk than in cheeses made with pasteurized milk (Kruskal-Wallace; $p = 0.004$).

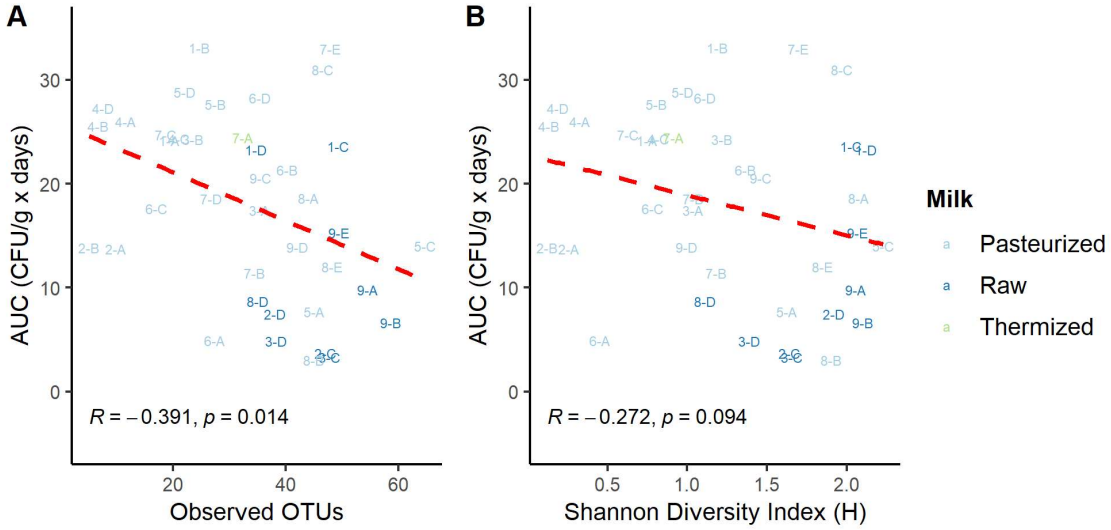

Figure 4.2 – Scatter plot and linear regression (dashed line) of the growth of *L. monocytogenes vs.* species richness (A) and Shannon diversity (B). Pearson correlations are shown at the bottom left corner of each plot, and the cheese samples are coloured by milk treatment.

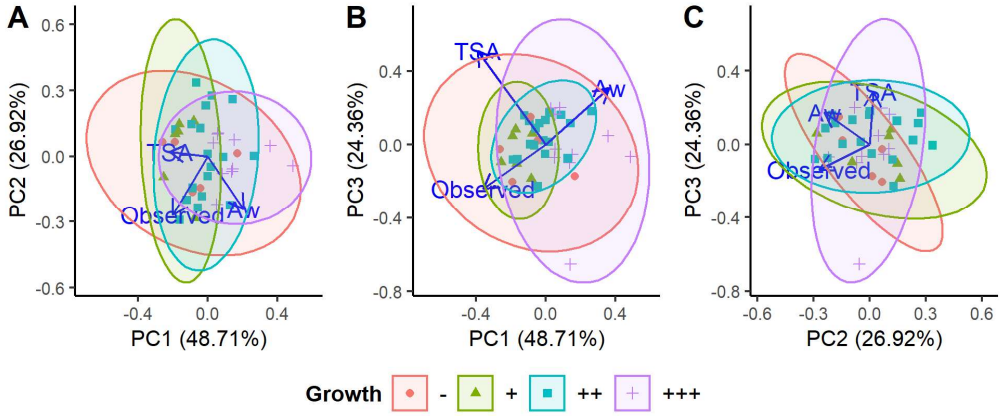

Figure 4.3 – Principal components analysis of total aerobic microbial count (TSA), water activity (a_w), and total observed OTUs measured for each cheese. Graphs show the relationship between PC1 and PC2 (A), PC1 and PC3 (B), and PC2 and PC3 (C). Cheeses are coloured and surrounded by ellipses based on growth category.

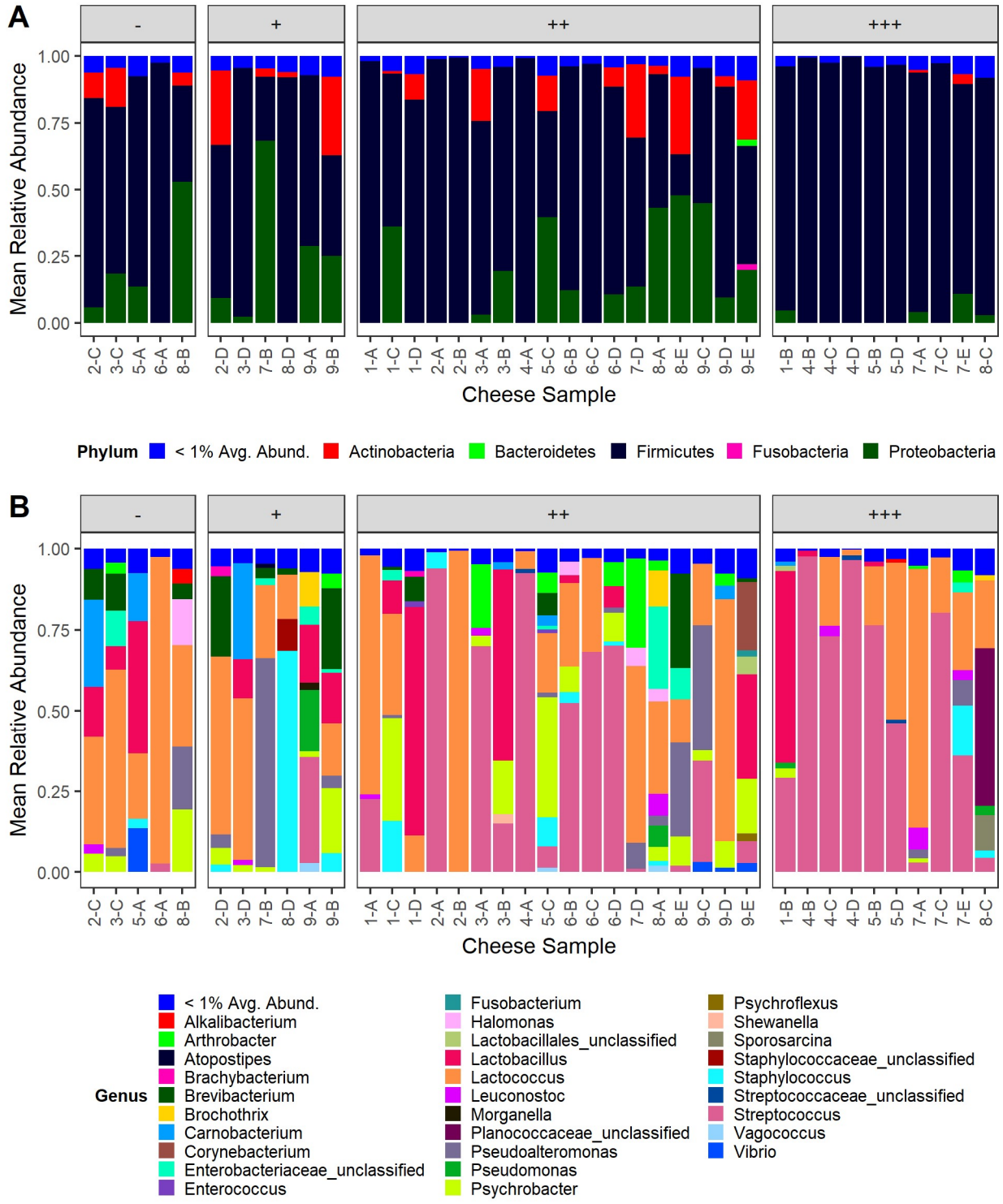

Figure 4.4 – Mean relative abundance of phyla (A) and genera (B) representing greater than 1% of total reads across all cheeses as measured by 16S rRNA gene targeted amplicon sequencing. Cheeses are grouped by growth category.

Table 4.2 – Relative abundances of the most common three phyla across the different categories of cheeses from this study. Values in the same column of the same subsection (*e.g.*, Milk, Origin) are significantly different (Wilcoxon test or Dunn's test; $p < 0.05$).

Category	n	Firmicutes	Proteobacteria	Actinobacteria
Milk				
Raw	10	71.0% ± 19.3%	15.8% ± 13.2%	12.8% ± 12.0%[a]
Pasteurized	28	80.1% ± 24.9%	15.3% ± 20.4%	4.6% ± 8.5%[b]
Thermized	1	92.7%	5.9%	1.4%
Origin				
Quebec	7	81.0% ± 25.0%	16.4% ± 23.9%	2.5% ± 4.1%
France	25	73.7% ± 24.1%	17.8% ± 18.5%	8.2% ± 10.8%
Denmark	2	91.5% ± 11.8%	6.2% ± 8.4%	2.4% ± 3.4%
British Columbia	3	84.7% ± 24.1%	9.5% ± 16.3%	5.7% ± 7.8%
Ontario	2	99.3% ± 0.4%	0.6% ± 0.4%	0.1% ± 0.1%
Rind				
Bloomy	32	89.2% ± 20.3%[a]	11.5% ± 16.6%[a]	5.6% ± 9.3%
Washed	7	56.2% ± 25.8%[b]	32.1% ± 17.7%[b]	11.0% ± 12.5%
Growth				
-	6	74.0% ± 23.1%[a]	19.4% ± 20.9%	6.5% ± 6.4%[a]
+	7	64.7% ± 28.4%[a]	23.6% ± 25.5%	11.6% ± 14.6%[a]
++	20	73.7% ± 23.9%[a]	17.8% ± 18.3%	8.3% ± 10.7%[a]
+++	10	96.0% ± 5.3%[b]	3.3% ± 4.0%	0.7% ± 1.5%[b]
Total (range)	39	78.1% ± 23.4% (18.7% - 100%)	15.2% ± 18.4% (0% - 69.5%)	6.6% ± 10.0% (0% - 31.1%)

Values are listed as the mean ± standard deviation

Streptococcus and *Lactococcus* were the two most common genera observed, represented by 28.6% ± 35.1% and 29.1% ± 27.6% relative abundance in cheeses, respectively (Fig. 4.4B). At finer taxonomic resolution, the over-representation of these two genera was shown to be the result of just one OTU each, with a relative abundance in the cheeses of 28.5% ± 35.0% and 27.4% ± 26.7% for the dominant *Streptococcus* and *Lactococcus* OTUs, respectively (Fig. 4.S1A&B). Other common genera representing greater than 1% relative abundance included *Lactobacillus*, *Psychrobacter*, *Pseudoalteromonas*, *Staphylococcus*, *Brevibacterium*, *Carnobacterium*, and *Arthrobacter.* The over-representation of *Streptococcus* and *Lactococcus* notwithstanding, the most abundant genus in many of the cheeses was not either of those two. The genus with highest relative abundance in some cheeses was *Lactobacillus* (4 cheeses), *Psychrobacter* (2 cheeses),

Pseudoalteromonas (2 cheeses), *Brevibacterium* (2 cheeses), and an unidentified member of the *Planococcaceae* family (1 cheese).

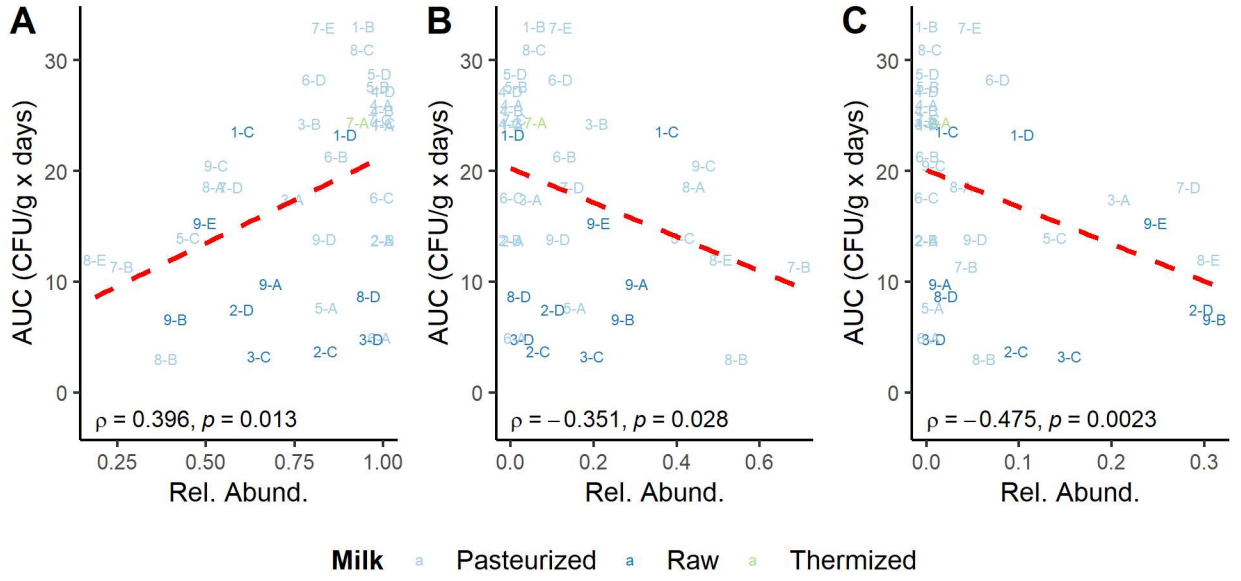

Figure 4.5 - Scatter plot and linear regression (dashed line) of the growth of *L. monocytogenes vs.* the relative abundance of Firmicutes (A), Proteobacteria (B), and Actinobacteria (C). Spearman rank correlations (ρ) are shown at the bottom left corner of each plot, and the cheese samples are coloured by milk treatment.

Significant correlations were observed between *L. monocytogenes* growth and the relative abundance of the genera *Streptococcus* and *Brevibacterium*, as summarized in Table 4.3. The relative abundance of *Streptococcus* was positively correlated with *L. monocytogenes* growth in cheeses (Spearman rank correlation; ρ = 0.606; *q*-value = 0.002). Additionally, an increasing trend in *Streptococcus* was observed across increasing growth categories of *L. monocytogenes*, with the high growth category having a significantly greater relative abundance of *Streptococcus* than both the no growth (Pairwise Wilcoxon rank sum test; *q*-value = 0.004) and low growth (Pairwise Wilcoxon rank sum test; *q*-value = 0.005) categories. Alternatively, the growth of *L. monocytogenes* was negatively correlated with the relative abundance of *Brevibacterium*

(Spearman rank correlation; ρ = -0.562; *q*-value = 0.004). Further, the average relative abundance of *Brevibacterium* was significantly lower in the high growth category than in both the low (Pairwise Wilcoxon rank sum test; *q*-value = 0.023) and no (Pairwise Wilcoxon rank sum test; *q*-value = 0.034) growth categories. The genus *Lactococcus* also showed a decreasing trend with increasing *L. monocytogenes* growth, with the average relative abundance in the no growth category being 47%, compared to the 23% to 25% average relative abundance observed in the other three growth categories. However, this between-category variation in the relative abundance of *Lactococcus* was not significant (Kruskal-Wallace; $p = 0.25$), and no significant correlation was observed between the AUC of *L. monocytogenes* growth and *Lactococcus* relative abundance (Spearman rank correlation; ρ = -0.310; *q*-value = 0.34) in cheeses.

Metagenomic sequencing was used to conduct taxonomic analysis at the species level (Fig 4.6). The two most abundant species were *Lactococcus cremoris* and *Lactococcus lactis*, which represented an average of 20.3% ± 19.0% and 13.7% ± 13.6% of reads in each cheese, respectively. *Streptococcus thermophilus* and *Brevibacterium aurantiacum* were also common, with an average of 7.6% ± 11.9% and 6.9% ± 13.3% of reads in each cheese, respectively. Other species representing >1% of total reads in cheeses included *Pseudoalteromonas translucida*, *Staphylococcus xylosus*, *Pseudomonas taetrolens*, *Pseudoalteromonas nigrifaciens*, *Hafnia paralvei*, *Corynebacterium variabile*, *Mammaliicoccus vitulinus*, *Lacticaseibacillus paracasei*, *Latilactobacillus curvatus*, *Lactiplantibacillus plantarum*, *Lactobacillus delbrueckii*, *Brochothrix thermosphacta*, and an unidentified species of *Psychrobacter*. The most abundant species in most of the cheeses was either *Lactococcus cremoris* (4 cheeses) or *Lactococcus lactis* (3 cheeses). Of the remaining cheeses, two showed *Brevibacterium aurantiacum* as the species with the highest

relative abundance, and *Hafnia paralvei*, *Staphylococcus xylosus*, *Pseudomonas taetrolens*, *Pseudoalteromonas translucida*, *Corynebacterium variabile*, and an unidentified species of *Psycrhobacter* were the most abundant species in one cheese each

Table 4.3 – Spearman rank correlations of *Listeria monocytogenes* growth with the relative abundances of the genera observed in cheeses.

Genus	Spearman Correlation	FDR adjusted p-value
Streptococcus	0.606	0.002
Brevibacterium	-0.562	0.004
Psychrobacter	-0.381	0.195
Listeria	0.362	0.195
Brachybacterium	-0.355	0.195
Lactococcus	-0.310	0.339
Leuconostoc	-0.293	0.372
Thermus	0.269	0.415
Pseudoalteromonas	-0.267	0.415
Corynebacterium	-0.251	0.458
Leucobacter	0.223	0.530
Halomonas	-0.223	0.530
Arthrobacter	-0.200	0.617
Brochothrix	-0.195	0.617
Pseudomonas	0.188	0.617
Morganella	-0.166	0.617
Fusobacterium	-0.165	0.617
Microbacterium	-0.163	0.617
Facklamia	-0.160	0.617
Psychroflexus	-0.155	0.617
Sporosarcina	-0.154	0.617
Mesonia	-0.143	0.650
Carnobacterium	-0.135	0.651
Staphylococcus	-0.132	0.651
Providencia	-0.127	0.651
Vagococcus	-0.106	0.741
Shewanella	0.101	0.741
Vibrio	-0.095	0.747
Atopostipes	0.074	0.814
Coprothermobacter	0.069	0.814
Marinobacter	-0.065	0.814
Cobetia	0.063	0.814
Alkalibacterium	-0.044	0.848
Weissella	-0.043	0.848
Pediococcus	-0.041	0.848
Enterococcus	0.034	0.861
Lactobacillus	0.006	0.969

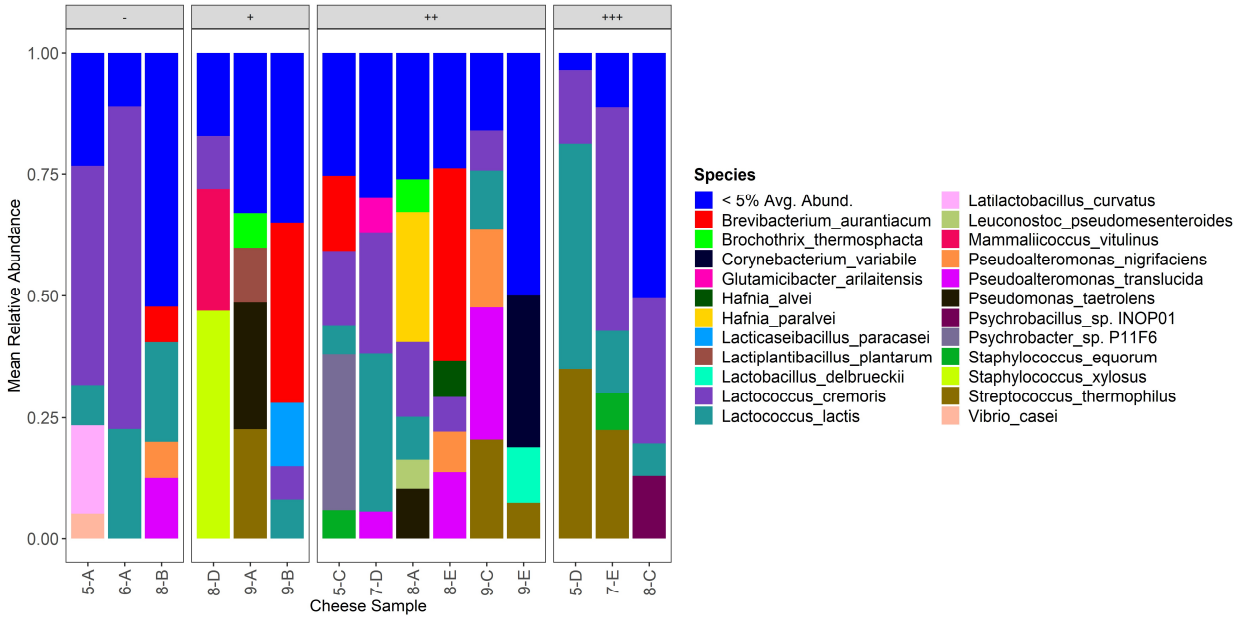

Figure 4.6 – Mean relative abundance of species representing greater than 5% of total reads across the cheeses using shotgun metagenomic sequencing. Cheeses are grouped by growth category.

4.3.4 Beta-diversity and differential abundance

The β-diversity of cheeses was measured using the weighted UniFrac distance method and is summarized in Fig. 4.7. A significant separation was observed between the *L. monocytogenes* growth categories (Fig. 4.7A; PERMANOVA; $p = 0.002$). Specifically, the high growth category was significantly different in microbial community structure than both the no and low growth categories (pairwise PERMANOVA; q-value = 0.024). As shown in Fig. 4.7E, an OTU of the genus *Streptococcus* clustered exclusively with cheeses of the high and medium growth categories. One OTU from the *Lactobacillus* genus also clustered outside the no and low growth categories. A significant difference in community structure was also observed between cheeses made with raw and pasteurized milk (Fig 4.7C; PERMANOVA; $p = 0.001$) and between bloomy and washed rind cheeses (Fig 4.7D; PERMANOVA; $p = 0.023$).

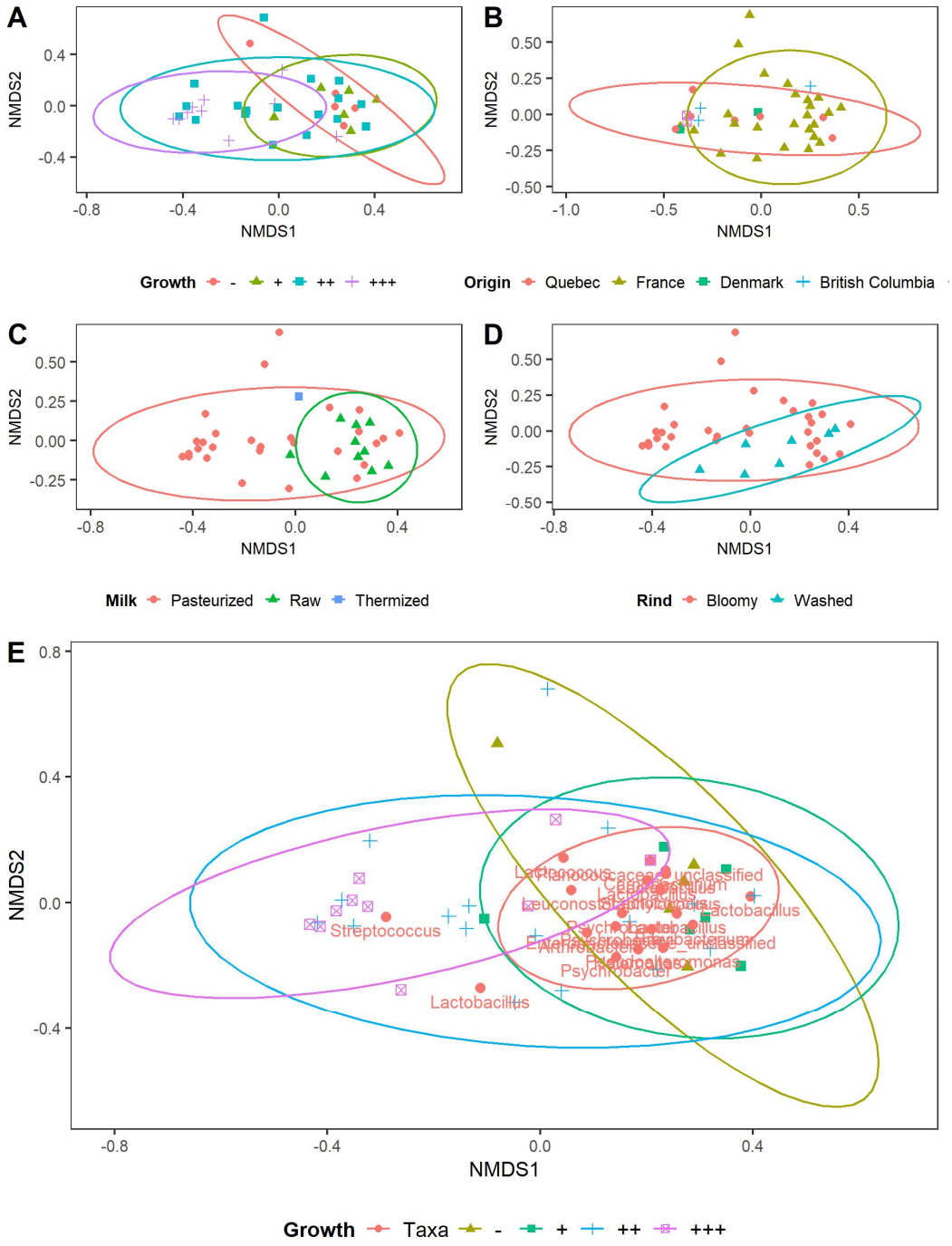

Figure 4.7 – Non-metric multidimensional scaling ordination of cheeses based on weighted-UniFrac distances calculated using all (A-D; stress = 0.15) or the twenty most abundant OTUs (E; stress = 0.16). Ellipses represent a multivariate t-distribution for each growth category(A,E), cheese origin (B), milk treatment (C), and rind type (D). Ellipses are only presented for sample groups with ≥ 4 samples represented. Genera are labelled for the most common OTUs.

Differential abundance analysis of the 16S rRNA gene data identified five prevalent OTUs that were differentially abundant with respect to the growth of *L. monocytogenes* in the cheeses. The Spearman rank correlation between *L. monocytogenes* growth and these OTUs was then calculated (Table 4.4). An OTU of the genus *Streptococcus* was positively correlated with pathogen growth, while four other OTUs were negatively correlated with pathogen growth. Negatively correlated OTUs included two members of the genus *Lactococcus*, one *Brevibacterium* sp., and an unidentified member of the *Brevibacteriaceae* family. Scatter-plot analysis (Fig. 4.S2) confirmed that these correlations were not the result of outliers but represented true trends in the data. The positively correlated *Streptococcus* OTU (Otu00001) was one of the most abundant OTUs observed (Fig. 4.S1A). The most abundant species of *Streptococcus* observed in the shotgun metagenomic sequencing data was *St. thermophilus*, implying this to be the species identity of Otu00001. Similarly, the negatively correlated OTU of *Brevibacterium* (Otu00003) is also a highly abundant OTU, and the most abundant OTU of the Actinobacteria phylum (Fig. 4.S1D), suggesting it represents the species *B. aurantiacum* which was the most abundant species of the Actinobacteria phylum in the shotgun metagenomic sequencing data. The OTUs from the *Lactococcus* genus that were negatively correlated with *L. monocytogenes* growth were not present in high relative abundance compared to other OTUs of *Lactococcus* (Fig. 4.S1B). Therefore it is unlikely that they represent *L. lactis* or *L. cremoris*, the most abundant species of *Lactococcus* observed in the shotgun metagenomics data. Other observed species of *Lactococcus* included *L. raffinolactis*, *L. piscium*, and *L. garvieae*, but there is not enough evidence to suggest which *Lactococcus* species may be represented by the OTUs in question.

Differential abundance analysis of the 16S rRNA gene data was also conducted across the cheese origin, milk treatment, and rind type (Fig. 4.S3). Of note is that the OTUs of *Streptococcus* and *Brevibacterium*, which were differentially abundant based on *L. monocytogenes* growth, were also differentially abundant relative to milk treatment. The *Streptococcus* OTU (Otu00001) was present at greater relative abundance in pasteurized milk cheeses than in raw milk cheeses, whereas the *Brevibacterium* OTU (Otu00003) was more prevalent in raw milk cheeses than in pasteurized milk cheeses. Additionally, the *Streptococcus* OTU (Otu00001) was also differentially abundant with respect to cheese origin. Specifically, the OTU had a lower relative abundance in the cheeses from France than in those from either Ontario or Denmark (Dunn's test; $p < 0.05$).

Table 4.4 – Spearman rank correlations of *Listeria monocytogenes* growth with the abundances of differentially abundant OTUs identified using the ANCOM-BC algorithm.

OTU	Genus	Spearman Correlation	FDR adjusted p-value
Otu00001	*Streptococcus*	0.606	< 0.0001
Otu00003	*Brevibacterium*	-0.561	0.0002
Otu00046	Unclassified *Brevibacteriaceae*	-0.614	< 0.0001
Otu00058	*Lactococcus*	-0.407	0.010
Otu00061	*Lactococcus*	-0.452	0.004

In contrast, no prevalent species were identified as being differentially abundant relative to *L. monocytogenes* growth in cheese in the metagenomics data. This lack of observed differential abundance may be the result of a loss of statistical power due to the reduced number of cheeses sampled. Therefore, species identity confirmation of the 16S rRNA gene data in relation to differential abundance could not be confirmed.

4.3.5 Strain analysis

Strain-level analysis was conducted to determine if strain differences were associated with *L. monocytogenes* growth, or any other qualitative category (Fig. 4.S4). Only *Lactobacillus curvatus* and *Pseudoalteromonas nigrifaciens* showed strain-specific trends between growth categories, but neither trend was strong enough to make decisive conclusions, an no statistical analysis could be conducted. Specifically, *P. nigrifaciens* was able to be identified to the strain level in five cheeses. The three cheeses from the medium growth category clustered separately from the cheeses from the low and no growth category, but not enough cheeses possessed a resolved strain of *P. nigrifaciens* to conduct statistical analysis between the growth categories. Similarly, a *L. cruvatus* strain was identified in three cheeses, one each from no, low, and medium growth categories. While these cheeses all clustered separately based on growth, not enough cheeses possessed a resolved strain of *L. cruvatus* to allow for statistical analysis.

One species, *Lactococcus lactis,* was resolved to the strain level in thirteen different cheeses. No trend related to growth of *L. monocytogenes* was observed, but there was a significant difference based on the cheese origin (PERMANOVA; $p = 0.048$). Specifically, eight out of the nine cheeses from Europe (France and Denmark) clustered together in a separate clade from the four cheeses collected from Canada (Quebec and British Columbia). Further taxonomic analysis showed that, *L. cremoris* was the dominant species of *Lactococcus* in cheese from Europe, while all cheeses from Canada had *L. lactis* as the most abundant species of *Lactococcus*. The one exception was cheese 9-B which possessed roughly equal concentrations of both species, and clustered with the Canadian cheeses even though it was produced in France.

4.4 Discussion

This experiment investigated how the taxonomic composition of the SRC microbiome can affect the growth of *L. monocytogenes*. Targeted amplicon sequencing of the 16S rRNA gene identified an average of 35 OTUs per cheese sample, which is similar to previous community structure profiling studies of SRC (Dugat-Bony et al., 2016; Gérard et al., 2021). Raw milk cheeses were expected to have a more diverse microbiome than pasteurized or thermized cheeses (O. O'Sullivan & Cotter, 2017), which matches the average results observed in this current study; however, several pasteurized milk cheeses showed greater diversity than other raw milk cheeses, and the greatest diversity was observed in a cheese made from pasteurized milk. This increased diversity in pasteurized milk cheeses might be a product of the cheese production environment. A large proportion of the microbiota in cheeses is acquired from the cheese production environment (Cotter & Beresford, 2017), meaning many of the observed OTUs could have colonized the cheeses after pasteurization of the milk. Additionally, previous research has observed that while pasteurization reduced the bacterial population in milk, the total species richness was not reduced relative to raw milk (Quigley et al., 2013). It is possible that low levels of many of the raw milk microorganisms are below detectable levels by culture-based methods after pasteurization but are still present enough to be detected by molecular methods (*e.g.*, metataxonomic sequencing).

Species richness, measured as the total number of observed OTUs, was significantly correlated with a reduced level of *L. monocytogenes* growth. This factor was not correlated with TAMC, suggesting that a larger diversity of different microbial species, instead of just a larger population of native microbes, might decrease the ability of *L. monocytogenes* to grow. However, the inclusion of species richness in a multilinear model along with TAMC and a_w did not increase the

predictive power of the model compared to when it was not included, implying that it is not an important predictive variable. Indeed, the cheese in the current experiment with the highest number of observed OTUs, was assigned to the medium growth category. Similarly, while a recent study of apple packaging facilities found increasing occurrence of *L. monocytogenes* to be associated with a lower overall alpha-diversity in the facility microbiome (Tan et al., 2019), several studies on cheese ripening consortia have shown that composition, and not species richness, was the most important factor related to antilisterial effects in the respective cheeses (reviewed in Montel et al., 2014).

A likely explanation for the observed correlation between *L. monocytogenes* growth and reduced species richness in cheese could be that the relationship is confounded with the effect of milk treatment. Raw milk cheeses, which showed a lower average *L. monocytogenes* growth compared to pasteurized milk cheeses, had a higher average species richness. Further, the three pasteurized milk cheeses with the highest richness (higher than many of the raw milk cheeses) were all members of the medium and high growth groups, suggesting that milk treatment and not species richness is the true predictor of *L. monocytogenes* growth. While the species richness may not play a role directly, it is reasonable to suggest that increased richness improves the likelihood that species possessing antilisterial abilities are present. High species richness might also imply that there is less opportunity for contaminating *L. monocytogenes* to gain a foothold due to a more varied use of nutrients by the native population.

Microbial community profiling identified Firmicutes as the dominant phylum in the cheeses. This observation was consistent with previous studies that identified Firmicutes as the most abundant

phylum in both raw and pasteurized milk (Quigley et al., 2013), as well as most cheeses (Falardeau et al., 2019; Gérard et al., 2021; Quigley et al., 2012). The overabundance of Firmicutes may be attributed to the fact that most starter cultures used in cheesemaking are members of the Firmicutes phylum (Parente et al., 2017). Indeed, three of the four most abundant species observed (*Lc. lactis*, *Lc. cremoris*, and *St. thermophilus*) are common members of defined starter cultures for cheesemaking, and the *Streptococcus* and *Lactococcus* genera have been suggested to be especially suited to the cheese environment (Kamimura et al., 2020). Other common Firmicutes genera observed in the cheeses of this study included *Lactobacillus* (now split into multiple new genera including *Lacticaseibacillus*, *Latilactobacillus*, and *Lactiplantibacillus*; Zheng et al., 2020), *Carnobacterium*, and *Staphylococcus* (including the novel genus *Mammaliicoccus*; Madhaiyan et al., 2020). *Lactobacillus* spp. and *Carnobacterium* spp. are common NSLAB in soft cheeses (Henri-Dubernet et al., 2008; Monnet et al., 2015), with non-starter lactobacilli being important to flavour development (Montel et al., 2014). *Staphylococcus* is a common member of the cheese rind microbiota (Monnet et al., 2015; Wolfe et al., 2014), likely attributed to the brine used during the salting step (Marino et al., 2017). *Staphylococcus* spp. may be important for developing the organoleptic properties of these cheeses (Parente et al., 2017) and may also contribute the characteristic pigmentation on washed rind SRC (Jérôme Mounier et al., 2017).

The high relative abundance of *Streptococcus* in the cheese samples was unexpected since mesophilic starter cultures (*i.e. Lactococcus* spp.) are typically used in the production of Camembert, and other mold-ripened cheeses (Spinnler, 2017). *Streptococcus* has been previously observed as a dominant genus in cheeses where it was not included as a starter culture (Gérard et al., 2021), which suggests that it could be a part of the NSLAB. However, recent interest in the

production of "stabilized" or "solubilized" SRC has led to the addition of *S. thermophilus* as a starter culture. This stabilization process increases the shelf life of the cheeses by controlling rate of acidification (through the inclusion of *S. thermophilus*), leading to a higher pH at draining, an increase on mineral content, and a firmer resulting paste (Batty et al., 2019). Therefore, the high representation of the *Streptococcus* in a portion of the cheeses from this study likely indicates that such cheeses were produced using the stabilization process.

The two other most abundant phyla were Proteobacteria, represented by species of *Psychrobacter*, *Pseudoalteromonas*, and *Pseudomonas*; and Actinobacteria, represented by species of *Brevibacterium*, *Corynebacterium*, and *Arthrobacter*. The large standard deviations in relative abundance associated with these two phyla, however, imply that these results are not consistent across all the cheeses, and that these results should be analyzed with caution. Despite this inter-cheese variation in the relative abundances of these two phyla, these bacteria have all been previously identified as part of the cheese rind community (Wolfe et al., 2014). They are also known to populate the house microbiota in cheese production facilities (Bokulich & Mills, 2013; Goerges et al., 2008). *Psychrobacter* spp. have been previously isolated form both washed and bloomy rind SRC (Monnet et al., 2015), and are important to flavour development (Jérôme Mounier et al., 2017). These psychrotolerant and halotolerant bacteria are believed to enter as part of the raw milk microbiome (Coton et al., 2012), and were observed in the milk samples collected as part of Chapter 2. Species of *Pseudoalteromonas* are halophilic bacteria associated with marine environments and are suggested to colonize the cheese from brine solution used during the salting step (Delcenserie et al., 2014; Wolfe et al., 2014). *Pseudomonas* spp. are psycrhophilic spoilage bacteria commonly associated with raw milk (Ercolini et al., 2009; Raats et al., 2011), but the most

abundant species observed in this study, *P. taetrolens*, is also important to flavour production in bloomy rind SRC (Spinnler, 2017). The Actinobacteria *Brevibacterium*, *Corynebacterium*, and *Arthrobacter* are common secondary starter cultures associated with washed rind cheeses (Jérôme Mounier et al., 2017), but are also commonly found on bloomy rind cheeses (Spinnler, 2017). The most common genus of Actinobacteria in SRC is *Brevibacterium*, especially *B. aurantiacum* and *B. linens,* which are important for flavour production through the generation of volatile sulfur compounds (Sourabié et al., 2012). Additionally, both *Brevibacterium* and *Arthrobacter* are known to produce compounds (*e.g.*, carotenoids) responsible for characteristic pigmentation on the surface of washed rind cheeses (Galaup et al., 2005; Sutthiwong et al., 2014).

The Firmicutes phylum was positively correlated with the growth of *L. monocytogenes* in the cheeses. But this correlation was driven primarily by the genus *Streptococcus*; particularly by a highly abundant OTU likely representing *S. thermophilus*. As described above, *S. thermophilus* was added for the production of "stabilized" or "solubilized" SRC. It is possible that many of the cheeses with the highest growth of *L. monocytogenes* were produced using the stabilized process (*i.e.*, large scale production cheeses), meaning that the correlation between *Streptococcus* and *L. monocytogenes* growth is not causative, but that the high relative abundance of *Streptococcus* is merely indicative of a favourable environment for *L. monocytogenes* growth. If this is the case, it would mean that the stabilization process may produce cheeses that are at higher risk for *L. monocytogenes* growth than cheese produced through traditional methods. It should also be noted, however, that the *S. thermophilus* OTU had a significantly higher relative abundance in cheeses made with pasteurized milk than those made with raw milk, confounding this observed correlation. Further, not all cheeses with high *L. monocytogenes* growth had high relative abundances of

Streptococcus, but all were made with pasteurized milk. Additional targeted experiments should be conducted to assess the effect of the stabilization process on the growth of *L. monocytogenes* in the finished cheese.

Despite the positive correlation between Firmicutes and *L. monocytogenes* growth, two OTUs of *Lactococcus* actually showed a strong negative correlation with growth of the pathogen. A recent study of Belgian cheeses also found a negative correlation between the relative abundance of *Lactococcus* and the growth of *L. monocytogenes* in washed rind SRC (Gérard et al., 2021). Bacteriocins produced by *Lc. lactis* have regularly shown to inhibit the growth of *L. monocytogenes* in soft cheeses (reviewed in Falardeau et al., 2021), but based on the relative abundance of the potentially inhibitory OTUs observed in this study, they are unlikely to represent *Lc. lactis*. Of the three other species of *Lactococcus* observed in this study, *Lc. raffinolactis*, *Lc. piscium*, and *Lc garvieae*, both *Lc. piscium* and *Lc. garvieae* have previously shown antimicrobial action against *L. monocytogenes*. Strains of *Lc. piscium* isolated from modified atmosphere packaged salmon were inhibitory against *L. monocytogenes in vitro* (Matamoros et al., 2009) as well as in cooked shrimp (Fall et al., 2010). These strains of *Lc. piscium* were non-bacteriocinogenic, but no clear mechanism of inhibition was deduced. Similarly, *Lc. garvieae* from raw milk was previously credited with antilisterial activity observed in cheeses produced from the milk (Saubusse et al., 2007). A bacteriocin produced by select strains of *Lc. garvieae*, garvieacin Q, is known to inhibit *L. monocytogenes* (Tosukhowong et al., 2012), and has been identified in a strain collected from raw milk cheese (Flórez et al., 2012). Additionally, *Lc garvieae* was able to inhibit *Staphylococcus aureus*, another Gram-positive foodborne pathogen, in a milk matrix (Alomar et al., 2008).

The Actinobacteria phylum was negatively correlated with *L. monocytogenes* growth, primarily due to the strong negative correlation between the growth of *L. monocytogenes* and an abundant OTU of *Brevibacterium* assumed to be *B. aurantiacum*. *B. linens* has been shown to produce multiple antiliserial bacteriocins (Maisnier-Patin & Richard, 1995; Motta & Brandelli, 2002; Valdés-Stauber & Scherer, 1994), and has been suggested to also possess non-bacteriocinogenic activity against *L. monocytogenes* (Eppert et al., 1997). Since the description of this antilisterial activity, the *B. linens* group has been separated into three new species (Gavrish et al., 2004), including *B. aurantiacum*, which is now considered to be the most abundant species of *Brevibacterium* in SRC (Jérôme Mounier et al., 2017). Since the above-described strains of *B. linens* were all isolated from cheeses, it is reasonable to assume that they would now be considered *B. aurantiacum*. Indeed, the *B. linens* strain described by Motta & Brandelli (2002), is now listed as *B. aurantiacum* Gavrish et al. (ATCC 9175). Therefore, it is reasonable to suggest that the negative correlation between *L. monocytogenes* growth and the *Brevibacterium* OTU observed in this study might share similar mechanisms of action to those previous studies involving *B. linens*. Alternatively, as the raw milk cheeses from the current study possessed a higher average relative abundance of both Actinobacteria and *Brevibacterium*, the negative correlations with *L. monocytogenes* growth could be confounded with the differences in *L. monocytogenes* growth between milk treatments.

While no trends were observed between *L. monocytogenes* growth and the specific strains of the most abundant bacterial species in the cheeses, a geographic trend was observed for between strains of *Lc. lactis*. This geographic difference might be related to an inconsistent

species/subspecies classification within the *Lactococcus* genus. Until recently, *Lc. lactis* subsp. *lactis* and *Lc. lactis* subsp. *cremoris* were part of a single species but have now been split into two separate species: *Lc. lactis* and *Lc. cremoris* (T. T. Li et al., 2019). While the Kraken2 database appears to identify the two as different species (as demonstrated in Fig. 4.6), the PanPhlAn software only contains a pangenome for *Lc. lactis*, suggesting the PanPhlAn software still treats the two species as subspecies of *Lc. lacis*. This discrepancy in the PanPhlAn software could lead to these two species being viewed as separate strains based on their different gene profiles. This hypothesis is further supported by the fact that *Lc. cremoris* was more abundant than *Lc. lactis* in the individual cheeses from Europe, and that *Lc. lactis* was more abundant than *Lc. cremoris* in the individual cheeses from Canada. The reason for the geographic separation, however, is still unclear, and to the best of my knowledge, this is the first documentation of the differential abundance of *Lactococcus* species across cheeses from different regions. Differentiation between the two subspecies was traditionally based on phenotypic characteristics, with *Lc. lactis* subsp. *lactis* possessing the ability to grow at 4°C, tolerate up to 4% NaCl, and hydrolyze arginine, whereas *Lc. lactis* subsp. *cremoris* does not (Olivia McAuliffe, 2018). Both can be used as mesophilic starter cultures, but *Lc. lactis* subsp. *cremoris* is believed to result in less defects in the cheese such as bitterness (Olivia McAuliffe, 2018). It is possible that differences in cheesemaking practices between the two continents could preferentially select for one species over the other (*e.g.*, ripening temperatures or salt content), but not enough is known about the production of the individual cheeses from this study to make any definitive conclusions.

Care should be taken when analyzing the microbiome data presented, primarily due to the compositional nature of these microbiome data. Specifically, the negative correlation bias

associated with compositional data means that an observed (but not true) increase in one taxon may really be the result of a true decrease in the amount of another (Gloor et al., 2017). In the context of this study, this means that negative correlations between *L. monocytogenes* growth and the relative abundance of *Brevibacterium* or *Lactococcus* could really be artifacts resulting from decreasing relative abundances of *Streptococcus*, or *vice versa*. Therefore, enumeration methods (*e.g.*, qPCR) should be used to confirm these findings in the future. Another thing to consider with this data is that the presence of *L. monocytogenes* in cheese can also affect the microbiome of the cheese itself (Salazar et al., 2021), suggesting that the microbiomes observed in this study might not completely reflect the real-world scenario of *L. monocytogenes* contamination in the same cheeses. To confirm the findings of the current study, future studies should investigate these antilisterial effects *in vitro*, and possibly *in situ* using defined microbial communities. Further work should also be conducted to investigate the possible mechanisms behind these effects on *L. monocytogenes* growth.

4.5 Conclusion

The microbiome of SRC can be variable, and may affect, or at least predict, the growth of *L. monocytogenes* in the respective cheese. Increasing species richness may increase the likelihood that *L. monocytogenes* will be inhibited, but likely only due to an increased chance of antilisterial species being present, such as the species of *Brevibacterium* and *Lactococcus* identified in this current study. The production process itself may also affect *L. monocytogenes* growth, with industrialized cheese production processes possibly leading to a higher food safety risk. Future research with functionally profile the metagenomes of these cheeses to attempt to elucidate possible mechanisms of action for the observed differences in antilisterial potential.

Chapter 5: Genome-Resolved Metagenomics of Cheese Microbiomes

5.1 Introduction

The growth of *L. monocytogenes* in cheeses may be affected through the functional or metabolic organization of the native microbiota independent of taxonomy, including nutrient acquisition and provisioning, or the production of inhibitory compounds. Competition for nutrients, also known as the Jameson effect, has been shown to inhibit the growth of *L. monocytogenes* in co-culture, provided the inhibiting culture was inoculated at a higher initial concentration than the concentration of *L. monocytogenes* (Mellefont et al., 2008). Competition-driven inhibition of *L. monocytogenes* has also been observed with biofilm cultures from cheese ripening shelves (Guillier et al., 2008), and the depletion of manganese by lactobacilli has resulted in the inhibition of *Listeria* spp. in cottage cheese (van Gijtenbeek et al., 2021).

Inhibition of *L. monocytogenes* may also be mediated through the production of antimicrobial compounds from competing microorganisms. Bacteriocins are ribosomally-synthesized, antimicrobial peptides produced by bacteria that often target closely related taxa (Cleveland et al., 2001). Many bacteriocins, especially from LAB, have been investigated for their ability to inhibit the growth of *L. monocytogenes* in soft cheeses, with varying success (reviewed in Falardeau et al., 2021). Other antimicrobial proteins may also occur, such as the metalloprotease pseudoalterin (Tang et al., 2020), which was recently identified in members of the cheese microbiome (Walsh et al., 2020). Finally, the presence of organic acids, particularly lactic and acetic acids, have been identified as important mechanisms for the inhibition of *L. monocytogenes* in ripened cheeses (Callon et al., 2011, 2014; Millet et al., 2006).

Metagenomic sequencing enables function-driven investigation of microbial communities that might not be possible using traditional, culture-based methods (New & Brito, 2020). By aligning reads against known databases of enzymes and metabolic pathways, the relative abundance of these functions can be inferred and compared across metagenomes (Beghini et al., 2021). Additionally, metagenome assembled genomes (MAGs) can be constructed by binning together related contigs from metagenome assemblies (Bowers et al., 2017). The construction of MAGs provides a more resolved layer of analysis, linking predicted gene functions with more accurate taxonomic labels (Setubal, 2021). Therefore, the objectives of this study were to i) investigate the metagenomes of cheeses for genes and/or pathways that are differentially abundant relative to *L. monocytogenes* growth, ii) construct and classify MAGs from the cheese metagenomes, and iii) identify MAGs possessing any functional determinants of antilisterial activity in the cheeses.

5.2 Materials and methods

5.2.1 Cheese samples

Functional analysis and the construction of MAGs was conducted on all cheeses that were subjected to metagenomic sequencing (Table 3.1). The methods and results of sequencing and quality filtering were described previously in Chapter 4.

5.2.2 Functional profiling of cheese metagenomes

Functional profiling of cheese metagenomes was conducted using HUMAnN software (version 3.0.0; Beghini et al., 2021), and following the creator's recommended guidelines and default parameters. As recommended by the creators, the functional profiling was conducted using unassembled reads after quality filtering. Gene families were annotated using the UniRef90

database (Suzek et al., 2015), and grouped by EC number. Pathways were annotated using the MetaCyc database (Caspi et al., 2020). For both functional analyses, the results of each sample were normalized to copies per million (CPM) before statistical analysis.

The identification of putative bacteriocin-encoding genes in cheese metagenomes was assessed by aligning the metagenomic assemblies against the BACTIBASE database (Hammami et al., 2010) using BLASTx (Altschul et al., 1990). Only hits with *E* value < 10^{-5} and similarity > 50% were retained. In cases where multiple hits to the BACTIBASE database were observed on a single contig, only the hit with the highest bitscore was retained.

5.2.3 Assembly, binning, and classification of MAGs

Metagenome assembled genomes were constructed using the MetaWRAP metagenomic wrapper suite (version 1.3.2; Uritskiy et al., 2018) following the creator's recommended guidelines. Sample replicates for each individual cheese were co-assembled and binned together to improve MAG construction. Briefly, assemblies were prepared for each individual cheese using metaSPAdes (Nurk et al., 2017). Assemblies were then binned using CONCOCT (Alneberg et al., 2014), MetaBAT2 (Kang et al., 2015), and MaxBin2 (Wu et al., 2016) binning software before consolidating the bins into MAGs using metaWRAP's bin_refinement module with minimum completion and maximum contamination set to 70% and 5%, respectively. High quality MAGs (HQMAGs) manifesting greater than 90% completion and less than 5% contamination (Bowers et al., 2017) were selected for downstream analysis. Contamination and completion were assessed in metaWRAP using CheckM (Parks et al., 2015). The resulting MAGs were taxonomically classified using the Genome Taxonomy Database Toolkit (GTDB-Tk; version 1.7.0; Chaumeil et

al., 2020), and annotated using Prokka (version 1.14.6; Seemann, 2014) under default parameters. The coverage of each MAG was calculated using CoverM read coverage and relative abundance calculator (version 0.6.1; https://github.com/wwood/CoverM).

To identify bacteriocin-encoding genes in constructed MAGs, BLASTx alignment was used with cheese-specific subsets of the BACTIBASE database, including only those bacteriocins previously identified in the respective cheese metagenomes. In cases where hits against multiple bacteriocins were identified over the same stretch of DNA in a specific MAG, only the hit with the highest bitscore was included in final analysis. The presence of pseudoalterin in constructed MAGs was assessed by aligning the pseudoalterin amino acid sequence (Tang et al., 2020) to the MAG assemblies using tBLASTn. As with the bacteriocin BLASTx analysis, the minimum threshold for pseudoalterin identity was *E* value < 10^{-5} and similarity > 50%.

5.2.4 Data analysis

Data wrangling and statistical analysis was conducted using R software (version 4.1.0; R Foundation for Statistical Computing, Vienna, Austria [https://www.Rproject.org/]) with the assistance of the tidyverse collection of packages (version 1.3.1; Wickham et al., 2019) and the phyloseq package (version 1.36.0; McMurdie & Holmes, 2013). For analysis of the data from the HUMAnN functional profiling software, the output was converted to a phyloseq object by treating gene families or pathways as OTUs. Differential abundance analysis of gene families and pathways was conducted using the ANCOMBC package (version 1.2.2; Lin & Peddada, 2020). For Spearman rank correlation, Wilcoxon rank sum test, and Kruskal-Wallace test, the significance level (α) was set at 0.05.

5.3 Results

5.3.1 Functional profile of cheese metagenomes

5.3.1.1 Enzyme and pathway analysis

Of the 137,937,980 paired end reads remaining after quality filtering, 8.9% (8.9% ± 2.1% in each cheese) were successfully mapped to 1,818 UniRef90 gene families with unique EC numbers. Additionally, 5.2% of reads (5.1% ± 1.0% in each cheese) were able to be integrated into 390 different MetaCyc reaction pathways.

Differential abundance analysis was used to identify gene families or metabolic pathways that were differentially abundant relative to the growth of *L. monocytogenes* in the respective cheeses (Fig. 5.1). Differentially abundant gene families and metabolic pathway relative to milk treatment and rind type are shown in Figs. 5.S1 – 5.S4. Of the gene families observed, only one, EC 2.1.1.64 (3-demethylubiquinol 3-O-methyltransferase), was identified as significantly negatively correlated with the growth of *L. monocytogenes* (Fig 5.1A; Spearman rank correlation; $\rho = -0.475$; $p = 0.076$). Two MetaCyc pathways were differentially abundant with respect to *L. monocytogenes* growth (Fig. 5.1B): both the (GLUCUROCAT pathway; Spearman rank correlation, $\rho = -0.520$, $p = 0.047$) and the formaldehyde oxidation I pathway (RuMP pathway; Spearman rank correlation, $\rho = -0.476$, $p = 0.073$) were negatively correlated with the growth of *L. monocytogenes* in the analyzed cheeses. Additionally, the relative abundance of the RuMP pathway was higher in raw milk cheeses than in pasteurized milk cheeses (Fig 5.S3; Wilcoxon test, $p = 0.005$), except for a single pasteurized milk cheese from the no growth category.

Since the production of lactic acid has been previously associated with *L. monocytogenes* inhibition in cheeses, the correlations between *L. monocytogenes* growth and the abundance of the homolactic and heterolactic fermentation pathways was assessed. As shown in Fig. 5.2, no significant correlation was observed between the abundance of the combined or individual lactic acid formation pathways.

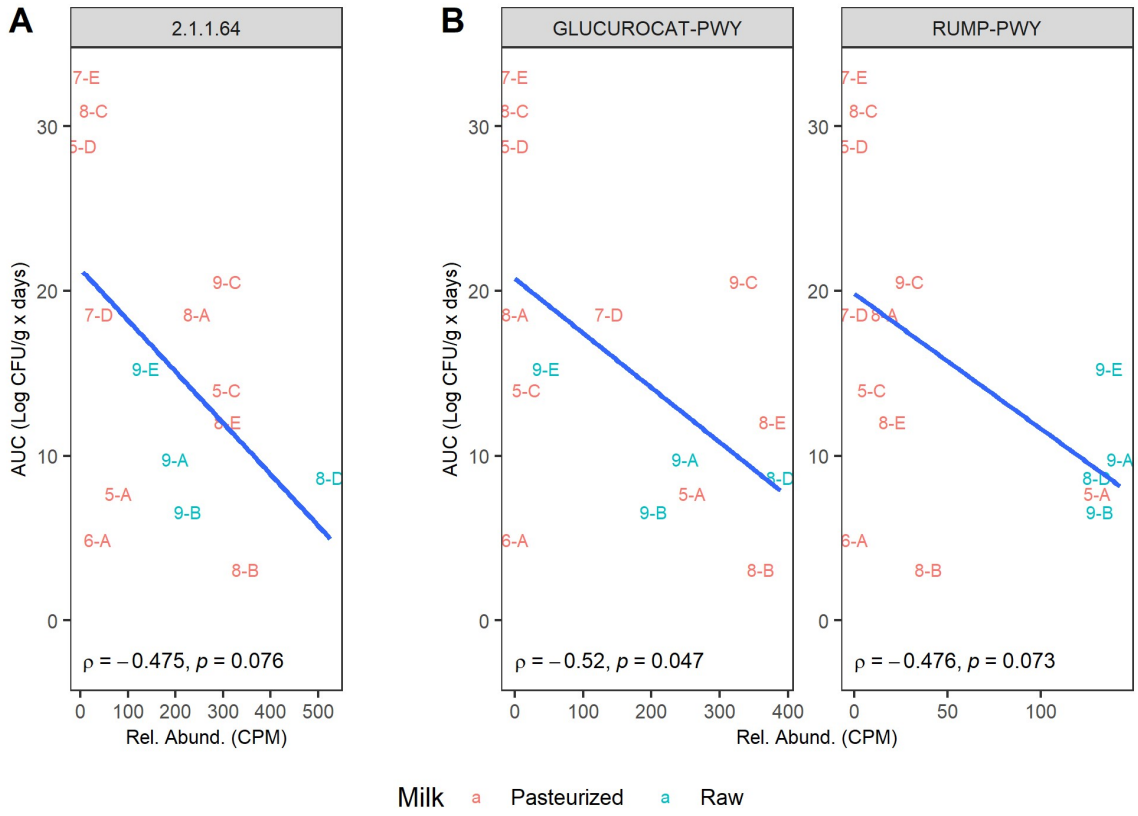

Figure 5.1 – Scatter plots showing the correlation between the growth of *L. monocytogenes* in cheeses and their copies per million reads (CPM) of enzymes (A; EC number) and MetaCyc pathways (B). Linear model regression is shown for each plot (blue line), with the Spearman's rank correlation (ρ) shown at the bottom. EC 2.1.1.64 = 3-demethylubiquinol 3-O-methyltransferase; GLUCUROCAT-PWY = β-D-glucuronosides degradation pathway; RUMP-PWY = formaldehyde oxidation I pathway.

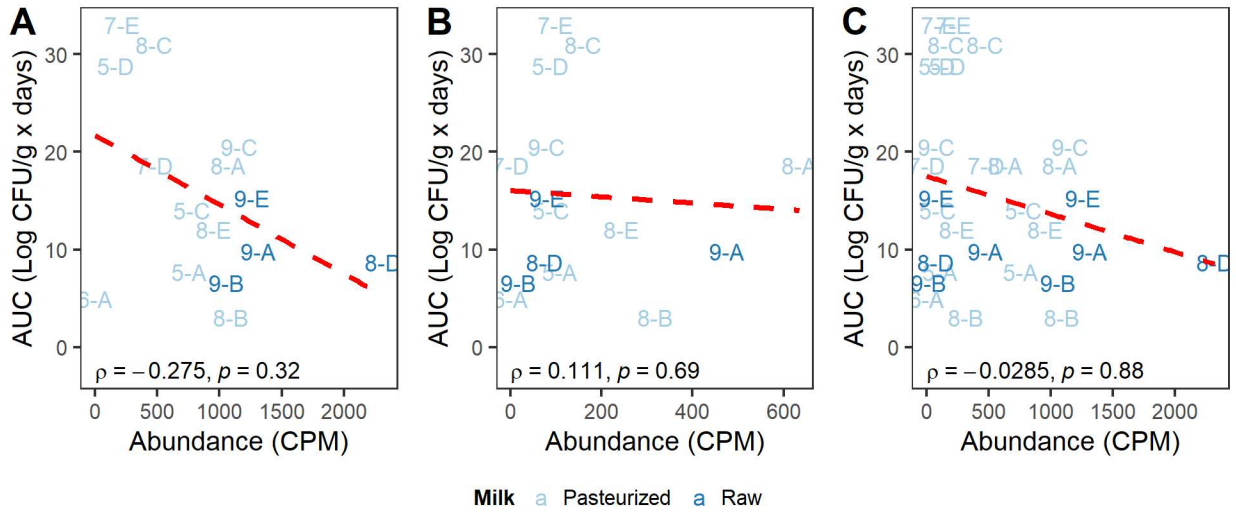

Figure 5.2 - Scatter plots showing the correlation between the growth of *L. monocytogenes* in cheeses and their copies per million reads (CPM) of the homolactic fermentation pathway (A), heterolactic fermentation pathway (B), and the combined abundance of both the homolactic and heterolactic fermentation pathways (C). Spearman's rank correlation (ρ) shown at the bottom of each plot.

5.3.1.2 Identification of bacteriocin-encoding genes

To investigate the possible presence of antimicrobial peptides among the cheeses, a BLAST search was used to identify bacteriocin-encoding genes in the respective metagenome assemblies. A summary of the identified bacteriocin-encoding genes in each cheese is shown in Fig. 5.3, with a summary of their presence across growth category, milk treatment, and rind type shown in Table 5.1.

The two most common bacteriocin-encoding genes observed were those for Lactococcin-A and Enterocin Xβ, which were identified in 13 and 10 cheeses, respectively. Further, both Lactococcin-A and Enterocin Xβ were identified in cheeses from each growth category, milk treatment and rind type. Lactococcin-B and Nisin U were also identified in each growth category, milk treatment and rind type, but were less common, with each being present in only six cheeses.

Linocin M18 was also common, being observed in seven cheeses, but only from the no, low, and medium growth categories. As shown in Fig. 5.4, no significant trends were observed between the number of observed bacteriocins and the growth of *L. monocytogenes* (Spearman Rank correlation; ρ = -353; p = 0.20), or between the number of observed bacteriocins across growth categories of cheeses (Kruskal-Wallis test; p = 0.46). The average number of unique bacteriocins observed, however, was significantly greater (Wilcoxon test, p = 0.048) in washed rind cheeses (7.4 ± 3.1 different bacteriocins) than in bloomy rind cheeses (4.0 ± 2.9 different bacteriocins). No trends in the number of unique bacteriocin-encoding genes were observed relative to cheese origin (Kruskal-Wallis test; p = 0.15) or milk treatment (Wilcoxon test; p = 0.26).

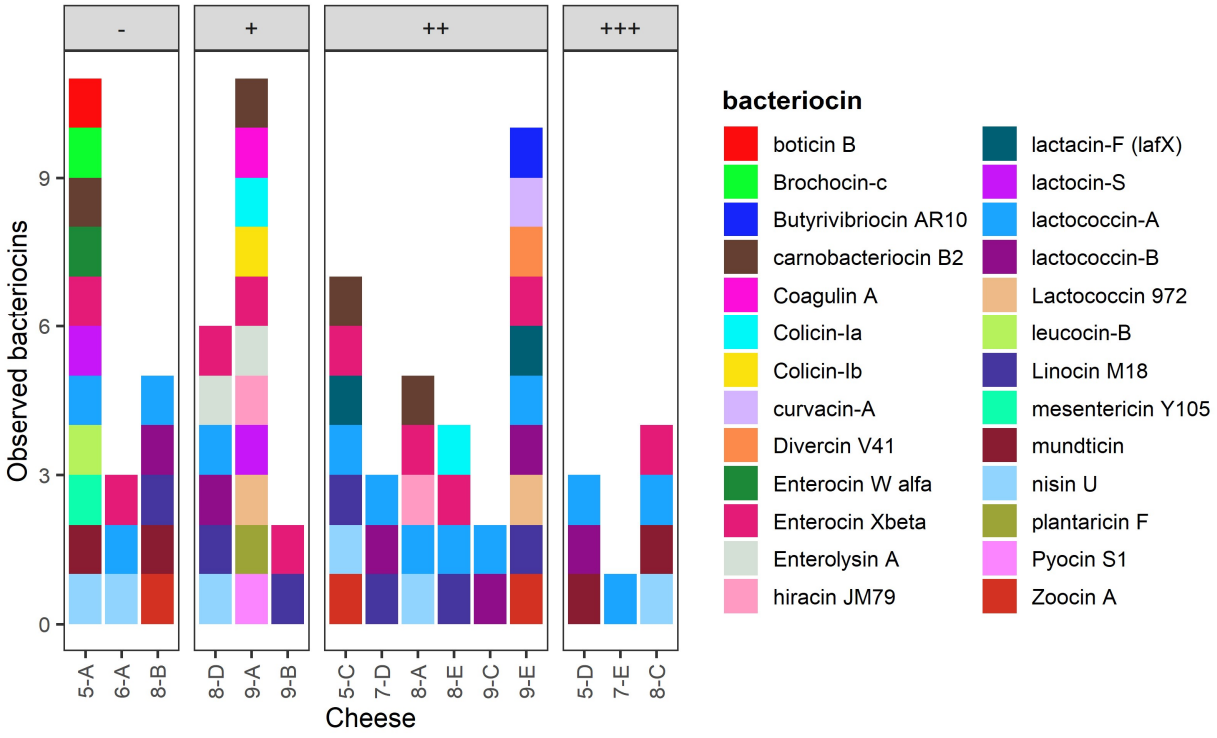

Figure 5.3 - The number of unique bacteriocin-encoding genes observed in each cheese metagenome, according to the BACTIBASE database. Cheeses are grouped by growth category.

Table 5.1 - Presence (marked as an "X") of bacteriocins in each cheese grouping.

	Growth Category				Milk Treatment		Rind Type	
Bacteriocin	**-**	**+**	**++**	**+++**	**Pasteurized**	**Raw**	**Bloomy**	**Washed**
boticin B	X				X		X	
Brochocin-c	X		X		X		X	
Butyrivibriocin AR10			X			X		X
carnobacteriocin B2	X	X	X		X	X	X	X
Coagulin A		X				X		X
Colicin-Ia		X	X		X	X		X
Colicin-Ib		X				X		X
curvacin-A			X			X		X
Divercin V41			X			X		X
Enterocin W alfa	X				X		X	
Enterocin Xbeta	X	X	X	X	X	X	X	X
Enterolysin A		X				X	X	X
hiracin JM79		X	X		X	X	X	X
lactacin-F (lafX)			X		X	X		X
lactocin-S	X	X			X	X	X	X
lactococcin-A	X	X	X	X	X	X	X	X
lactococcin-B	X	X	X	X	X	X	X	X
Lactococcin 972		X	X			X		X
leucocin-B	X				X		X	
Linocin M18	X	X	X		X	X	X	X
mesentericin Y105	X				X		X	
mundticin	X			X	X		X	X
nisin U	X	X	X	X	X	X	X	X
plantaricin F		X				X		X
Pyocin S1		X				X		X
Zoocin A	X		X		X	X		X

Growth categories were based on total increase of *L. monocytogenes* over 12 days and were defined as no growth (< 1 log CFU increase; "-"), low growth (1 log – 2 log CFU increase; "+"), medium growth (2 log – 3.5 log CFU increase; "++"), and high growth (> 3.5 log CFU increase; "+++").

A subset of the observed bacteriocin-encoding genes was observed strictly in the no and/or low growth categories of cheeses. Genes for production of boticin B, enterocin Wα, leucocin-B, and mesentericin Y105 were only present in cheeses from the no growth category, while genes to produce coagulin A, colicin-Ib, enterolysin A, plantaricin F, and pyocin S1 were only present in cheeses from the low growth category. Only lactocin-S was observed in both the no and low growth categories. It should be noted, however, that almost all the bacteriocin-encoding genes that were observed strictly in the no/low growth categories were only present in a single cheese; one from each growth category. The only exceptions were enterolysin A and lactocin-S, which

were each observed in two cheeses. Further, all these single hit bacteriocin genes were from the same cheese within their respective growth category.

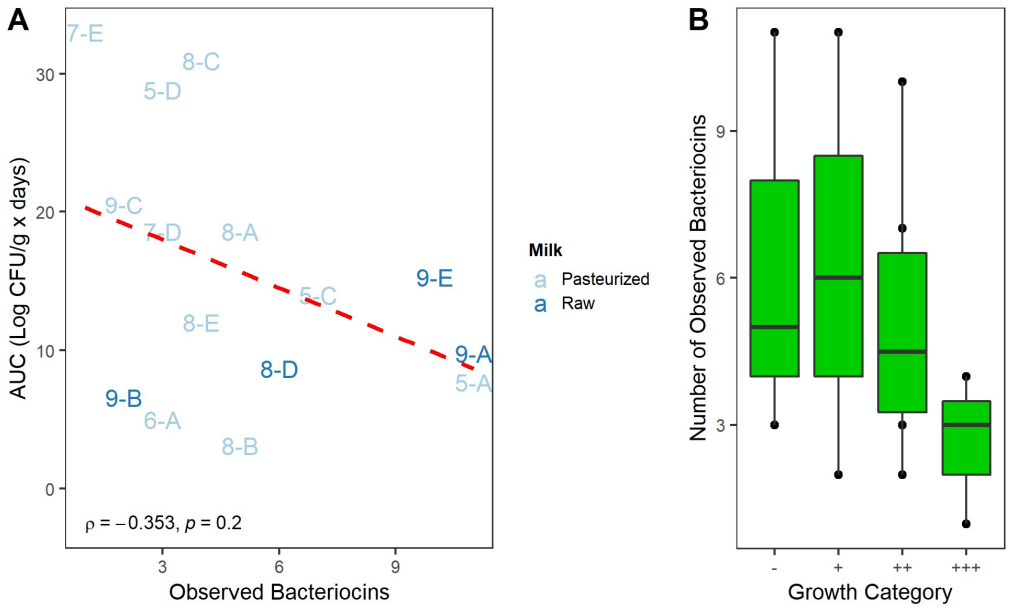

Figure 5.4 – The number of observed bacteriocins relative to the growth of *L. monocytogenes* in cheeses (A), and the growth category of cheeses (B). Spearman rank correlation is shown at the bottom left of plot A.

5.3.2 Metagenome assembled genomes

Metagenomic binning was used to construct MAGs using the metaWRAP pipeline. A total of 125 MAGs of sufficient quality (completion > 70%; contamination < 5%) were constructed from the 15 cheeses, including 106 HQMAGs (Figure 5.S5; Table 5.S1). The number of MAGs in each cheese ranged from 2 to 16, with an average of 8.3 for each cheese. On average, the MAGs represented a relative coverage of 65.4% ± 15.0% from each cheese's sequenced metagenome (Fig 5.5). Among these MAGs, there were 72 Firmicutes, 30 Proteobacteria, 21 Actinobacteria, and one each from Fusobacteria and Bacteroidetes. A total of 109 MAGs were successfully assigned

to the species level, representing 45 different species (Fig 5.6). The remaining 16 MAGs were unable to be assigned at the species level, sharing less than 95% average nucleotide identity (ANI) with the closest reference, and suggesting the possibility that these MAGs may represent novel species. Of these potentially novel species, seven belonged to the genus *Psychrobacter*, three to *Sporosarcina*, two to *Carnobacterium*, and one each were assigned to the genera *Fusobacterium*, *Galactobacter*, *Vagococcus*, and *Vibrio* (Table 5.S1). Importantly, almost all of these novel species MAGs were HQMAGs, except for one MAG each from the *Psychrobacter* and *Carnobacterium* genera.

The numbers of MAGs assigned to each species are shown in Fig 5.6. The most common species assigned to the MAGs was *Lactococcus lactis*, represented by 15 different MAGs across 12 cheeses. It should be noted that the GTDB still treats *Lc. lactis* and *Lc. cremoris* as a single species, suggesting that the MAGs classified as *Lc. lactis* could represent either species. Indeed, three cheeses each had two MAGs assigned to *Lc. lactis*, which may mean one *Lc. lactis* and one *Lc. cremoris*. For the purposes of this chapter, this taxonomic differentiation is not important. The next most common species assigned to the MAGs were *Streptococcus thermophilus* and *Leuconostoc pseudomesenteroides*, assigned to seven MAGs each; and *Brevibacterium aurantiacum*, *Lactococcus laudensis*, and *Glutamibacter ardleyensis*, assigned to six MAGSs each.

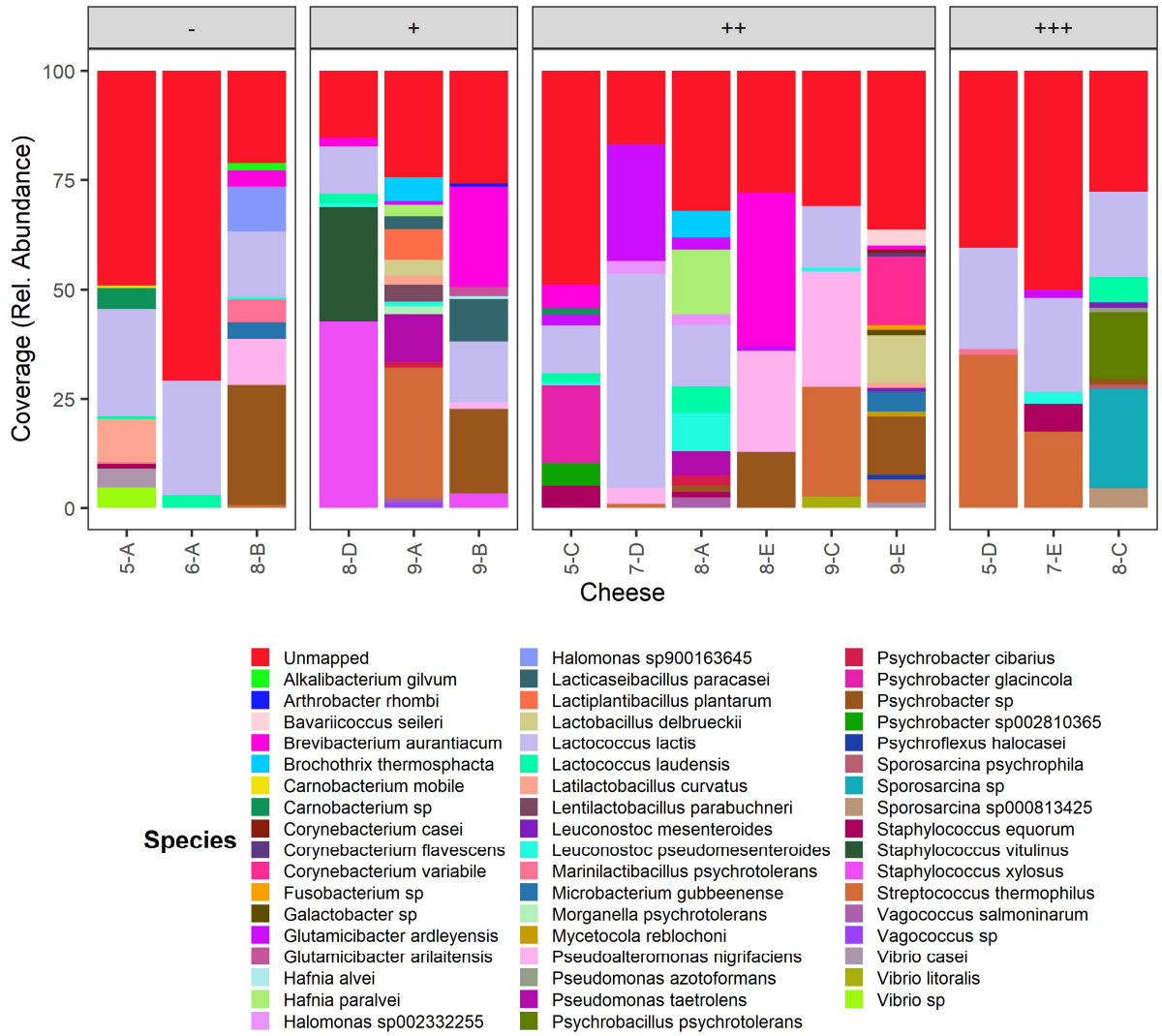

Figure 5.5 - Relative coverage and taxonomic identity of each bin observed in each cheese. The unmapped group represents sequence reads that were not part of any of the identified bins. Cheeses are grouped by growth category.

The occurrence of each identified species in the assembled MAGS relative to growth category, milk treatment, and rind type is presented in Table 5.2. *Lc. lactis*, *Lc. laudensis*, *S. thermophilus*, and *Leu. Pseudomesenteroides* were the only species observed across all growth categories, milk treatment, and rind type. MAGs from four species were only observed in the no growth category and were each present in only a single cheese: *Alkalibacterium gilvum*, *Carnobacterium mobile*,

Halomonas sp900163645, and an unidentified species of *Vibrio*. MAGs from 10 species were observed strictly in cheeses from the low growth category: *Lacticaseibacillus paracasei* and *Staphylococcus xylosus,* which were both observed in two cheeses; and *Arthrobacter rhombi*, *Glutamibacter arilaitensis*, *Hafnia alvei*, *Lactiplantibacillus plantarum*, *Lentilactobacillus parabuchneri*, *Morganella psychrotolerans*, *Staphylococcus vitulinus*, and an unidentified species of *Vagococcus*, each observed in a single cheese. There were no species-classified MAGs identified in both the no and low growth categories.

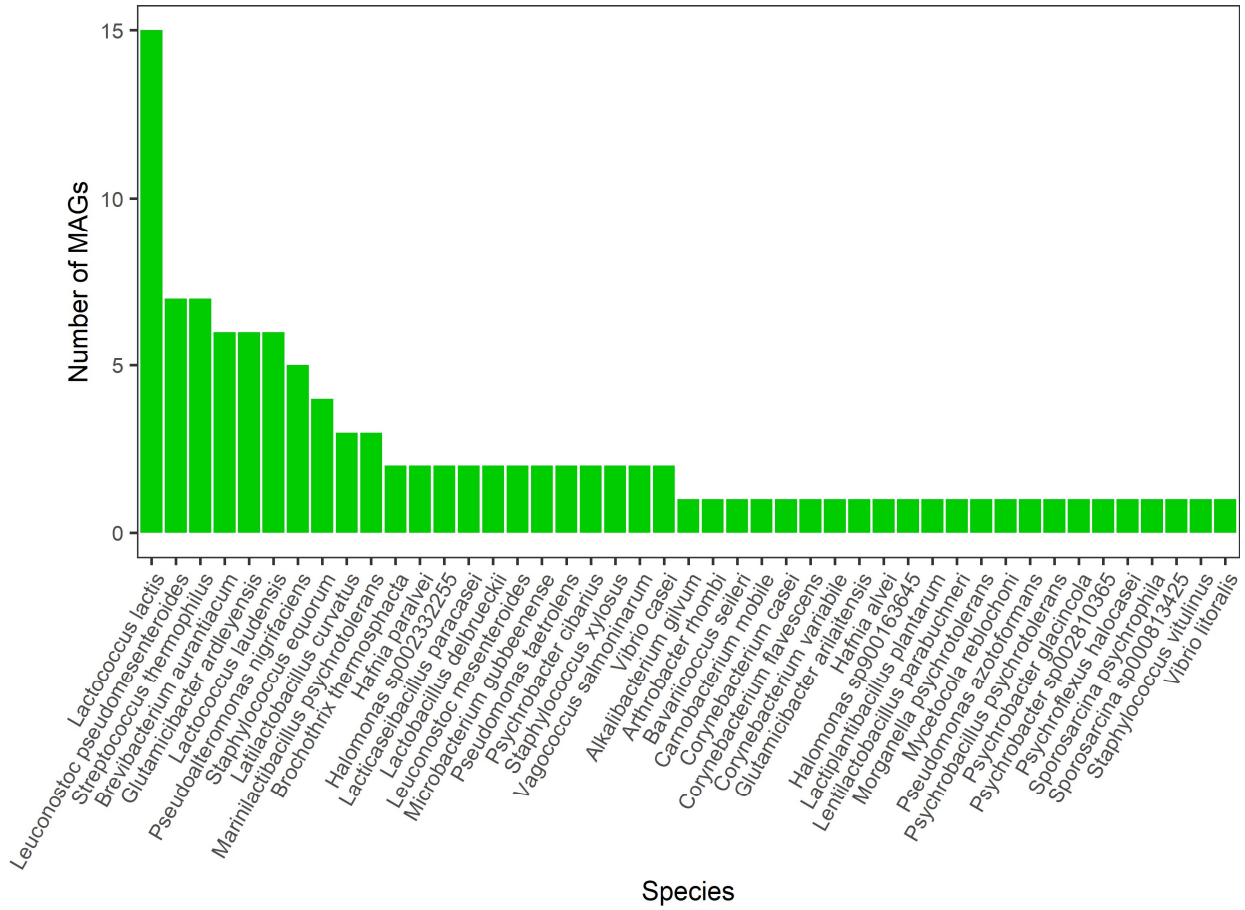

Figure 5.6 - Number of MAGS assigned to each species.

Table 5.2 - Identified MAGs in each cheese grouping (marked as an "X").

	Growth Category				Milk Treatement		Rind Type	
Species	**-**	**+**	**++**	**+++**	**Past.**	**Raw**	**Bloomy**	**Washed**
Alkalibacterium gilvum	X				X			X
Arthrobacter rhombi		X				X	X	
Bavariicoccus seileri			X			X		X
Brevibacterium aurantiacum	X	X	X		X	X	X	X
Brochothrix thermosphacta		X	X		X	X	X	X
Carnobacterium mobile	X				X		X	
Carnobacterium sp	X		X		X		X	X
Corynebacterium casei			X			X		X
Corynebacterium flavescens			X			X		X
Corynebacterium variabile			X			X		X
Fusobacterium sp			X			X		X
Galactobacter sp			X			X		X
Glutamicibacter ardleyensis		X	X	X	X	X	X	X
Glutamicibacter arilaitensis		X				X	X	
Hafnia alvei		X				X	X	
Hafnia paralvei		X	X		X	X	X	X
Halomonas sp2332255			X		X		X	
Halomonas sp9X63645	X				X			X
Lacticaseibacillus paracasei		X				X	X	X
Lactiplantibacillus plantarum		X				X		X
Lactobacillus delbrueckii		X	X			X		X
Lactococcus lactis	X	X	X	X	X	X	X	X
Lactococcus laudensis	X	X	X	X	X	X	X	X
Latilactobacillus curvatus	X	X	X		X	X	X	X
Lentilactobacillus parabuchneri		X				X		X
Leuconostoc mesenteroides			X	X	X	X	X	X
Leuconostoc pseudomesenteroides	X	X	X	X	X	X	X	X
Marinilactibacillus psychrotolerans	X			X	X		X	X
Microbacterium gubbeenense	X		X		X	X		X
Morganella psychrotolerans		X				X		X
Mycetocola reblochoni			X			X		X
Pseudoalteromonas nigrifaciens	X	X	X		X	X	X	X
Pseudomonas azotoformans				X	X		X	
Pseudomonas taetrolens		X	X		X	X	X	X
Psychrobacillus psychrotolerans				X	X		X	
Psychrobacter cibarius		X	X		X	X	X	X
Psychrobacter glacincola			X		X			X
Psychrobacter sp	X	X	X	X	X	X	X	X
Psychrobacter sp28X365			X		X			X
Psychroflexus halocasei			X			X		X
Sporosarcina psychrophila				X	X		X	
Sporosarcina sp				X	X		X	
Sporosarcina sp8X3425				X	X		X	
Staphylococcus equorum	X		X	X	X		X	X
Staphylococcus vitulinus		X				X	X	
Staphylococcus xylosus		X				X	X	
Streptococcus thermophilus	X	X	X	X	X	X	X	X
Vagococcus salmoninarum		X	X		X	X	X	X
Vagococcus sp		X				X		X
Vibrio casei	X		X		X	X	X	X
Vibrio litoralis			X		X		X	
Vibrio sp	X				X		X	

Growth categories were based on total increase of *L. monocytogenes* over 12 days at refrigerated temperatures and were defined as no growth (< 1 log CFU increase; "-"), low growth (1 log – 2 log CFU increase; "+"), medium growth (2 log – 3.5 log CFU increase; "++"), and high growth (> 3.5 log CFU increase; "+++").

Differences in the species classification of MAGs were observed between milk treatment and rind type groups (Table 5.2). Of the species-classified MAGS, 16 were unique to pasteurized milk cheeses, and 19 were unique to raw milk cheeses. Similarly, 14 and 18 unique species were observed in the bloomy and washed rind cheeses, respectively. Most of the milk treatment-specific species were found only in a single cheese, except for *Staphylococcus equorum*, *Marinilactibacillus psychrotolerans*, and *Halomonas sp002332255*, which were observed in four, three, and two pasteurized milk cheeses, respectively; and *Lacticaseibacillus paracasei*, *Lactobacillus delbrueckii*, and *Staphylococcus xylosus*, which were each observed in two raw milk cheeses. Of the rind type-specific MAG species, *Staphylococcus xylosus* and *Halomonas sp002332255* were observed in two different bloomy rind cheeses, while *Lactobacillus delbrueckii*, and *Microbacterium gubbeenense* were observed in two washed rind cheeses each. The other rind-specific MAG species were all each observed in only a single cheese.

5.3.3 Presence of bacteriocins and antilisteria-associated genes and metabolic pathways in metagenome assembled genomes.

5.3.3.1 Functional genes and pathways

To further investigate the genes and metabolic pathways that were identified as differentially abundant relative to *L. monocytogenes* growth in cheeses (Fig. 5.1), annotations of the identified MAGs were probed to identify species potentially possessing these functional characteristics. EC 2.1.1.64 was not identified in any of the constructed MAGs, but the gene associated with it, COQ3/ubiG, was identified in 79 MAGs classified as 34 different species, and present in every cheese. (Table 5.S2).

Analysis of the differentially abundant pathways provided more species-specific results compared to the gene family analysis (Table 5.3). The two initial genes in the RuMP pathway (rmpA and rmpB) were observed in 4 MAGs from four different cheeses, all of them classified as *Glutamibacter ardleyensis*. Interestingly, none of these cheeses were those that were identified as having a high abundance of the genes in the cheese metagenome (Fig 5.1B), and all the cheeses possessing MAGs containing the RuMP pathway were either from the medium or high growth category. The genes encoding the first two enzymes for the GLUCUROCAT pathway, *uidA* and *uxaC*, were identified in seven MAGs from seven different cheeses. Six of these MAGs were from the genus *Staphylococcus* (four *S. equorum* and two *S. xylosus*), and one was classified as *Lentilactobacillus parabuchneri*. Of these seven MAGs possessing the GLUCUROCAT pathway, one, three, two, and one were from cheeses from the no, low, medium, and high growth categories, respectively.

Table 5.3 – MAGs containing genes for pathways identified as differentially abundant with respect to *L. monocytogenes* growth in cheese.

Pathway	Cheese_ID	MAG	Species Classification
RUMP-PWY	7-D	bin.4	*Glutamicibacter ardleyensis*
	7-E	bin.1	*Glutamicibacter ardleyensis*
	8-A	bin.2	*Glutamicibacter ardleyensis*
	8-E	bin.3	*Glutamicibacter ardleyensis*
GLUCUROCAT-PWY	5-A	bin.7	*Staphylococcus equorum*
	5-C	bin.3	*Staphylococcus equorum*
	7-E	bin.4	*Staphylococcus equorum*
	8-A	bin.11	*Staphylococcus equorum*
	8-D	bin.2	*Staphylococcus xylosus*
	9-A	bin.1	*Lentilactobacillus parabuchneri*
	9-B	bin.10	*Staphylococcus xylosus*

RUMP-PWY: formaldehyde oxidation I pathway
GLUCUROCAT-PWY: β-D-glucuronosides degradation pathway

5.3.3.2 Bacteriocin-encoding genes

To investigate the taxonomic source of the bacteriocins observed in section 5.3.1.2, a BLAST analysis was conducted to identify MAGs containing the previously observed bacteriocin-encoding genes (Fig. 5.7). Lactococcin-A was the most common bacteriocin detected, being present in nine MAGs, from nine cheeses, all of which were classified as *Lc. lactis*. Lactococcin-A genes were present in MAGs from at least one cheese from each growth category. Lactococcin-B was also observed, but only five times: four times from MAGs identified as *Lc. lactis*, and once from a MAG classified as *St. thermophilus*. The next most common bacteriocin-encoding genes were those for linocin-18 and nisin U, which were each present in all six MAGs classified as *Brevibacterium aurantiacum* and *Lactococcus laudensis*, respectively. Similarly, genes encoding mundticin were identified in all three MAGs classified as *Marinilactibacillus psychrotolerans*. Genes encoding five different plantaricin variants were all present in a single MAG classified as *Lactiplantibacillus plantarum*. Interestingly, a single MAG, identified as *Bavariicoccus seileri* possessed bacterion-producing genes for three different bacteriocins: lacticin-F, brochocin-c, and divercin V41. This multi-bacteriocin MAG was identified in a washed rind cheese produced from raw milk. All other bacteriocin-encoding gene hits were identified in single, individual MAGs.

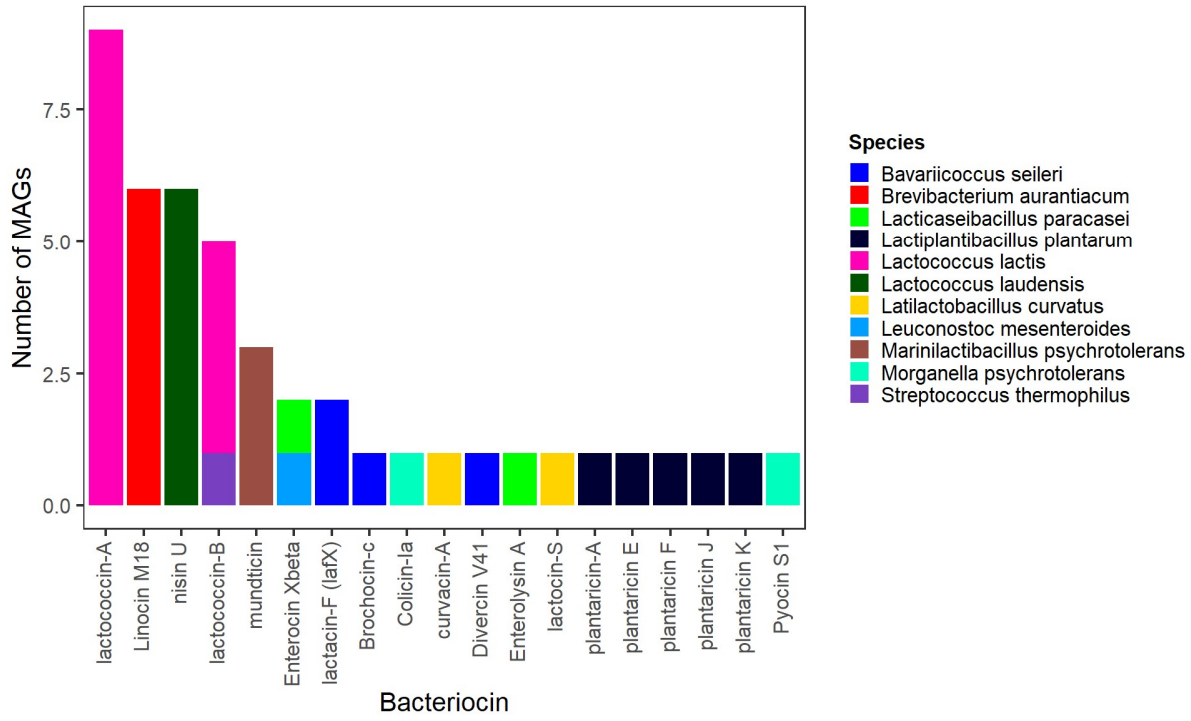

Figure 5.7 – The abundance of bacteriocin-encoding genes observed in the constructed MAGs.

5.4 Discussion

The purpose of this experiment was to identify and investigate functional aspects of the cheese metagenomes that are associated with the differential growth of *L. monocytogenes*. Differential abundance analysis of the unassembled reads identified one gene family and two metabolic pathways that were differentially abundant in the cheese metagenomes relative to *L. monocytogenes* growth. The differentially abundant gene family was an enzyme involved in ubiquinone biosynthesis, 3-demethylubiquinol 3-O-methyltransferase (EC 2.1.1.64). Ubiquinone, also known as coenzyme Q, is involved in the electron transport chain, and is widespread among microorganisms (Meganathan, 2001). The ubiquinone biosynthesis pathway involves two demethylation steps, catalyzed by enzymes EC 2.1.1.222 and EC 2.1.1.64; however both enzymes are produced from the same gene, COQ3/*ubi*G (Aussel et al., 2014). The COQ3/*ubiG* gene was

identified in 72 MAGs (34 species) spanning all the cheeses analyzed in this study, confirming the widespread occurrence of the ubiquinone biosynthesis pathway. While ubiquinone has shown some antibacterial effects previously (Cheng et al., 2011; Guo et al., 2020), the widespread occurrence of the COQ3/*ubiG* gene in many MAGs from all cheeses suggests that its differential abundance is more likely an artifact of the same enzyme having two classifications within the database. Perhaps, for unknown reasons, reads from inhibitory cheeses were more likely to be classified as the EC 2.1.1.64 gene family than the EC 2.1.1.222 gene family.

The RuMP pathway was also identified as being negatively correlated with the growth of *L. monocytogenes* in the cheeses. The RuMP pathway is important for the detoxification of formaldehyde in methylotrophic bacteria, but has also been observed in other taxa such as *Bacillus subtilis* and *Burkholderia cepacia* (Yurimoto et al., 2005). The genes encoding the initial two enzymes to the RuMP pathway were identified in four MAGs, all of which were classified as *Glutamibacter ardleyensis*. This observation is reasonable since the RuMP pathway has been identified previously in the *Arthrobacter* genus (Chistoserdova & Lidstrom, 2013), of which *Glutamicibacter* is a novel genus (Busse, 2016). Interestingly, the four MAGs possessing the RuMP pathway genes were all from cheeses of the medium and high growth categories. Further, the four *G. arsleyensis* MAGs were all from pasteurized milk cheeses, whereas a higher relative abundance of the RuMP pathway was observed in raw milk cheese metagenomes than in metagenomes from pasteurized milk cheeses. This discrepancy between MAGs and metagenomes implies that this pathway might be more abundant in lesser, undetected species in the raw milk cheeses. Interestingly, the single pasteurized milk cheese with high relative abundance of this

pathway was a member of the no growth category. This outlier in the pasteurized milk cheese highlights that RuMP pathway may indeed have some antilisterial potential.

It is unclear why the presence of the RuMP pathway might be associated with *L. monocytogenes* inhibition as formaldehyde is generally considered toxic to bacteria (N. H. Chen et al., 2016). One possibility is that the pathway may affect the presence of other important nutrients. For instance, the first enzyme in the pathway requires the presence of a divalent metal ion for stability, with Mn^{2+} or Mg^{2+} being preferred (Ferenci et al., 1974). Recent research has also identified Mn depletion by lactobacilli in cottage cheese as an important mechanism in *L. monocytogenes* inhibition (van Gijtenbeek et al., 2021), suggesting the possibility that the RuMP pathway could inhibit *L. monocytogenes* through the same mechanism. There is currently insufficient evidence to draw any specific conclusions about the antilisterial effects of the RuMP pathway, but future investigation into this pathway's relationship to *L. monocytogenes* growth may be warranted.

Another pathway that was negatively correlated with the growth of *L. monocytogenes* was the GLUCUROCAT pathway. According the the MetaCyc database (Caspi et al., 2020), this pathway is involved in the catabolism of the β-D-glucuronosides into forms that can be used for energy metabolism. The GLUCUROCAT pathway is primarily associated with *Escherichia coli*, with the gene associated with the initial enzyme (*uidA*) commonly used as a target for polymerase chain reaction-based detection of *E. coli* (Molina et al., 2015). In the current study, the first two genes of the pathway were observed in seven MAGs from three different species: *Staphylococcus equorum*, *Staphylococcus xylosus*, and *Lentilactobacillus parabuchneri*. The presence of this gene in these MAGS is not unprecedented as homologs of *uidA* have been previously identified in species of *Lactobacillus* (Russell & Klaenhammer, 2001) and *Staphylococcus* (Arul et al., 2008).

Like the RuMP pathway, many of the identified MAGs were from cheeses that did not show a high relative abundance of the GLUCUROCAT pathway in their metagenome relative to the other cheeses. Additionally, three out of four cheeses with the highest identified relative abundance of the GLUCUROCAT pathway did not possess any MAGs containing the gene for the initial enzyme. As with the RuMP pathway, there is no clear reason why the GLUCUROCAT pathway might affect the growth of *L. monocytogenes*. A more detailed analysis of the genes and cofactors involved in the pathway is required before any clear conclusions about the GLUCUROCAT pathway can be drawn.

The pathways associated with homolactic and heterolactic fermentation did not show any significant correlation with *L. monocytogenes* growth. The production of lactic acid in soft and semi-soft cheeses has previously been associated with the inhibition of *L. monocytogenes* (Callon et al., 2011, 2014; Millet et al., 2006), but those studies were focused on contamination at the production stage, and not the retail stage. During the ripening of SRC, lactic acid is catabolized by fungi on the surface of the cheese, raising the pH, and making the cheese more hospitable to other ripening microorganisms (Jérôme Mounier et al., 2017; Spinnler, 2017). This reduction in lactic acid throughout ripening, along with the results of this study, imply that presence of lactic acid production pathways is not relevant to *L. monocytogenes* growth when contamination happens at the retail stage. Further, differences in expression of these pathways might be more important than merely their presence. An investigation of lactic acid concentrations of the cheeses is still necessary to rule out lactic acid production as affecting *L. monocytogenes* growth.

Bacteriocins are antibacterial peptides produced by bacteria, and have shown varied effectiveness in inhibiting *L. monocytogenes* in soft cheese (Falardeau et al., 2021). Bacteriocins provide an attractive option for the control of *L. monocytogenes* since they are often found in bacteria already associated with the cheese microbiome (Trmčić et al., 2008). Therefore, it was relevant to assess the presence of bacteriocins in the cheese metagenomes relative to the growth of *L. monocytogenes*. No clear trend was observed between the number of different bacteriocin-encoding genes present in cheeses and *L. monocytogenes* growth, implying that having a wide diversity of bacteriocins does not improve antimicrobial effects against a specific pathogen such as *L. monocytogenes*. Since, the antilisterial efficacy is variable across different bacteriocins (Falardeau et al., 2021), it seems more likely that the identity of the bacteriocins present would be more important than the number of bacteriocins.

Of the 26 unique bacteriocins implied by the presence of their genes in the cheese metagenomes, four of the five most common, lactococcin-A, lactococcin-B, enterocin Xβ and nisin U, were observed in cheeses from all four growth categories. Therefore, it is reasonable to assume that none of them have a strong ability to inhibit *L. monocytogenes* in the cheese environment. The other most common bacteriocin was linocin M18, identified in all MAGs classified as *Brevibacterium aurantiacum*, and which was noticeably absent from the high growth category. The relative abundance of *Brevibacterium aurantiacum* in the cheeses was negatively correlated with the growth of *L. monocytogenes* (Chapter 4), and perhaps linocin M18 was part of the reason for this antilisterial effect. Indeed, the inclusion of a linocin-producing strain of *Brevibacterium linens* (more likely *B. aurantiacum*; Gavrish et al., 2004) in the wash brine of a washed rind cheese previously resulted in an antilisterial effect (Eppert et al., 1997).

A variety of bacteriocin-encoding genes were present solely in cheese metagenomes from the no and low growth categories, but most of these were only observed in a single cheese. The lack of multiple occurrences of these bacteriocin-encoding genes makes it impossible to assess whether they truly provide a protective effect against *L. monocytogenes* growth, or their presence is merely a coincidence. That being said, three bacteriocins implied by the presence of their genes in metagenomics from the no growth category, enterocin Wα, (Sawa et al., 2012), leucocin-B, (Makhloufi et al., 2013), and mesentericin Y105 (Hechard et al., 1992), have all been demonstrated as inhibitory to *L. monocytogenes*. Since all three of these antilisterial bacteriocin-encoding genes were present in a single cheese, one or multiple could be responsible for the lack of *L. monocytogenes* growth. The only bacteriocin-encoding genes unique to the no/low growth category that were present in multiple cheeses were those responsible for the production of enterolysin A and lactocin-S. Enterolysin A has only shown minimal inhibition against *L. monocytogenes in vitro* (Nilsen et al., 2003), which makes it an unlikely that it is driving the *L. monocytogenes* inhibition observed in these cheeses. To the best of my knowledge, no published work has been assessing the effect of lactocin-S against *L. monocytogenes*, but some inhibitory potential was observed against *L. innocua* when grown in co-culture with a lactocin-S-producing strain of *Lactobacillus sake*. Overall, there is little evidence to conclude that bacteriocins are having a meaningful effect on the growth of *L. monocytogenes* in the cheeses.

It is important to acknowledge that the functional profiling of the cheese metagenomes in this study, including the identification of antimicrobial peptide associated genes, suffers from a variety of limitations. The first limitation is that the presence of a putative gene does not, in and of itself,

demonstrate that this gene is expressed. In order to demonstrate gene expression, metatranscriptomics or metaproteomics are required to measure presence and relative abundance of RNA transcripts or peptides, respectively (Allaband et al., 2019). Additionally, *in vitro* confirmation of the inhibitory effects of any expressed antimicrobial peptides is also necessary, since mutations within bacteriocin peptides have been shown to affect their antimicrobial efficacy (Kumariya et al., 2019). Therefore, it is likely that the bacteriocin-encoding genes identified in this study could differ in their ability to inhibit *L. monocytogenes* since they may share as little as 50% sequence homology with those of the BACTIBASE database. Finally, functional profiles of metagenomes are biased by the databases used for annotation and alignment. Since only known enzymes, peptides, and metabolic pathways can be identified using these databases (Prakash & Taylor, 2012), novel sequences with antilisterial effects may go unnoticed.

Among the 125 MAGs constructed from the 15 cheese metagenomes in this study, 96 were considered HQMAGs based on completeness and contamination. A few of these HQMAGs were assigned to species not previously identified in cheese: *Lactococcus laudensis*, *Glutamicibacter arleyensis*, *Spororsarcina psychrophila*, and *Morganella psychrotolerans*. Four HQMAGs from four different cheeses were classified as *Lc. laudensis*. *Lc. laudensis* was originally cultured from cow's milk in Italy and is more closely related to *Lc. raffinolactis* and *Lc. piscium* than to *Lc. lactis*. (Meucci et al., 2015). *G. arleyensis* was the species classification given to four HQMAGs from four different cheeses. *G. arlyenesis* (formerly *Arthrobacter* arlyensis; Busse, 2016) was initially isolated from lake sediment in the Antarctic (M. Chen et al., 2005), and is closely related to *G. arilaitensis* (Busse, 2016) which has been previously observed in cheese metagenomes (Walsh et al., 2020). *M. psychrotolerans* , a histamine producing microbe initially isolated from

seafood (Emborg et al., 2006), was observed in a single cheese. *M. psychrotolerans* is closely related to *M. morganii*, a species isolated from cheese previously (L. T. Ryser et al., 2021). One HQMAG classified as *Sporosarcina psychrohila*, and four other HQMAGs classified as *Sporosarcina* spp. were observed in a single cheese. *Sporosarcina* spp. are spore-forming bacilli and may be involved in dairy spoilage (Gopal et al., 2015). Members of the *Sporosarcina* genus have been previously identified in raw cow milk (Wolfgang et al., 2012) and on the farm environment (Huck et al., 2008), but to the best of my knowledge this genus has not been previously identified in cheese.

Fourteen HQMAGs were not able to be classified at the species level, suggesting the possibility that they could represent novel species. Six and one of these were from the *Psychrobacter* and *Vibrio* genera, respectively. Unclassified MAGs from these two genera were also observed in a previous meta-analysis of cheese metagenomes (Walsh et al., 2020) suggesting the possibility that novel species of *Psychrobacter* and *Vibrio* may exist in the cheese environment. It would be of value to compare the similarity between the unclassified MAGs observed between the two studies. It should be noted, however, that HQMAGs are not guaranteed to be composed of a single strain (Setubal, 2021), which could possibly lead to improper assignment at the species level. Therefore, further detection of the novel and unique species observed in this study is warranted before they should be considered a confirmed part of the cheese microbiome.

5.5 Conclusion

Functional profiling of the cheese metagenome and constructed MAGs was conducted to identify potential mechanisms leading to differential growth of *L. monocytogenes* in SRC. The RuMP and

GLUCUROCAT pathways may be associated with the inhibition of *L. monocytogenes*, possibly due to the depletion of nutrients inside the cheese microcosm, but a clear mechanism was not apparent. Bacteriocin-encoding genes were widespread throughout the cheese metagenome, but no statistically significant association with antilisterial activity was observed. A small selection of bacteriocin-encoding genes was, however, only identified in a single cheese where little *L. monocytogenes* growth was observed. Future research should target the bacteria producing these bacteriocins to determine their roles in the inhibition of *L. monocytogenes*.

Chapter 6: Conclusion and Future Directions

6.1 Conclusions

Listeria monocytogenes is a ubiquitous foodborne pathogen with the ability to grow at refrigerated temperatures and a higher risk of mortality relative to other common foodborne pathogens. *L. monocytogenes* poses a special risk of foodborne illness in soft ripened cheeses (SRC) due to a favourable pH and a_w compared to other cheeses, and several outbreaks of listeriosis related to soft cheeses globally highlight the need to control the growth of *L. monocytogenes* in SRC. The biocontrol of *L. monocytogenes* in soft cheeses has been investigated using a variety of mechanisms, including the addition of bacteriocins, bacteriocin-producing cultures, and bacteriophage to the cheeses, but with limited success throughout the shelf life of the cheeses; especially if contamination occurs after ripening. Therefore, novel techniques are still required to control the growth of *L. monocytogenes* in SRC. Despite the accepted risk of *L. monocytogenes* growth in SRC, many of these cheeses have shown partial or complete inhibition of the pathogen *in situ*, and variations in the cheese microbiota has been suggested as a possible mechanism for this differential growth of *L. monocytogenes*. Therefore, the primary objective of my PhD dissertation was to investigate how variations in the natural microbiota of SRC affect the growth of *L. monocytogenes* in the cheese.

An investigation into the microbial communities throughout the cheese production continuum identified a variety of microbial taxa in the finished cheeses that were not intentionally added by the cheesemaker. Many of these taxa were initially observed at the dairy farm environment, while some were associated with the production environment (*e.g.*, aging shelves). These findings demonstrated the effect that the dairy farm and processing environments can have on the cheese

microbiota and suggested that the microbial community of cheeses differs between locations and producers.

The growth of *L. monocytogenes* was investigated in SRC (n = 43) from various producers and was found to vary significantly between cheeses. Over 12 days of incubation at 8°C, inoculated *L. monocytogenes* (3 log CFU/g) increased an average of 2.5 ± 1.2 log CFU, but that increase ranged from 0 to 5.4 log CFU across the 43 cheeses. Of note was that SRC produced with raw milk allowed, on average, less *L. monocytogenes* growth than cheeses produced using pasteurized milk. Analysis of physicochemical characteristics and viable microbial plate counts of the cheeses identified a_w and total aerobic microbial count (TAMC) as being significantly positively and negatively correlated with the growth of *L. monocytogenes*, respectively. Further, TAMC was higher in raw milk cheeses, suggesting a possible mechanism for the observed differences in *L. monocytogenes* growth between raw and pasteurized milk cheeses. The TAMC and a_w of cheeses only explained 26% of the variation in *L. monocytogenes* growth across cheeses, however, suggesting that other factors, such as the taxonomic and functional profiles of the cheese microbiome, may also affect the growth of *L. monocytogenes* in SRC.

The taxonomic profile of the microbial communities in the cheeses was investigated using a combination of 16S rRNA targeted amplicon sequencing (n = 39) and shotgun metagenomic sequencing (n = 15). The species richness of the cheese microbiota, measured as the total number of observed operational taxonomic units (OTUs) was significantly negatively correlated with the growth of *L. monocytogenes* and was independent of TAMC. Since high species richness was also observed in cheeses with relatively high *L. monocytogenes* growth, it is likely that the association

between *L. monocytogenes* inhibition and species richness is due to an increased likelihood of antilisterial species being present rather than just the number of unique species. Four bacterial species were identified that were differentially abundant relative to the growth of *L. monocytogenes*. The relative abundance of *Streptococcus thermophilus* was positively correlated with the growth of *L. monocytogenes* growth in cheeses, while the relative abundances of *Brevibacterium aurantiacum* and two unidentified species of *Lactococcus* were negatively correlated with *L. monocytogenes* growth. The inclusion of *S. thermophilus* as a starter culture is more common in the production of "stabilized" SRC, which may indicate that SRC made using the stabilization process (*i.e.*, industrially produced SRC) are more favourable to the growth of *L. monocytogenes* than traditionally produced SRC.

Finally, functional profiling of the cheese metagenomes (n = 15) was used to identify genes and metabolic pathways that are associated with the inhibition of *L. monocytogenes* in SRC. Two metabolic pathways were identified that were significantly negatively correlated with *L. monocytogenes* growth in the cheeses: a formaldehyde oxidation pathway and a β-D-glucuronosides degradation pathway. No definitive mechanism was apparent for the association between these metabolic pathways and the inhibition of *L. monocytogenes* growth, but I hypothesize that it may be related to nutrient depletion in the cheese environment. The presence of the gene encoding the bacteriocin linocin M18 was observed in all metagenomically assembled genomes (MAGs) that were classified as *Brevibacterium aurantiacum* and may explain that species' correlation with *L. monocytogenes* inhibition. A variety of other bacteriocin-encoding genes were present solely in cheeses with no or low growth of *L. monocytogenes*, but since they

were all identified in one or two cheeses, no statistically significant associations could be drawn between their occurrence and the inhibition of *L. monocytogenes*.

In summary, multiple characteristics of the cheeses were associated with the inhibition of *L. monocytogenes*, including physicochemical characteristics (*i.e.*, a_w); microbial characteristics (*i.e.*, TAMC); and the presence of specific species, metabolic pathways, and possibly bacteriocin-encoding genes. None of these observed characteristics, however, was a universal predictor of *L. monocytogenes* inhibition, suggesting that each no and low growth cheese may have been inhibitory in its own unique way. The future of this research should therefore focus on combining these inhibitory traits to develop cheeses that are both inhibitory to *L. monocytogenes*, but still maintain a desired sensory quality in the finished product.

In addition to the identification of antilisterial features of the cheese microbiome, 125 MAGs were constructed, including 96 high quality MAGs (HQMAGs). Species classification of the HQMAGs identified four species not previously identified in cheese: *Lactococcus laudensis*, *Glutamicibacter arleyensis*, *Spororsarcina psychrophila*, and *Morganella psychrotolerans*. Addionally, fourteen HQMAGs of the genera *Psychrobacter*, *Vibrio*, *Sporosarcina*, *Carnobacterium*, *Fusobacterium*, *Galactobacter*, and *Vagococcus* could not be classified at the species level, suggesting the possibility they represent novel species.

This research contributed to our understanding of the characteristics of SRC and the SRC microbiome that affect the growth of *L. monocytogenes* in the cheeses, specifically post-ripening, at the retail level. Previous research in this area has focused primarily on the growth of *L.*

monocytogenes during cheese production and ripening, but to the best of my knowledge, this is the first study investigating a large collection of SRC collected from retail. Additionally, this is only the second study to make use of targeted amplicon sequencing and the first to use shotgun metagenomic sequencing to investigate the SRC microbiome with respect to the growth potential of *L. monocytogenes*. The results of this study will be valuable to the development of cheesemaking techniques that prevent the growth of *L. monocytogenes* in the finished cheeses. Finally, the assembly of 125 MAGs from the cheese metagenomes, including species not previously identified in cheese, and multiple potentially novel species, are a valuable addition to the overall understanding of the cheese microbiome.

6.2 Future directions

The research provided here can be a steppingstone for future studies related to the growth of *L. monocytogenes* in SRC. The first future study would be the isolation of specific microorganisms from SRC that are inhibitory to *L. monocytogenes*. During the current study, up to 96 colonies from each of the viable plate counts were collected for each cheese with below average levels of *L. monocytogenes* growth. This collection of isolates should be screened for their ability to inhibit *L. monocytogenes in vitro*. Any isolates that are inhibitory to *L. monocytogenes* should be screened for the production of bacteriocins, and their genomes should be screened for the presence of the metabolic pathways identified as differentially abundant in this current study. Through this screening process, it might be possible to experimentally test the microbiome associations identified in this dissertation.

Once a collection of antilisterial cheese-associated bacteria is identified, research should focus on the development of SRC possessing these isolates. This study should investigate whether these antilisterial isolates establish themselves in the cheese when they are added as a starter culture, and whether they provide added inhibition to *L. monocytogenes* in finished cheeses relative to SRC produced without the addition of these isolates. The described project would hopefully result in the development of a starter-culture of SRC production that is inherently inhibitory to *L. monocytogenes* in the finished cheese.

Another future study would be to investigate whether the positive correlation between the relative abundance of *S. thermophilus* and *L. monocytogenes* growth is the result of mutualism between the two species, or if the increased pathogen growth is due to the stabilization process in industrial SRC production. This study would require the production of SRC following both the traditional method and the stabilization method, as well as the production of stabilized SRC without the addition of *S. thermophilus*. The growth of *L. monocytogenes* in these cheeses could then be compared following the protocol described in Chapter 3 of this dissertation. The results of this described study would be of value to both regulators and large cheese manufactures since it could show that the risk of *L. monocytogenes* in SRC is affected by the production process.

A study should also be conducted to further probe the correlations observed in the functional analysis, such as with metabolic pathways and bacteriocin production. One of the limitations of DNA-based analysis is that the presence of a gene does not necessarily mean that it is expressed, therefore, experiments that demonstrate expression and metabolite production are necessary. First, RNA-based analysis (*i.e.*, RNA-seq or RT-PCR) can be used to compare gene expression across

cheeses. Second, metabolomics and/or proteomic can be used to measure the concentration of the end-point metabolites in these cheeses. These two experiments would provide confirmatory evidence that the identified pathways and/or metabolites are active within the cheeses, and that the activity is associated with *L. monocytogenes* inhibition.

Finally, the sequence data from this current study will be shared with the wider scientific community for future analysis. As the library of known genes, metabolic pathways, and antimicrobial peptides increases, future analysis of these data may identify additional genomic features associated with *L. monocytogenes* inhibition in the cheese matrix.

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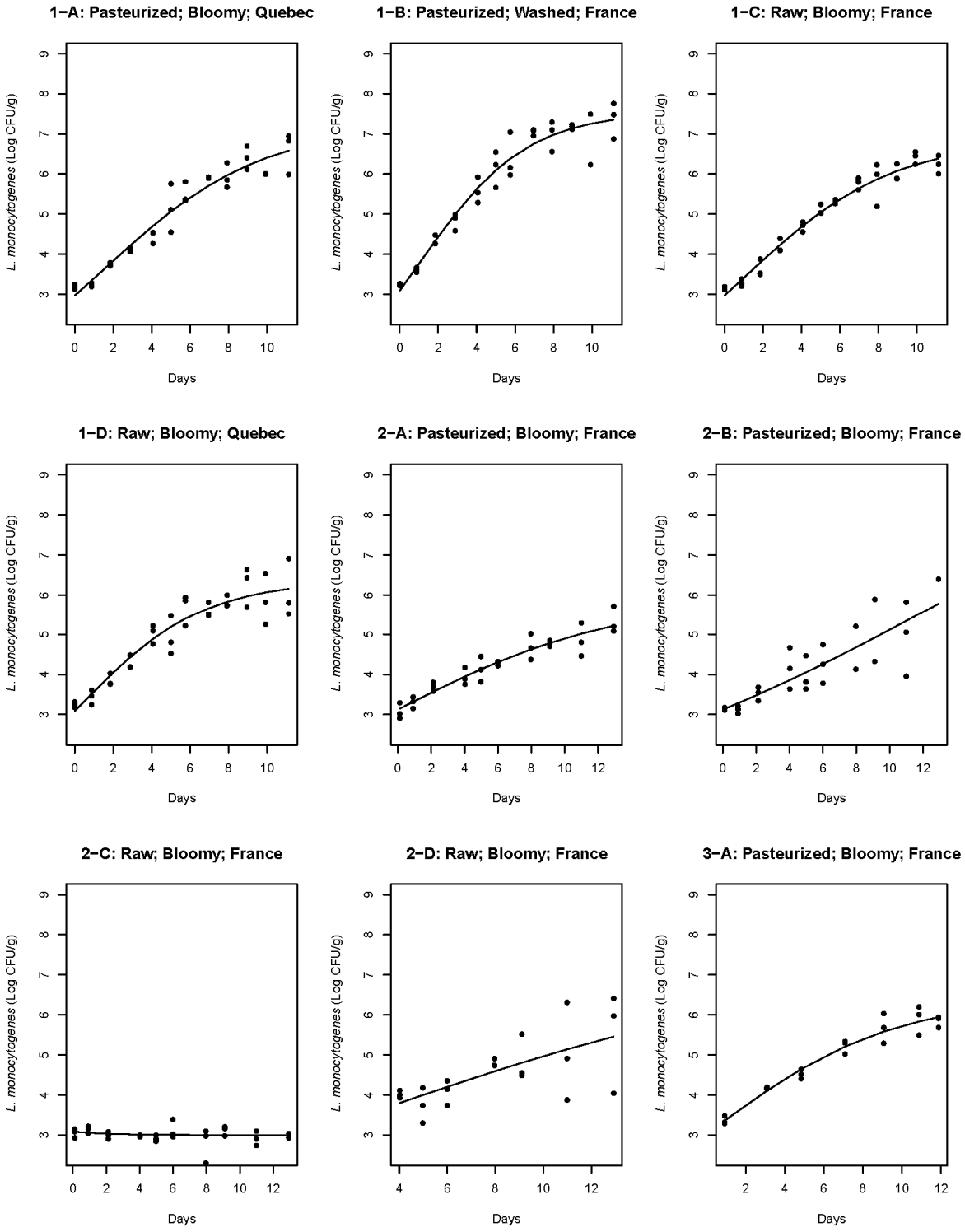
Appendix A: Chapter 3 Supplemental Figure

Figure 3.S1 (Continued)



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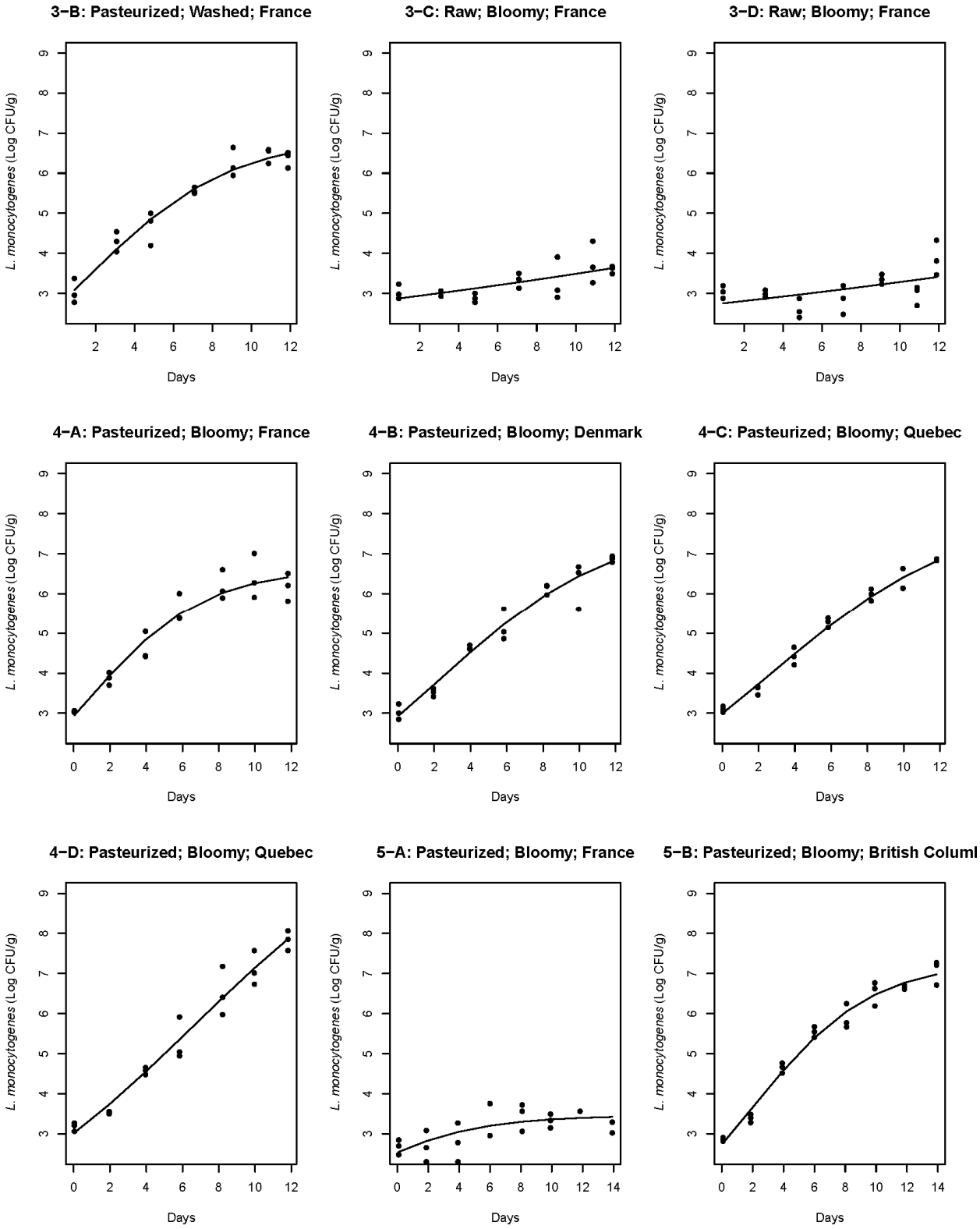

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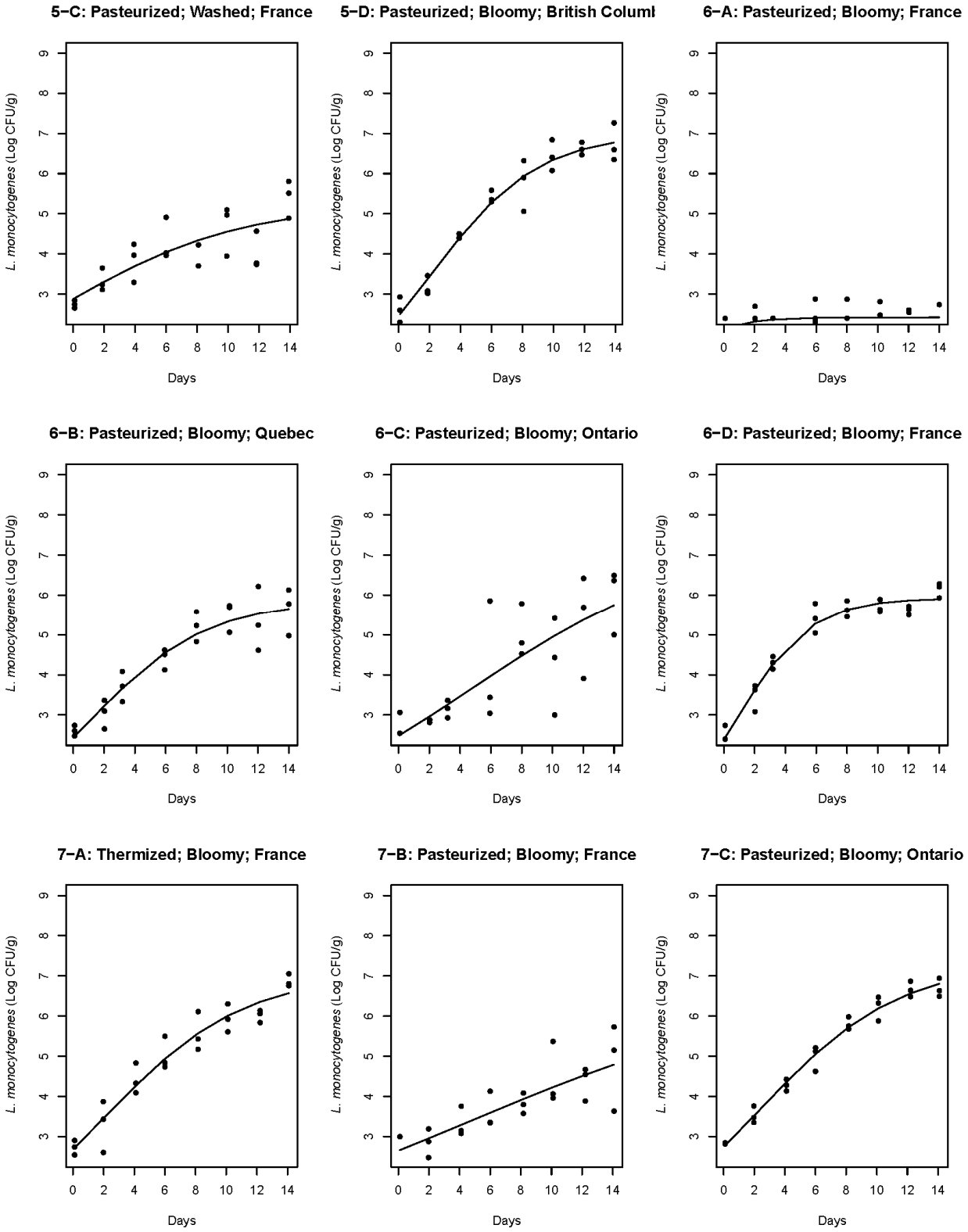

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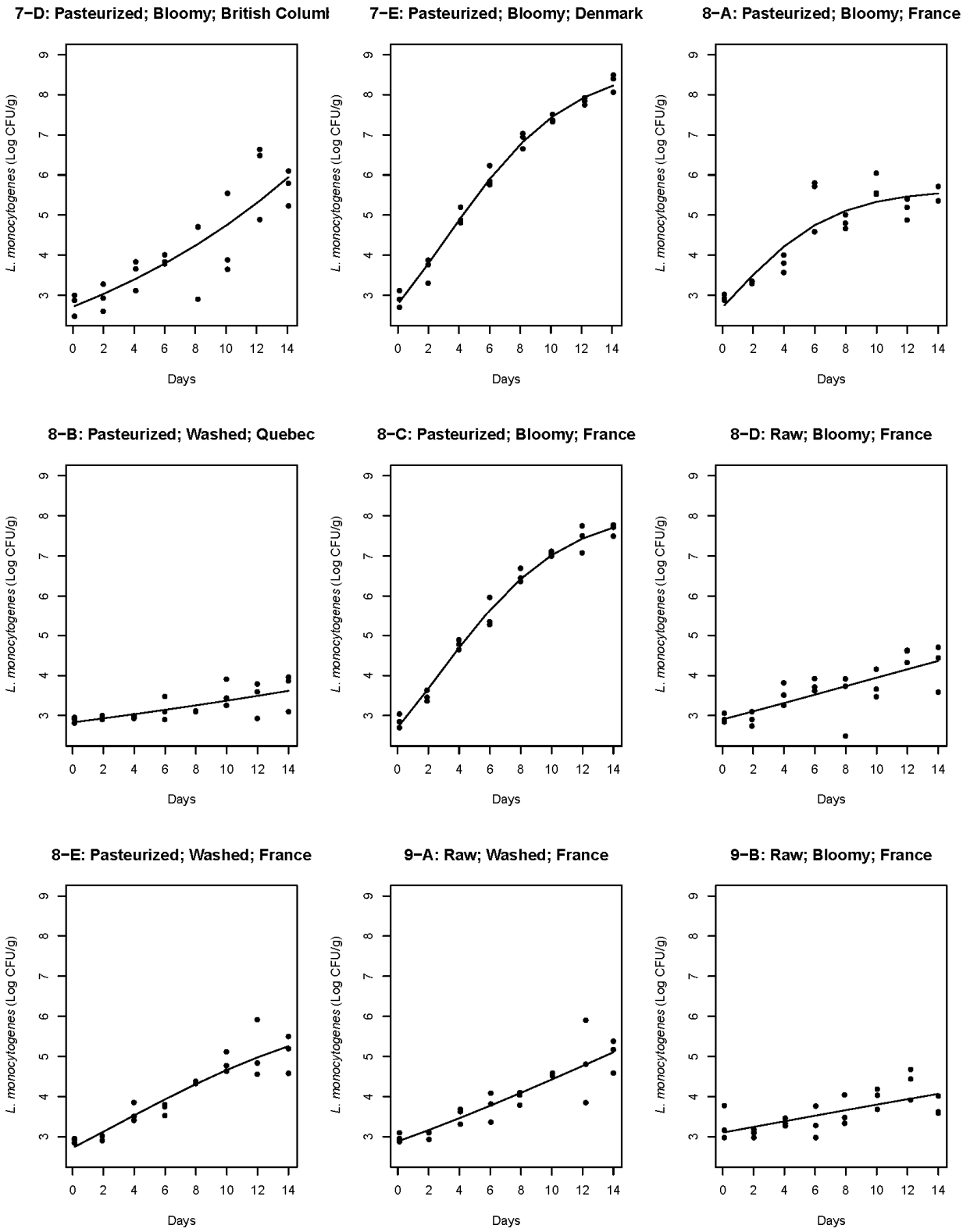

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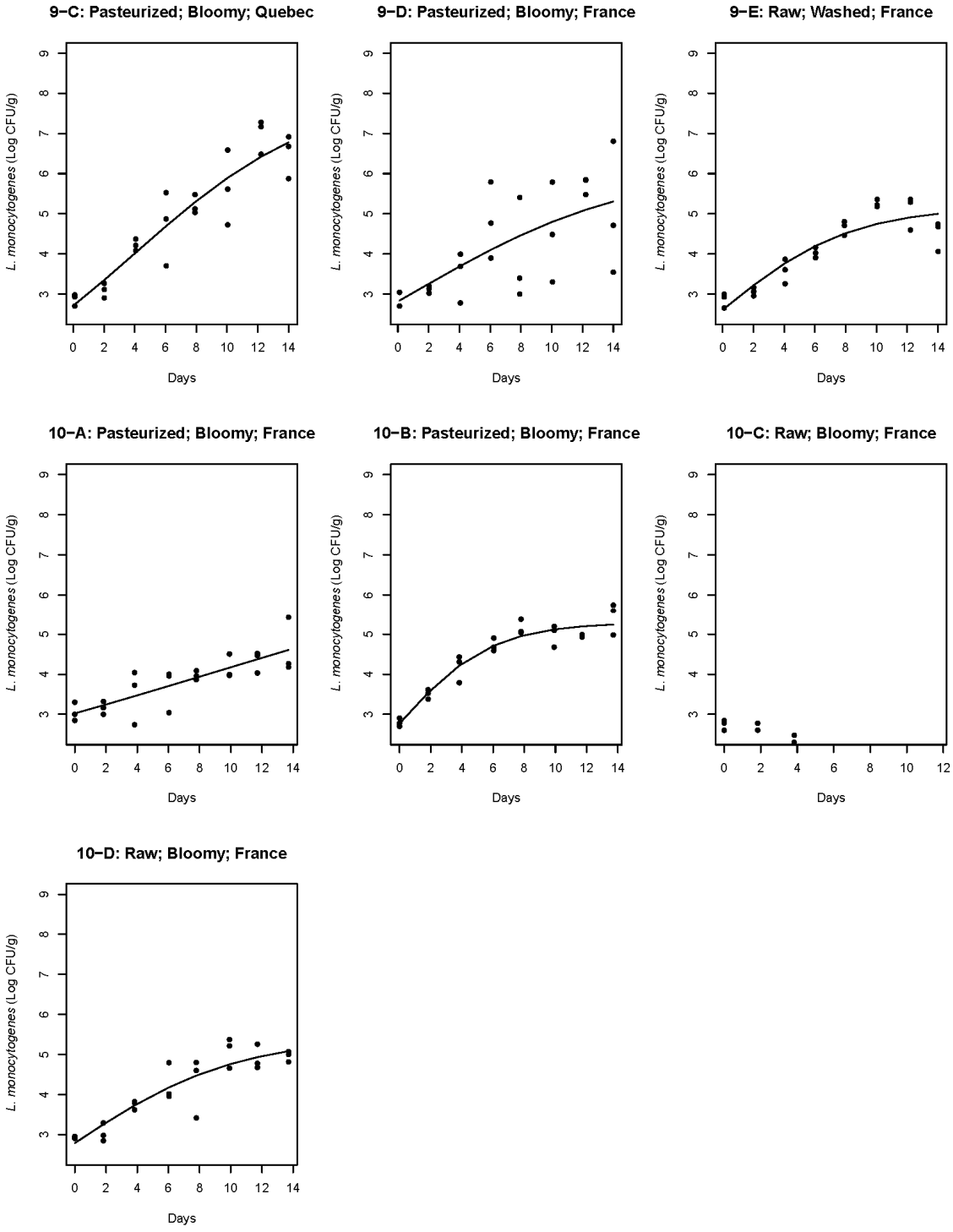


Figure 3.S1 Modelled growth curves of *L. monocytogenes* in each cheese. Models were created using logistic regression.

Appendix B: Chapter 4 Supplemental Figures

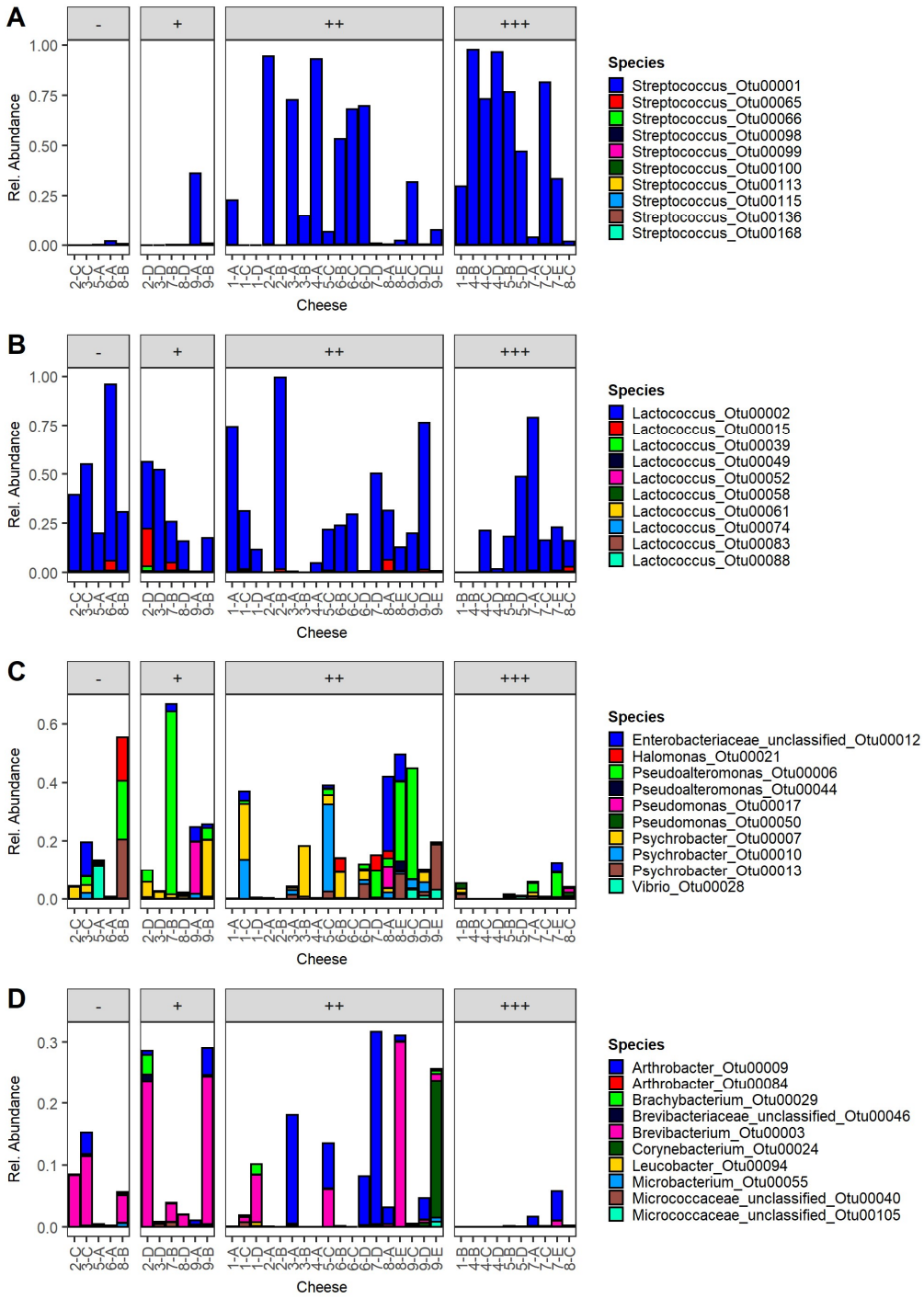


Figure 4.S1 Relative abundance of OTUs of the genera *Streptococcus* (A) and *Lactococcus* (B), and well as the Proteobacteria (C) and Actinobacteria (D) phyla. Cheeses are grouped by growth category.

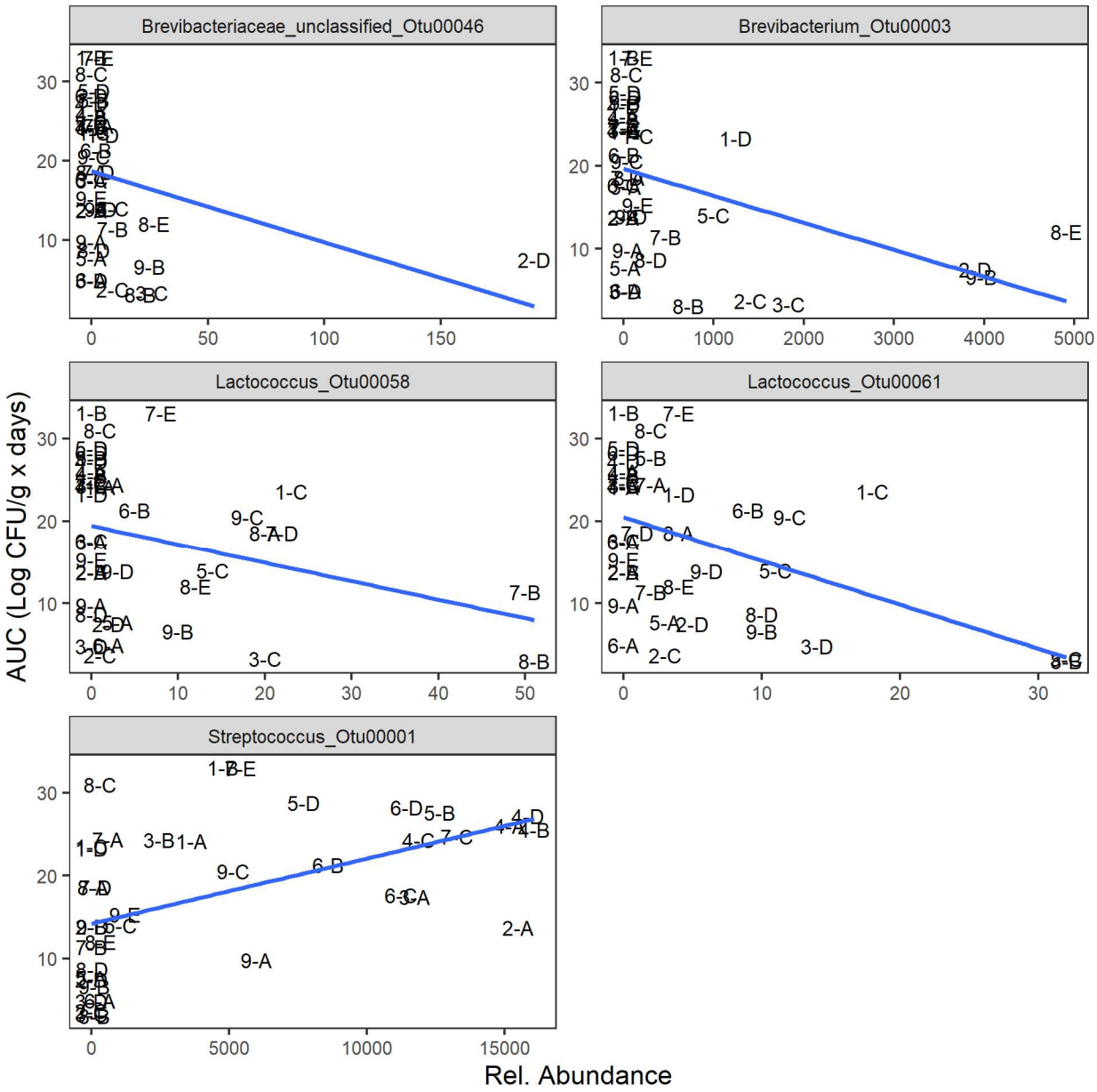
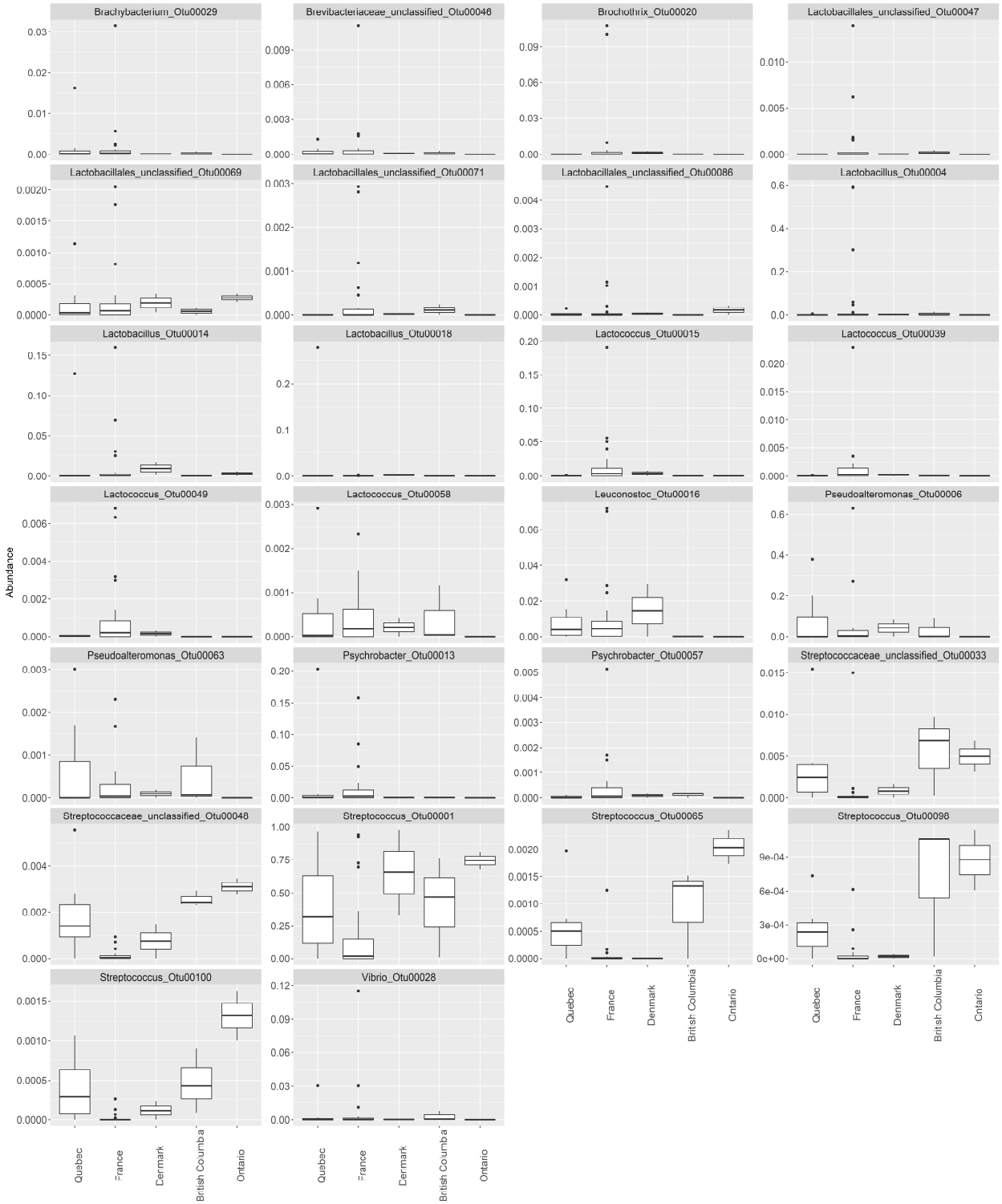


Figure 4.S2 Scatter plot and linear regression (blue line) of the growth of *L. monocytogenes vs.* the relative abundance of differentially abundant OTUs identified using the ANCOM-BC algorithm.

Figure 4.S3 (Continued)



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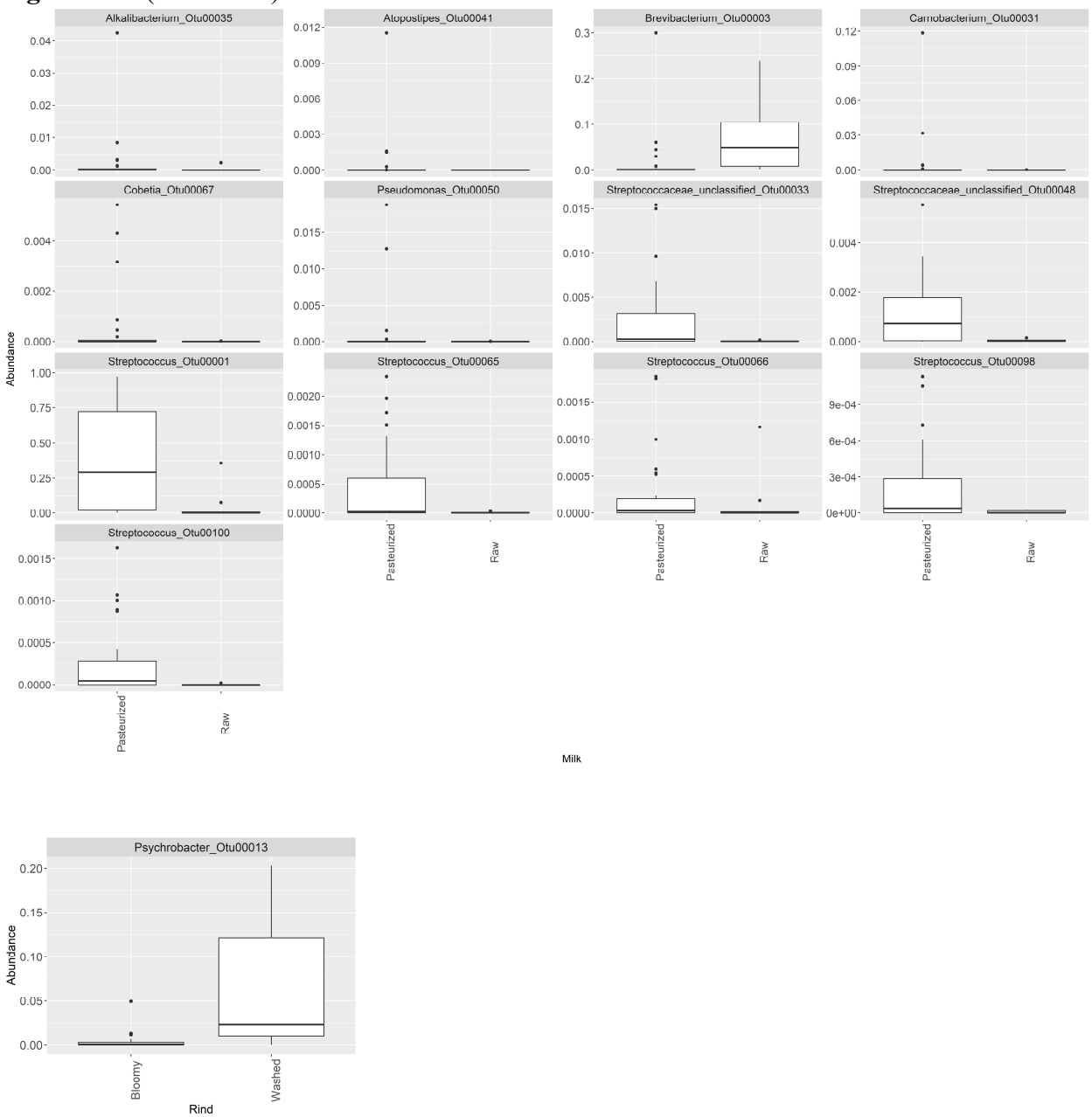
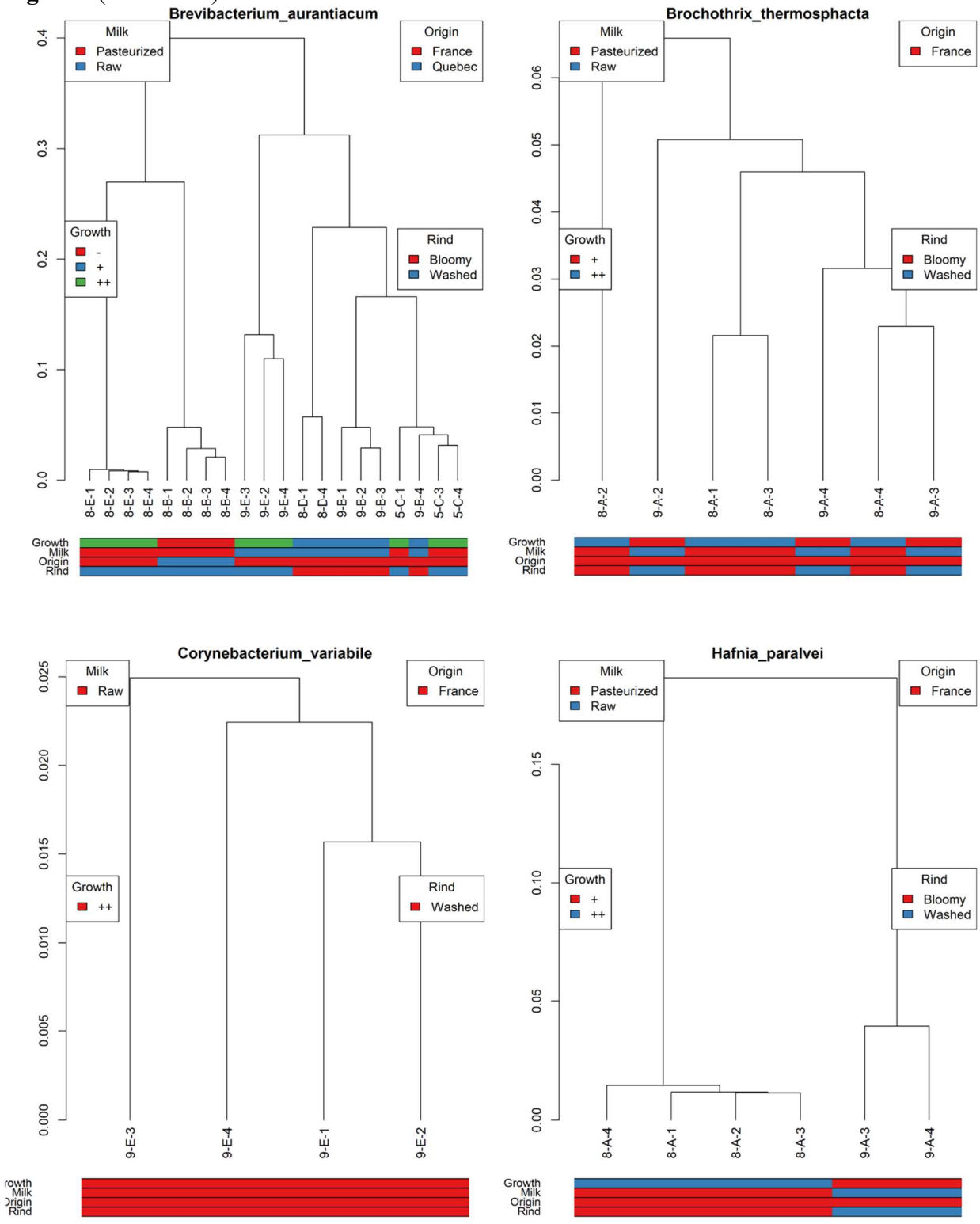


Figure 4.S3 Box plots of the relative abundance of OTUs identified as differentially abundant between cheese origin, milk treatment, and rind type**.**

Fig. 4.S4(Continued)



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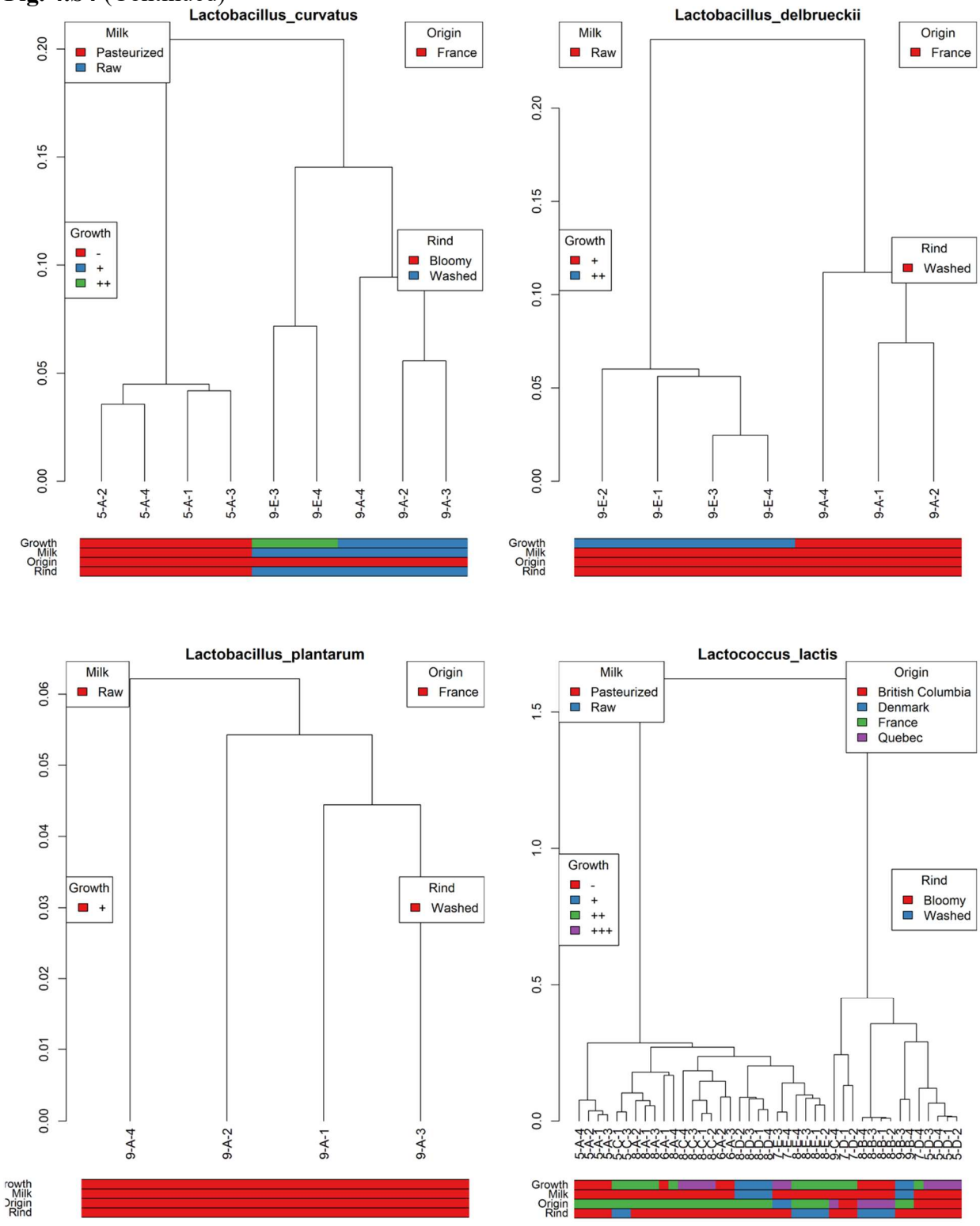

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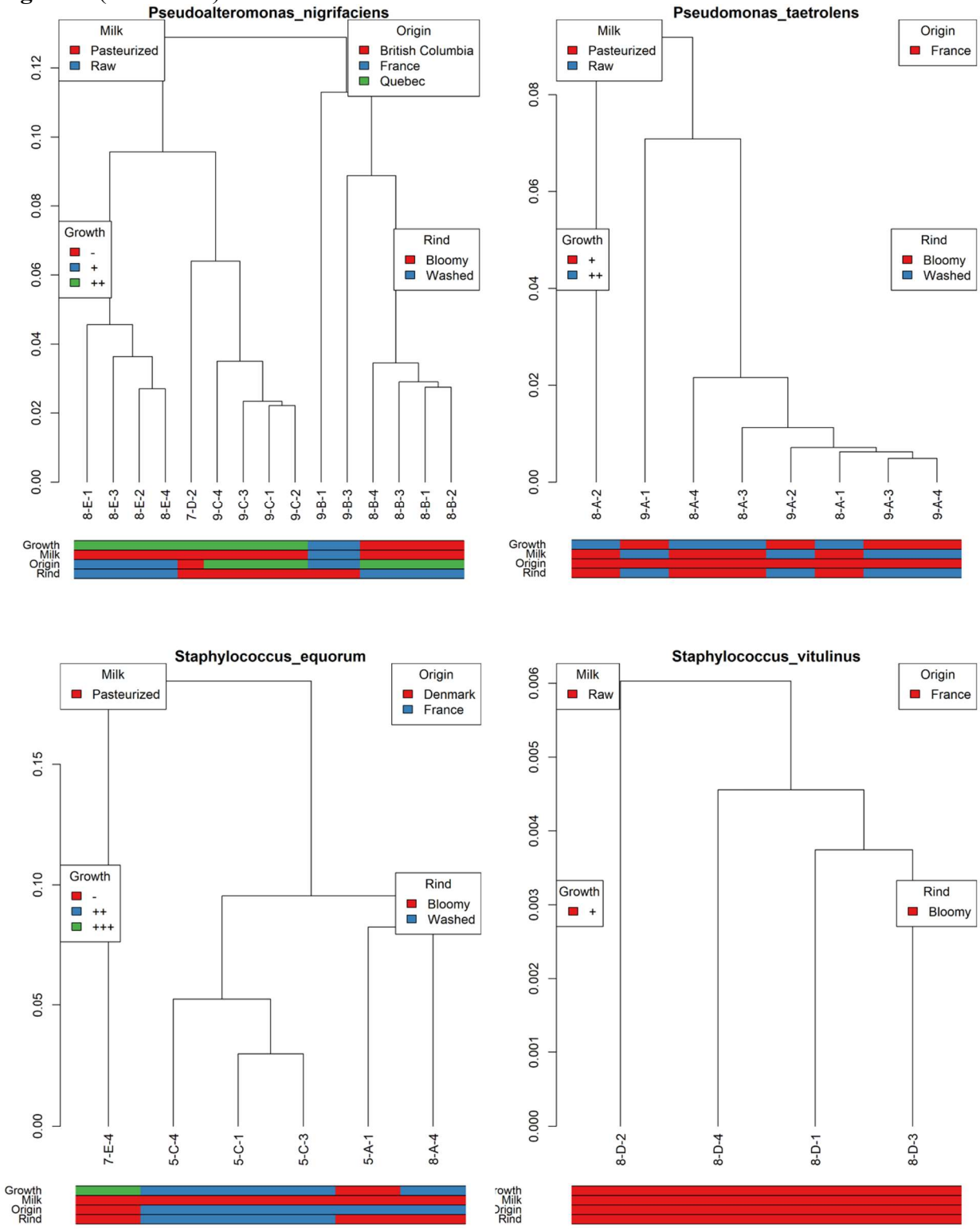

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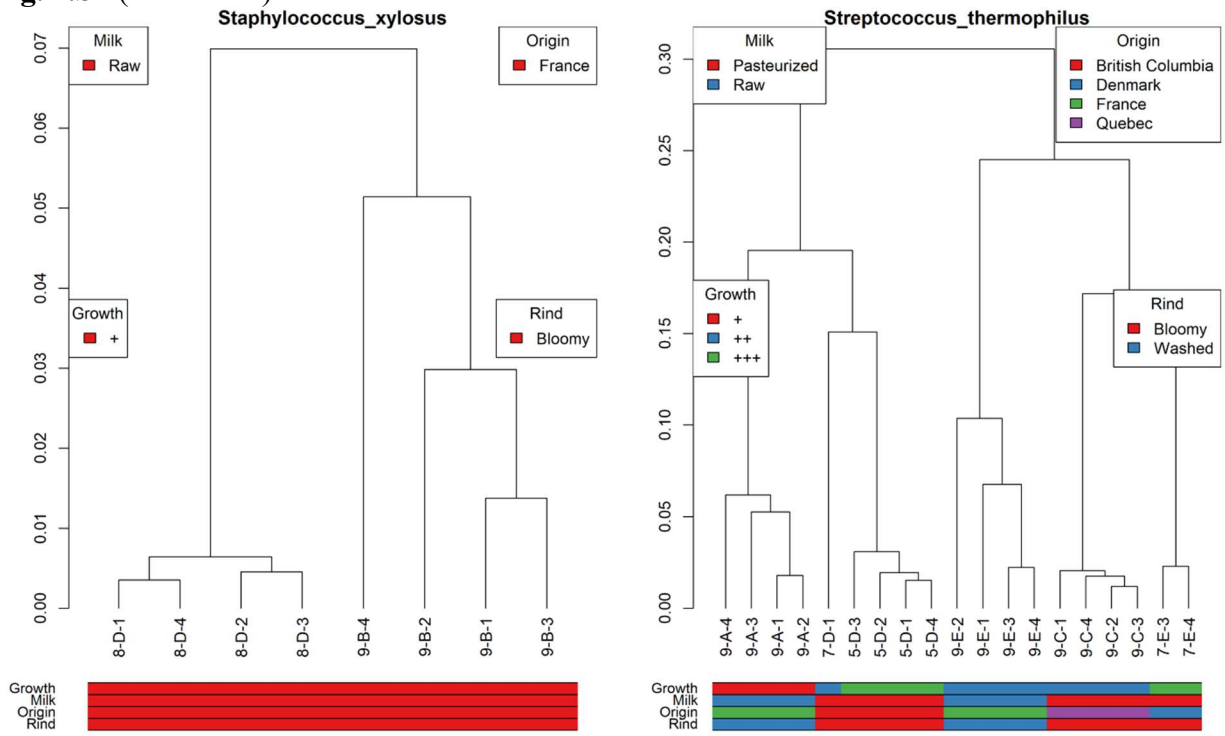

Figure 4.S4 Dendrograms of strain-level analysis of the species present at > 1% relative abundance. Strains were defined based on presence/absence of selected genes, and similarity was measured using Bray-Curtis distance.

Appendix C: Chapter 5 Supplemental Figures and Tables

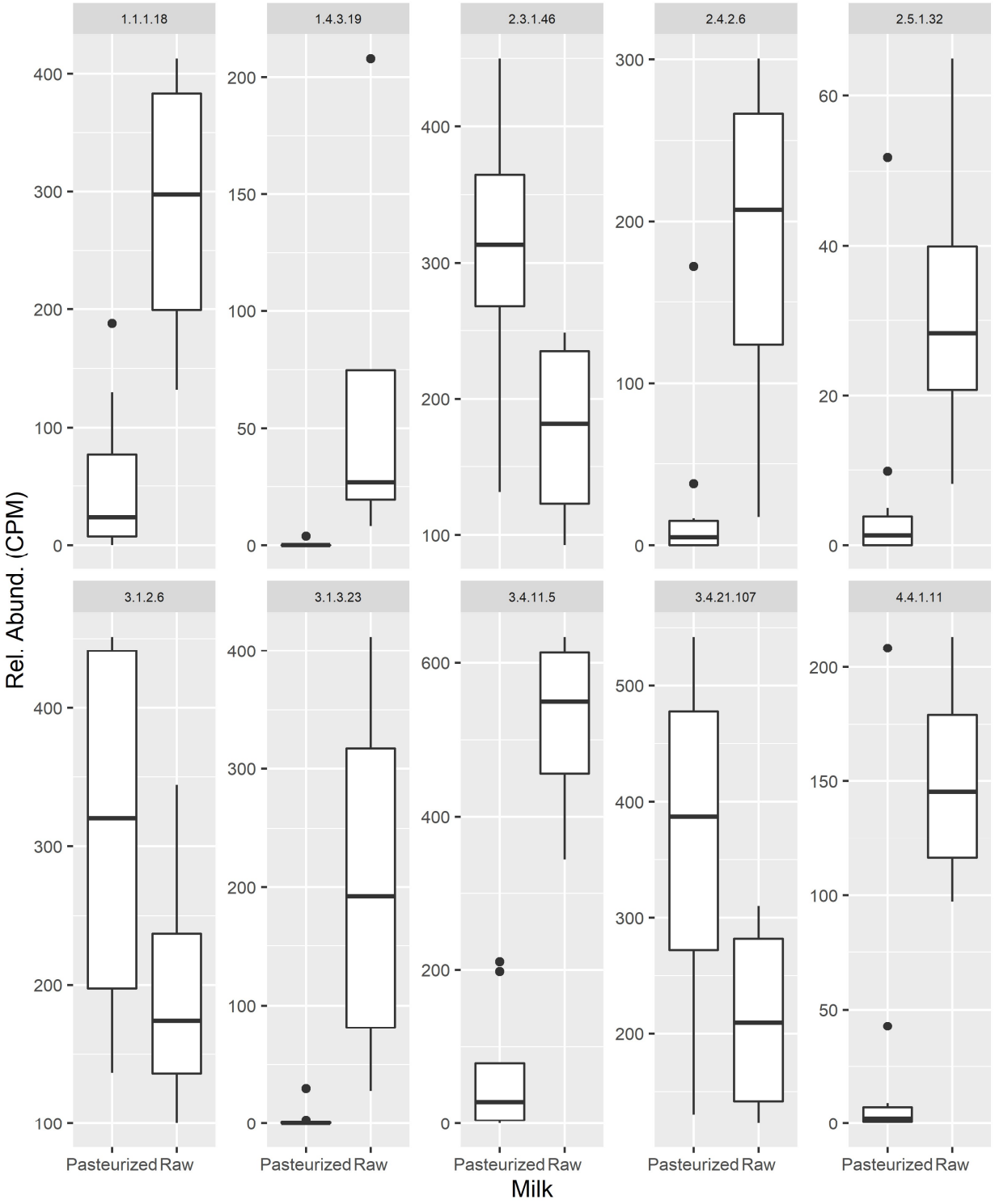

Figure 5.S1 Copies per million reads (CPM) of enzymes (EC number) that were identified as differentially abundant between cheeses produced by pasteurized or raw milk.

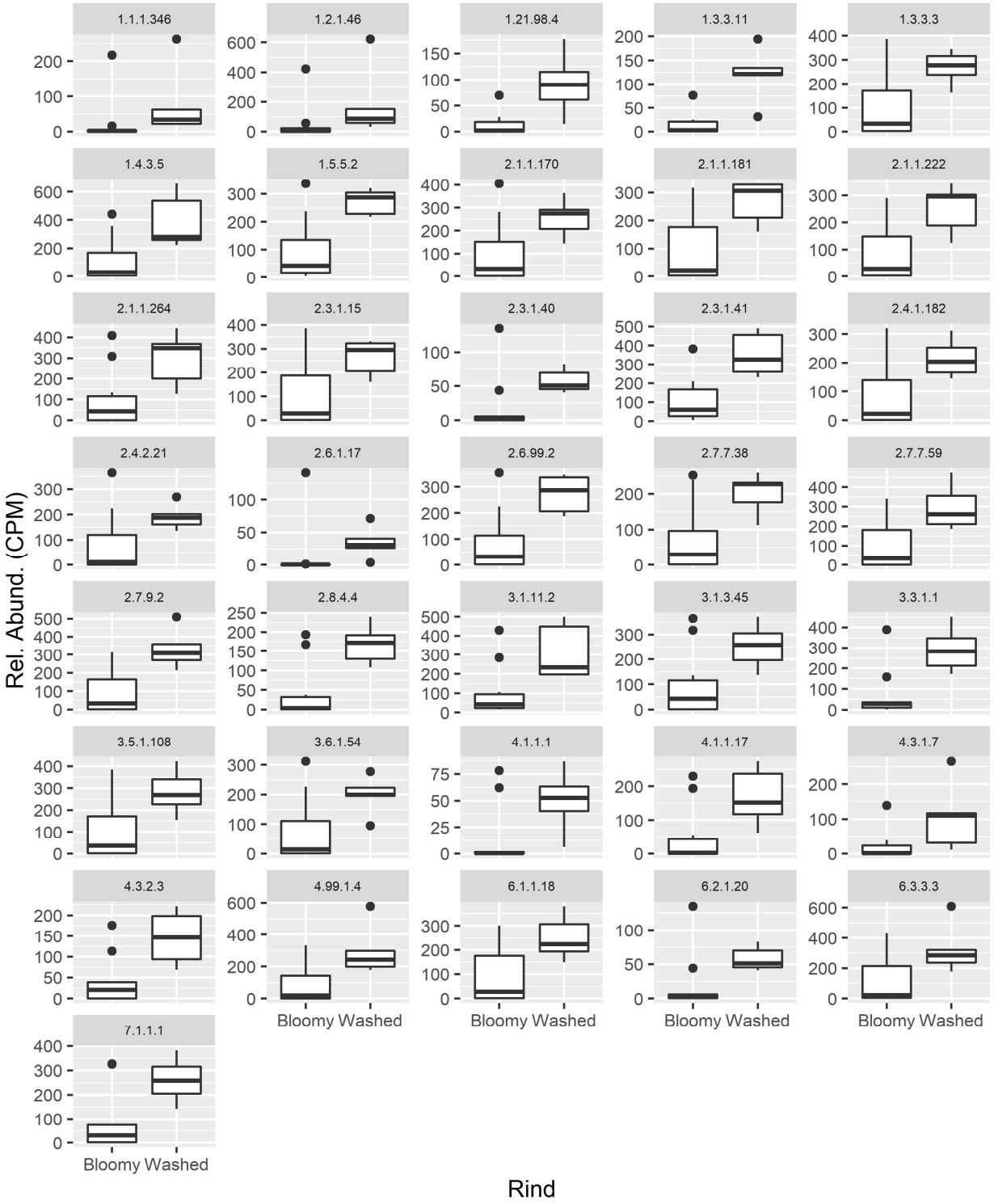

Figure 5.S2 Copies per million reads (CPM) of enzymes (EC number) that were identified as differentially abundant between bloomy and washed rind cheeses.

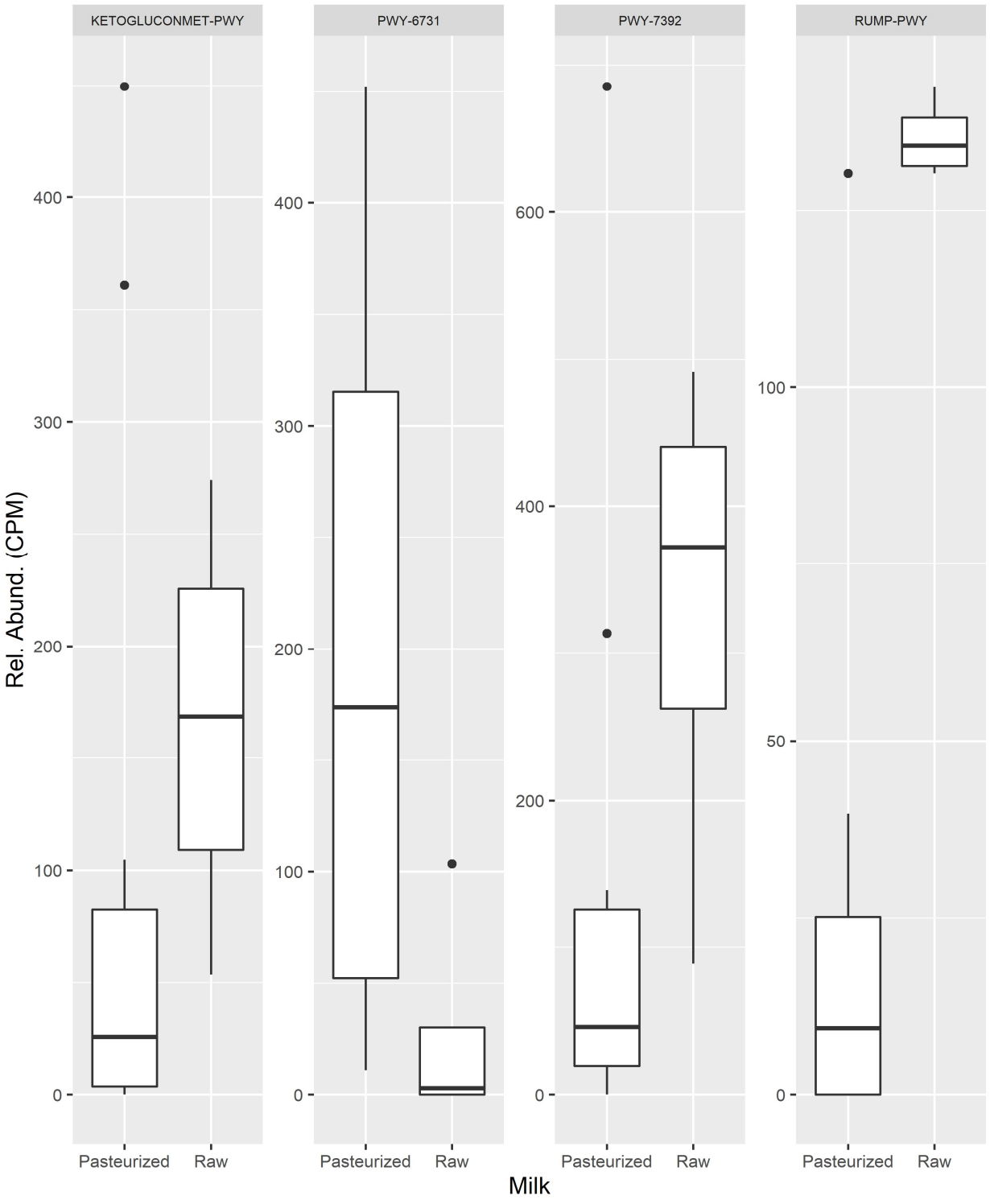

Figure 5.S3 Copies per million reads (CPM) of MetaCyc pathways that were identified as differentially abundant between cheeses produced by pasteurized or raw milk.

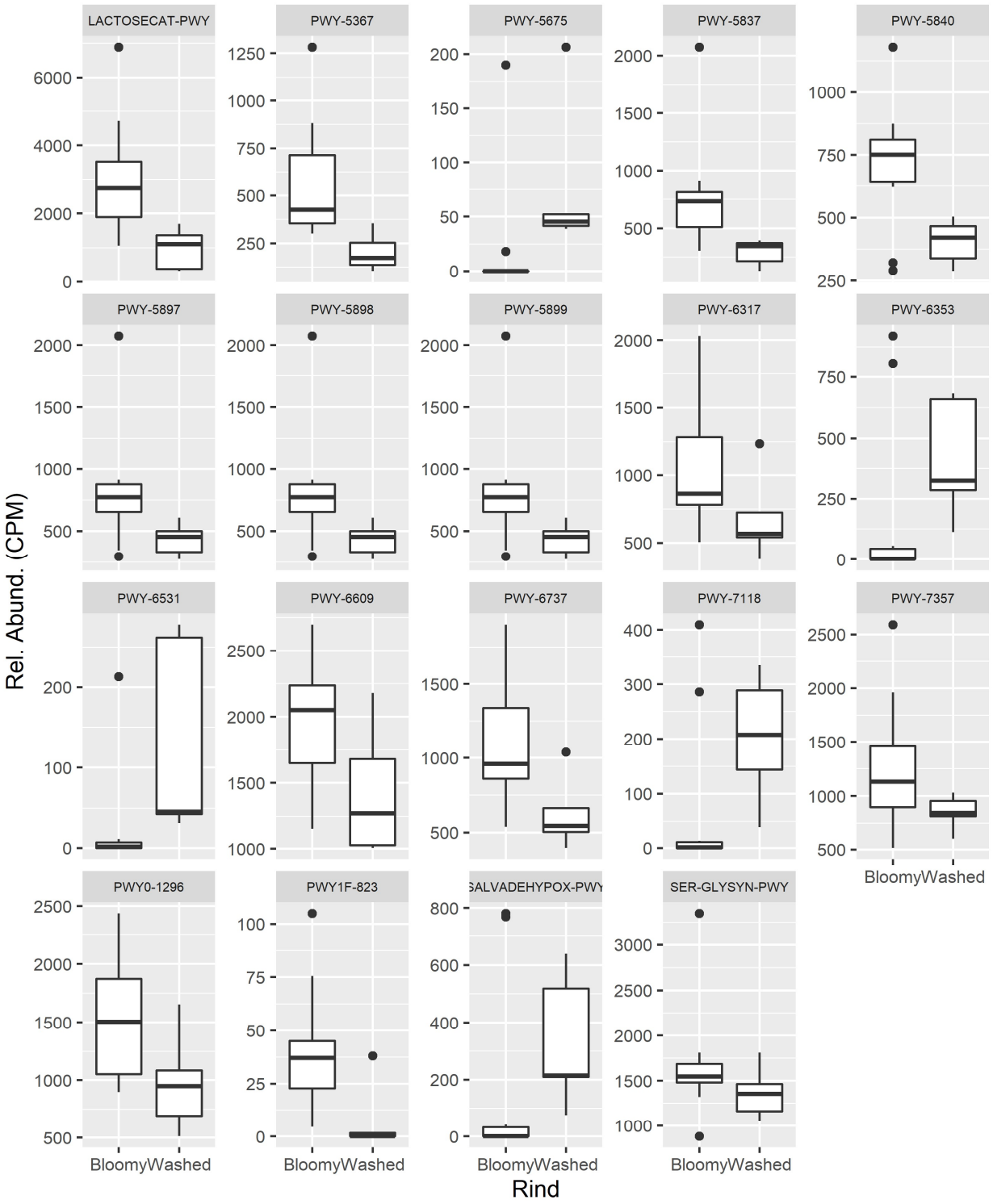

Figure 5.S4 Copies per million reads (CPM) of MetaCyc pathways that were identified as differentially abundant between bloomy and washed rind cheeses.

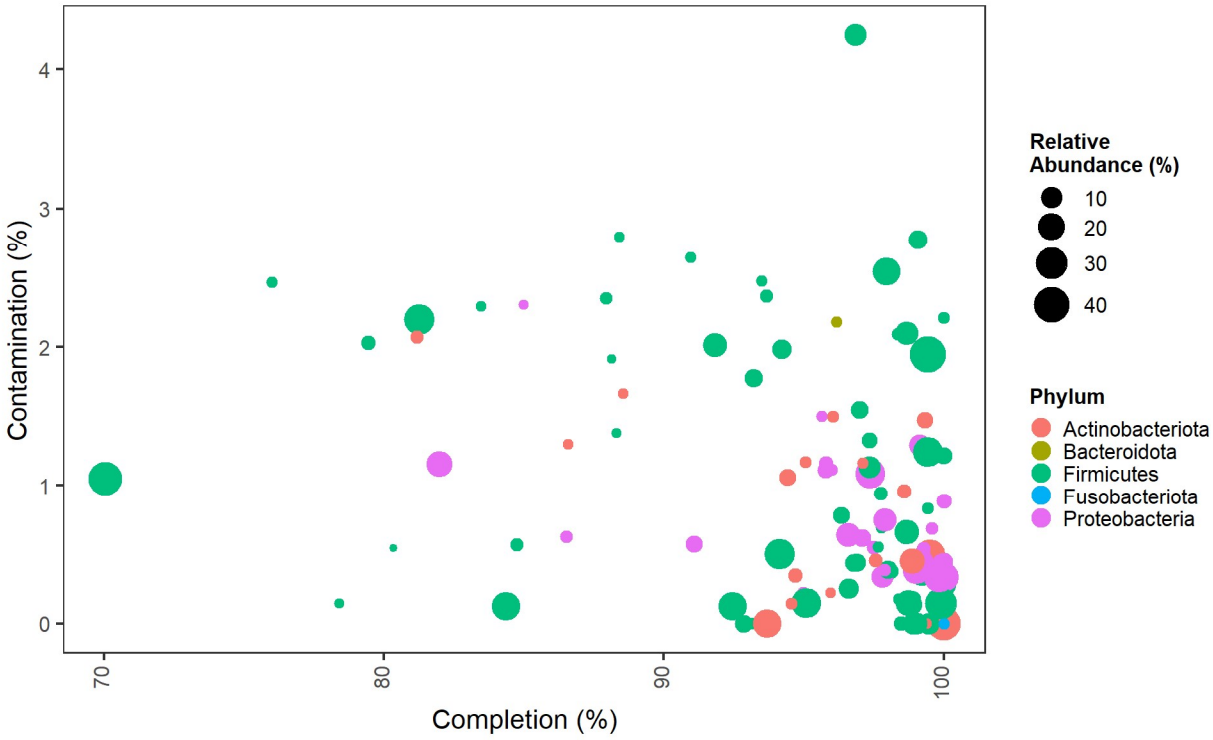

Figure 5.S5 – Scatterplot of contamination vs. completion of identified MAGs from cheeses. The size of the points is relative to the relative abundance within their respective metagenome, and they have been coloured based on the classified Phylum.

Table 5.S1 Identified MAGs from cheeses.

Cheese_ID	MAG	Completeness (%)	Contamination (%)	Phylum	Species	Reference	Reference ANI
5-A	bin.1	99.2	0.4	*Firmicutes*	*Lactococcus lactis*	GCF_002078765.2	99.67
5-A	bin.2	98.9	0.0	*Firmicutes*	*Latilactobacillus curvatus*	GCF_004101845.1	98.75
5-A	bin.3	96.4	0.8	*Firmicutes*	*Carnobacterium sp*	N/A	NA
5-A	bin.4	80.3	0.5	*Firmicutes*	*Marinilactibacillus psychrotolerans*	GCF_900101525.1	98.11
5-A	bin.5	91.1	0.6	*Proteobacteria*	*Vibrio sp*	GCF_009601765.2	80.65
5-A	bin.6	88.2	1.9	*Firmicutes*	*Carnobacterium mobile*	GCF_000744825.1	97.99
5-A	bin.7	97.8	0.7	*Firmicutes*	*Staphylococcus equorum*	GCF_900458565.1	99.34
5-A	bin.8	95.8	1.1	*Proteobacteria*	*Vibrio casei*	GCF_002218025.2	99.25
5-A	bin.9	88.3	1.4	*Firmicutes*	*Lactococcus laudensis*	GCF_013449735.1	98.39
5-C	bin.1	96.9	4.2	*Firmicutes*	*Lactococcus lactis*	GCF_002078765.2	99.3
5-C	bin.2	100.0	0.0	*Actinobacteriota*	*Brevibacterium aurantiacum*	GCF_900169065.1	98.03
5-C	bin.3	99.4	0.0	*Firmicutes*	*Staphylococcus equorum*	GCF_900458565.1	99.56
5-C	bin.4	83.5	2.3	*Firmicutes*	*Leuconostoc pseudomesenteroides*	GCF_000297375.1	99.92
5-C	bin.5	97.1	0.6	*Proteobacteria*	*Psychrobacter* sp002810365	GCF_002810365.1	95.8
5-C	bin.6	94.7	0.3	*Actinobacteriota*	*Glutamicibacter ardleyensis*	GCA_014644555.1	97.89
5-C	bin.7	84.8	0.6	*Firmicutes*	*Lactococcus laudensis*	GCF_013449735.1	98.4
5-C	bin.8	82.0	1.2	*Proteobacteria*	*Psychrobacter glacincola*	GCF_001411745.2	97.02
5-C	bin.9	87.9	2.4	*Firmicutes*	*Carnobacterium sp*	N/A	NA
5-D	bin.1	84.4	0.1	*Firmicutes*	*Lactococcus lactis*	GCF_900099625.1	98.73
5-D	bin.2	98.4	2.1	*Firmicutes*	*Marinilactibacillus psychrotolerans*	GCF_900101525.1	97.57
5-D	bin.3	70.1	1.0	*Firmicutes*	*Streptococcus thermophilus*	GCF_010120595.1	98.78
6-A	bin.1	81.3	2.2	*Firmicutes*	*Lactococcus lactis*	GCF_002078765.2	99.56
6-A	bin.2	98.1	0.4	*Firmicutes*	*Lactococcus laudensis*	GCF_013449735.1	99.57
7-D	bin.1	93.5	2.5	*Firmicutes*	*Streptococcus thermophilus*	GCF_010120595.1	98.57
7-D	bin.2	99.7	0.3	*Proteobacteria*	*Pseudoalteromonas nigrifaciens*	GCF_002221505.1	98.68
7-D	bin.3	92.5	0.1	*Firmicutes*	*Lactococcus lactis*	GCF_900099625.1	98.62
7-D	bin.4	99.5	0.5	*Actinobacteriota*	*Glutamicibacter ardleyensis*	GCA_014644555.1	96.96
7-D	bin.5	94.1	0.5	*Firmicutes*	*Lactococcus lactis*	GCF_002078765.2	97.7
7-D	bin.6	100.0	0.9	*Proteobacteria*	*Halomonas* sp002332255	GCF_002332255.1	99.46

Table 5.S1 (Continued)

Cheese_ID	MAG	Completeness (%)	Contamination (%)	Phylum	Species	Reference	Reference ANI
7-E	bin.1	81.2	2.1	*Actinobacteriota*	*Glutamicibacter ardleyensis*	GCA_014644555.1	96.92
7-E	bin.2	98.8	0.1	*Firmicutes*	*Streptococcus thermophilus*	GCF_010120595.1	98.51
7-E	bin.3	98.0	2.5	*Firmicutes*	*Lactococcus lactis*	GCF_002078765.2	99.5
7-E	bin.4	98.9	0.1	*Firmicutes*	*Staphylococcus equorum*	GCF_001747895.1	97.48
7-E	bin.5	79.4	2.0	*Firmicutes*	*Leuconostoc pseudomesenteroides*	GCF_000297375.1	99.92
8-A	bin.10	98.5	0.0	*Firmicutes*	*Vagococcus salmoninarum*	GCF_003987495.1	97.36
8-A	bin.11	99.4	0.8	*Firmicutes*	*Staphylococcus equorum*	GCF_900458565.1	99.6
8-A	bin.12	91.8	2.0	*Firmicutes*	*Lactococcus lactis*	GCF_002078765.2	99.43
8-A	bin.1	100.0	0.2	*Firmicutes*	*Leuconostoc pseudomesenteroides*	GCF_000297375.1	99.96
8-A	bin.2	98.6	1.0	*Actinobacteriota*	*Glutamicibacter ardleyensis*	GCA_014644555.1	97.28
8-A	bin.3	98.9	0.0	*Firmicutes*	*Brochothrix thermosphacta*	GCF_000620985.1	98.88
8-A	bin.4	100.0	0.4	*Proteobacteria*	*Halomonas* sp002332255	GCF_002332255.1	98.02
8-A	bin.5	99.2	0.4	*Proteobacteria*	*Hafnia paralvei*	GCF_001655005.1	99.04
8-A	bin.6	100.0	0.4	*Proteobacteria*	*Pseudomonas taetrolens*	GCF_900475285.1	99.52
8-A	bin.7	96.9	0.4	*Firmicutes*	*Lactococcus laudensis*	GCF_013449735.1	98.48
8-A	bin.8	97.5	0.5	*Proteobacteria*	*Psychrobacter cibarius*	GCF_900016235.2	97.03
8-A	bin.9	86.5	0.6	*Proteobacteria*	*Psychrobacter sp*	N/A	NA
8-B	bin.10	99.3	1.5	*Actinobacteriota*	*Microbacterium gubbeenense*	GCF_000422745.1	98.87
8-B	bin.11	78.4	0.1	*Firmicutes*	*Leuconostoc pseudomesenteroides*	GCF_000297375.1	99.62
8-B	bin.1	93.7	2.4	*Firmicutes*	*Alkalibacterium gilvum*	GCF_900109085.1	98.99
8-B	bin.2	97.4	1.1	*Proteobacteria*	*Psychrobacter sp*	GCF_003217155.1	83.84
8-B	bin.3	97.0	1.5	*Firmicutes*	*Marinilactibacillus psychrotolerans*	GCF_900101525.1	97.88
8-B	bin.4	100.0	0.3	*Firmicutes*	*Lactococcus lactis*	GCF_900099625.1	98.44
8-B	bin.5	95.0	0.2	*Proteobacteria*	*Psychrobacter sp*	GCF_002810365.1	79.46
8-B	bin.6	88.4	2.8	*Firmicutes*	*Streptococcus thermophilus*	GCF_010120595.1	98.71
8-B	bin.7	99.1	1.3	*Proteobacteria*	*Halomonas* sp900163645	GCF_900163645.1	95.51
8-B	bin.8	97.8	0.3	*Proteobacteria*	*Pseudoalteromonas nigrifaciens*	GCF_002221505.1	98.9
8-B	bin.9	100.0	0.0	*Actinobacteriota*	*Brevibacterium aurantiacum*	GCF_900169065.1	97.61
8-C	bin.10	95.6	1.5	*Proteobacteria*	*Pseudomonas azotoformans*	GCF_900103345.1	98.98

Table 5.S1 (Continued)

Cheese_ID	MAG	Completeness (%)	Contamination (%)	Phylum	Species	Reference	Reference ANI
8-C	bin.11	98.7	2.1	*Firmicutes*	*Sporosarcina sp*	N/A	NA
8-C	bin.1	93.2	0.0	*Firmicutes*	*Leuconostoc mesenteroides*	GCF_000014445.1	98.87
8-C	bin.2	97.3	1.1	*Proteobacteria*	*Psychrobacter sp*	GCF_003217155.1	83.96
8-C	bin.3	97.4	1.3	*Firmicutes*	*Sporosarcina sp*	N/A	NA
8-C	bin.4	91.0	2.6	*Firmicutes*	*Sporosarcina psychrophila*	GCF_001590685.1	98.09
8-C	bin.5	99.9	0.0	*Firmicutes*	*Lactococcus lactis*	GCF_002078765.2	99.24
8-C	bin.6	96.8	0.4	*Firmicutes*	*Lactococcus laudensis*	GCF_013449735.1	98.51
8-C	bin.7	98.7	0.7	*Firmicutes*	*Psychrobacillus psychrotolerans*	GCF_900115805.1	99.04
8-C	bin.8	100.0	1.2	*Firmicutes*	*Sporosarcina* sp000813425	GCF_000813425.1	97.31
8-C	bin.9	98.0	0.4	*Firmicutes*	*Sporosarcina sp*	GCF_013184495.1	78.24
8-D	bin.1	97.7	0.9	*Firmicutes*	*Lactococcus laudensis*	GCF_013449735.1	99.49
8-D	bin.2	99.4	1.9	*Firmicutes*	*Staphylococcus xylosus*	GCF_002732165.1	97.82
8-D	bin.3	98.4	0.2	*Firmicutes*	*Leuconostoc pseudomesenteroides*	GCF_000297375.1	99.75
8-D	bin.4	99.4	1.2	*Firmicutes*	*Staphylococcus vitulinus*	GCF_002902265.1	98.91
8-D	bin.5	97.4	1.1	*Firmicutes*	*Lactococcus lactis*	GCF_002078765.2	99.29
8-D	bin.6	100.0	0.0	*Actinobacteriota*	*Brevibacterium aurantiacum*	GCF_900169065.1	97.84
8-E	bin.1	100.0	0.0	*Actinobacteriota*	*Brevibacterium aurantiacum*	GCF_900169065.1	97.66
8-E	bin.2	97.9	0.7	*Proteobacteria*	*Psychrobacter sp*	GCF_003217155.1	83.78
8-E	bin.3	96.1	1.5	*Actinobacteriota*	*Glutamicibacter ardleyensis*	GCA_014644555.1	97.47
8-E	bin.4	100.0	0.3	*Proteobacteria*	*Pseudoalteromonas nigrifaciens*	GCF_002221505.1	98.59
9-A	bin.10	99.0	0.0	*Firmicutes*	*Lactobacillus delbrueckii*	GCF_001433875.1	97.11
9-A	bin.11	86.6	1.3	*Actinobacteriota*	*Glutamicibacter ardleyensis*	GCA_014644555.1	98.01
9-A	bin.12	100.0	2.2	*Firmicutes*	*Vagococcus sp*	GCF_004795745.1	79.29
9-A	bin.13	99.3	0.5	*Proteobacteria*	*Morganella psychrotolerans*	GCF_001676155.1	95.44
9-A	bin.14	99.9	0.4	*Proteobacteria*	*Hafnia paralvei*	GCF_001655005.1	99.11
9-A	bin.15	76.0	2.5	*Firmicutes*	*Leuconostoc pseudomesenteroides*	GCF_000297375.1	98.74
9-A	bin.1	98.9	0.0	*Firmicutes*	*Lentilactobacillus parabuchneri*	GCF_001435315.1	97.51
9-A	bin.2	99.5	0.0	*Firmicutes*	*Latilactobacillus curvatus*	GCF_004101845.1	98.86
9-A	bin.3	98.9	0.2	*Firmicutes*	*Lacticaseibacillus paracasei*	GCF_000829035.1	98.91

Table 5.S1 (Continued)

Cheese_ID	MAG	Completeness (%)	Contamination (%)	Phylum	Species	Reference	Reference ANI
9-A	bin.4	97.7	0.6	*Firmicutes*	*Vagococcus salmoninarum*	GCF_003987495.1	97.34
9-A	bin.5	98.9	0.0	*Firmicutes*	*Brochothrix thermosphacta*	GCF_000620985.1	98.98
9-A	bin.6	99.6	0.7	*Proteobacteria*	*Psychrobacter cibarius*	GCF_900016235.2	97.03
9-A	bin.7	99.1	2.8	*Firmicutes*	*Lactiplantibacillus plantarum*	GCF_014131735.1	98.9
9-A	bin.8	100.0	0.1	*Proteobacteria*	*Pseudomonas taetrolens*	GCF_900475285.1	99.52
9-A	bin.9	99.9	0.1	*Firmicutes*	*Streptococcus thermophilus*	GCF_010120595.1	98.37
9-B	bin.10	99.4	1.9	*Firmicutes*	*Staphylococcus xylosus*	GCF_002732165.1	97.82
9-B	bin.1	88.5	1.7	*Actinobacteriota*	*Arthrobacter rhombi*	GCF_900163545.1	98.8
9-B	bin.2	99.0	0.4	*Proteobacteria*	*Psychrobacter sp*	N/A	NA
9-B	bin.3	97.6	0.5	*Actinobacteriota*	*Glutamicibacter arilaitensis*	GCF_000197735.1	98.96
9-B	bin.4	99.5	0.0	*Firmicutes*	*Lacticaseibacillus paracasei*	GCF_000829035.1	98.28
9-B	bin.5	93.7	0.0	*Actinobacteriota*	*Brevibacterium aurantiacum*	GCF_900169065.1	98.84
9-B	bin.6	97.9	0.4	*Proteobacteria*	*Pseudoalteromonas nigrifaciens*	GCF_002221505.1	98.48
9-B	bin.7	85.0	2.3	*Proteobacteria*	*Hafnia alvei*	GCF_900095695.1	99.92
9-B	bin.8	92.8	0.0	*Firmicutes*	*Lactococcus lactis*	GCF_002078765.2	99.37
9-B	bin.9	96.6	0.3	*Firmicutes*	*Lactococcus lactis*	GCF_900099625.1	98.37
9-C	bin.1	95.8	1.2	*Proteobacteria*	*Vibrio litoralis*	GCF_000426765.1	98.43
9-C	bin.2	94.2	2.0	*Firmicutes*	*Lactococcus lactis*	GCF_900099625.1	98.69
9-C	bin.3	99.5	0.0	*Firmicutes*	*Leuconostoc pseudomesenteroides*	GCF_000297375.1	99.89
9-C	bin.4	95.1	0.1	*Firmicutes*	*Streptococcus thermophilus*	GCF_010120595.1	98.6
9-C	bin.5	93.2	1.8	*Firmicutes*	*Lactococcus lactis*	GCF_002078765.2	97.51
9-C	bin.6	99.8	0.3	*Proteobacteria*	*Pseudoalteromonas nigrifaciens*	GCF_002221505.1	98.61
9-E	bin.10	97.1	1.2	*Actinobacteriota*	*Brevibacterium aurantiacum*	GCF_900169065.1	98.75
9-E	bin.11	98.7	0.7	*Firmicutes*	*Leuconostoc mesenteroides*	GCF_000014445.1	99.16
9-E	bin.12	96.2	2.2	*Bacteroidota*	*Psychroflexus halocasei*	GCF_900107595.1	95.99
9-E	bin.13	94.6	0.1	*Actinobacteriota*	*Galactobacter sp*	N/A	NA
9-E	bin.14	96.0	0.2	*Actinobacteriota*	*Corynebacterium casei*	GCF_000550785.1	99.17
9-E	bin.15	96.6	0.6	*Proteobacteria*	*Psychrobacter sp*	GCF_003217155.1	84.01
9-E	bin.16	98.9	0.5	*Actinobacteriota*	*Corynebacterium variabile*	GCF_006539825.1	98.74

Table 5.S1 (Continued)

Cheese_ID	MAG	Completeness (%)	Contamination (%)	Phylum	Species	Reference	Reference ANI
9-E	bin.1	98.9	0.0	*Firmicutes*	*Bavariicoccus seileri*	GCF_000421665.1	99.09
9-E	bin.2	99.9	0.1	*Firmicutes*	*Streptococcus thermophilus*	GCF_010120595.1	98.56
9-E	bin.3	100.0	0.0	*Fusobacteriota*	*Fusobacterium sp*	GCF_010367435.1	94.26
9-E	bin.4	96.0	1.1	*Proteobacteria*	*Vibrio casei*	GCF_002218025.2	99.4
9-E	bin.5	98.9	0.0	*Firmicutes*	*Latilactobacillus curvatus*	GCF_004101845.1	98.84
9-E	bin.6	99.4	0.0	*Actinobacteriota*	*Corynebacterium flavescens*	GCF_008693105.1	99.39
9-E	bin.7	99.0	0.0	*Firmicutes*	*Lactobacillus delbrueckii*	GCF_001433875.1	97.14
9-E	bin.8	94.4	1.1	*Actinobacteriota*	*Microbacterium gubbeenense*	GCF_000422745.1	99.22
9-E	bin.9	95.1	1.2	*Actinobacteriota*	*Mycetocola reblochoni*	GCF_900163835.1	99.72

Table 5.S2 MAGs possessing the COQ3/*ubiG* gene associated with (EC 2.1.1.64/EC 2.1.1.222)

Cheese ID	MAG	Species
5-A	bin.1	*Lactococcus lactis*
5-A	bin.5	*Vibrio sp*
5-A	bin.7	*Staphylococcus equorum*
5-A	bin.8	*Vibrio casei*
5-C	bin.1	*Lactococcus lactis*
5-C	bin.2	*Brevibacterium aurantiacum*
5-C	bin.3	*Staphylococcus equorum*
5-C	bin.5	*Psychrobacter* sp002810365
5-C	bin.6	*Glutamicibacter ardleyensis*
5-C	bin.8	*Psychrobacter glacincola*
5-D	bin.1	*Lactococcus lactis*
6-A	bin.2	*Lactococcus laudensis*
7-D	bin.2	*Pseudoalteromonas nigrifaciens*
7-D	bin.3	*Lactococcus lactis*
7-D	bin.4	*Glutamicibacter ardleyensis*
7-D	bin.5	*Lactococcus lactis*
7-D	bin.6	*Halomonas* sp002332255
7-E	bin.1	*Glutamicibacter ardleyensis*
7-E	bin.3	*Lactococcus lactis*
7-E	bin.4	*Staphylococcus equorum*
8-A	bin.11	*Staphylococcus equorum*
8-A	bin.2	*Glutamicibacter ardleyensis*
8-A	bin.3	*Brochothrix thermosphacta*
8-A	bin.4	*Halomonas* sp002332255
8-A	bin.5	*Hafnia paralvei*
8-A	bin.6	*Pseudomonas taetrolens*
8-A	bin.6	*Pseudomonas taetrolens*
8-A	bin.8	*Psychrobacter cibarius*
8-A	bin.9	*Psychrobacter sp*
8-B	bin.2	*Psychrobacter sp*
8-B	bin.4	*Lactococcus lactis*
8-B	bin.5	*Psychrobacter sp*
8-B	bin.5	*Psychrobacter sp*
8-B	bin.7	*Halomonas* sp900163645
8-B	bin.8	*Pseudoalteromonas nigrifaciens*
8-B	bin.9	*Brevibacterium aurantiacum*
8-C	bin.10	*Pseudomonas azotoformans*
8-C	bin.10	*Pseudomonas azotoformans*
8-C	bin.2	*Psychrobacter sp*
8-C	bin.3	*Sporosarcina sp*
8-C	bin.4	*Sporosarcina psychrophila*
8-C	bin.7	*Psychrobacillus psychrotolerans*
8-C	bin.8	*Sporosarcina* sp000813425
8-C	bin.9	*Sporosarcina sp*
8-D	bin.5	*Lactococcus lactis*
8-D	bin.6	*Brevibacterium aurantiacum*
8-E	bin.3	*Glutamicibacter ardleyensis*
8-E	bin.4	*Pseudoalteromonas nigrifaciens*
9-A	bin.1	*Lentilactobacillus parabuchneri*
9-A	bin.11	*Glutamicibacter ardleyensis*

Table 5.S2 (Continued)

Cheese ID	MAG	Species

9-A	bin.13	*Morganella psychrotolerans*
9-A	bin.13	*Morganella psychrotolerans*
9-A	bin.14	*Hafnia paralvei*
9-A	bin.3	*Lacticaseibacillus paracasei*
9-A	bin.6	*Psychrobacter cibarius*
9-A	bin.8	*Pseudomonas taetrolens*
9-A	bin.8	*Pseudomonas taetrolens*
9-B	bin.1	*Arthrobacter rhombi*
9-B	bin.2	*Psychrobacter sp*
9-B	bin.3	*Glutamicibacter arilaitensis*
9-B	bin.4	*Lacticaseibacillus paracasei*
9-B	bin.5	*Brevibacterium aurantiacum*
9-B	bin.6	*Pseudoalteromonas nigrifaciens*
9-B	bin.7	*Hafnia alvei*
9-B	bin.8	*Lactococcus lactis*
9-B	bin.9	*Lactococcus lactis*
9-C	bin.1	*Vibrio litoralis*
9-C	bin.1	*Vibrio litoralis*
9-C	bin.2	*Lactococcus lactis*
9-C	bin.5	*Lactococcus lactis*
9-C	bin.6	*Pseudoalteromonas nigrifaciens*
9-E	bin.10	*Brevibacterium aurantiacum*
9-E	bin.12	*Psychroflexus halocasei*
9-E	bin.13	*Galactobacter sp*
9-E	bin.15	*Psychrobacter sp*
9-E	bin.16	*Corynebacterium variabile*
9-E	bin.4	*Vibrio casei*
9-E	bin.8	*Microbacterium gubbeenense*
9-E	bin.9	*Mycetocola reblochoni*