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# Microbiome and Physicochemical Features Associated with Differential *Listeria monocytogenes* Growth in Soft, Surface-Ripened Cheeses

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**ABSTRACT** Soft-ripened cheeses (SRCs) are at a higher risk for the growth of the foodborne pathogen *Listeria monocytogenes* due to favorable moisture content and pH compared to other cheeses. *L. monocytogenes* growth is not consistent across SRCs, however, and may be affected by physicochemical and/or microbiome characteristics of the cheeses. Therefore, the purpose of this study was to investigate how the physicochemical and microbiome profiles of SRCs may affect *L. monocytogenes* growth. Forty-three SRCs produced from raw ($n = 12$) or pasteurized ($n = 31$) milk were inoculated with *L. monocytogenes* ($10^3$ CFU/g), and the pathogen growth was monitored over 12 days at 8°C. In parallel, the pH, water activity ($a_w$), microbial plate counts, and organic acid content of cheeses were measured, and the taxonomic profiles of the cheese microbiomes were measured using 16S rRNA gene targeted amplicon sequencing and shotgun metagenomic sequencing. *L. monocytogenes* growth differed significantly between cheeses (analysis of variance [ANOVA]; $P < 0.001$), with increases ranging from 0 to 5.4 log CFU (mean of 2.5 ± 1.2 log CFU), and was negatively correlated with $a_w$. 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 competition. *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 aurantiacum* (Spearman correlation; $P = 0.0002$) and two *Lactococcus* spp. (Spearman correlation; $P < 0.01$). These results suggest that the cheese microbiome may influence the food safety in SRCs.

**IMPORTANCE** Previous studies have identified differences in *L. monocytogenes* growth between SRCs, but no clear mechanism has yet been elucidated. To the best of our knowledge, this is the first study to collect a wide range of SRCs from retail sources and attempt to identify key factors associated with pathogen growth. A key finding in this research was the positive correlation between the relative abundance of *S. thermophilus* and the growth of *L. monocytogenes*. The inclusion of *S. thermophilus* as a starter culture is more common in industrialized SRC production, suggesting that industrial production of SRC may increase the risk of *L. monocytogenes* growth. Overall, the results of this study further our understanding of the impact of $a_w$ and the cheese microbiome on the growth of *L. monocytogenes* in SRCs, hopefully leading toward the development of SRC starter/ripening cultures that can prevent *L. monocytogenes* growth.

**KEYWORDS** *Listeria monocytogenes*, soft-ripened cheese, cheese microbiome

*Listeria monocytogenes* is a psychrophilic foodborne pathogen, and one of the leading causes of foodborne-related deaths in the developed world (1). Due to its ability to persist in the food processing environment (2), *L. monocytogenes* is a common contaminant of ready-to-eat foods, resulting in many foodborne outbreaks associated with soft cheeses (3).

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Soft-ripened cheeses (SRCs) have a moisture on a fat-free basis (MFFB) of greater than 67% (4, 5) and can be either mold ripened (e.g., Brie and Camembert) or smear ripened (i.e., washed rind cheeses). Due to a favorable $a_w$ and pH in these cheeses, *L. monocytogenes* contamination can grow to dangerous levels, both during the ripening period (6, 7) and at the retail establishment (8). Various biocontrol strategies have been investigated for controlling the growth of this foodborne pathogen in these cheeses (e.g., bacteriocins and bacteriophages), but none have been able to completely prevent the growth of *L. monocytogenes* over the whole shelf-life of the cheese (3).

The growth rate of *L. monocytogenes* is not consistent across all SRCs, however, with certain washed-rind SRCs showing complete inhibition against *L. monocytogenes* growth despite favorable conditions of $a_w$ and pH (9). Previous research has suggested an effect of the milk treatment (10), ripening culture (11–14), and/or the production/concentration of organic acids (15–17), but no clear mechanism has yet been elucidated.

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 (18). Of importance is that (i) finished cheeses possess a nontrivial number of microbial taxa not inoculated by the cheesemaker (19–21), (ii) the final cheese microbiota is affected by the microbiome of the cheese processing facility (22), and (iii) many of the taxa present in the cheese processing facility and finished cheeses are also present in the dairy farm environment (23). Recent research has also highlighted the interaction between the cheese microbiome and *L. monocytogenes* in Gouda cheese made with unpasteurized milk (24). A variety of studies have been conducted to determine the microbial taxa associated with *L. monocytogenes* inhibition in surface-ripened cheeses (13–15, 25), but no clear mechanism has yet been elucidated.

The growth of *L. monocytogenes* in cheeses may also be affected through functional mechanisms of the native microbiota that are independent of taxonomy, such as the competition for nutrients (26–28) or the production of inhibitory compounds. Inhibitory compounds that affected the growth of *L. monocytogenes* include bacteriocins, which are ribosomally synthesized, antimicrobial peptides produced by bacteria that often target closely related taxa (29), other antimicrobial proteins, such as the metalloprotease pseudoalterin (30), which was recently identified in members of the cheese microbiome (31), and organic acids, particularly lactic and acetic acids, which have been identified as important mechanisms for the inhibition of *L. monocytogenes* in ripened cheeses (15–17).

The objectives of the current study were to (i) compare the growth of *L. monocytogenes* in a variety of SRCs at refrigerated temperatures, (ii) determine how much variation in *L. monocytogenes* growth can be explained by physicochemical differences (e.g., pH and $a_w$), and (iii) use a combination of viable plate counts, 16S rRNA gene targeted amplicon sequencing, and metagenomic sequencing to investigate microbial community differences between cheeses, with the goal of identifying microbial species or strains associated with *L. monocytogenes* inhibition.

## RESULTS

**Differential growth of *L. monocytogenes* across SRCs.** The growth of *L. monocytogenes* at 8°C in the cheeses was modeled over 12 days and measured by both the area under the growth curve (AUC) (Fig. 1A) and the total increase in pathogen concentration over the incubation period (see Table S1 in the supplemental material). The AUCs of *L. monocytogenes* growth differed significantly across cheeses (analysis of variance [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 and a mean increase of 2.5 $\pm$ 1.2 log CFU. Of the 43 cheeses assessed, 6, 7, 20, and 10 cheeses were assigned to the no-, low-, medium-, and high-growth groups, respectively (Table S1).

On average, cheeses made with raw milk showed significantly lower growth of *L. monocytogenes* than cheeses made with pasteurized milk (*t* test; $P = 0.001$) (Fig. 1B).

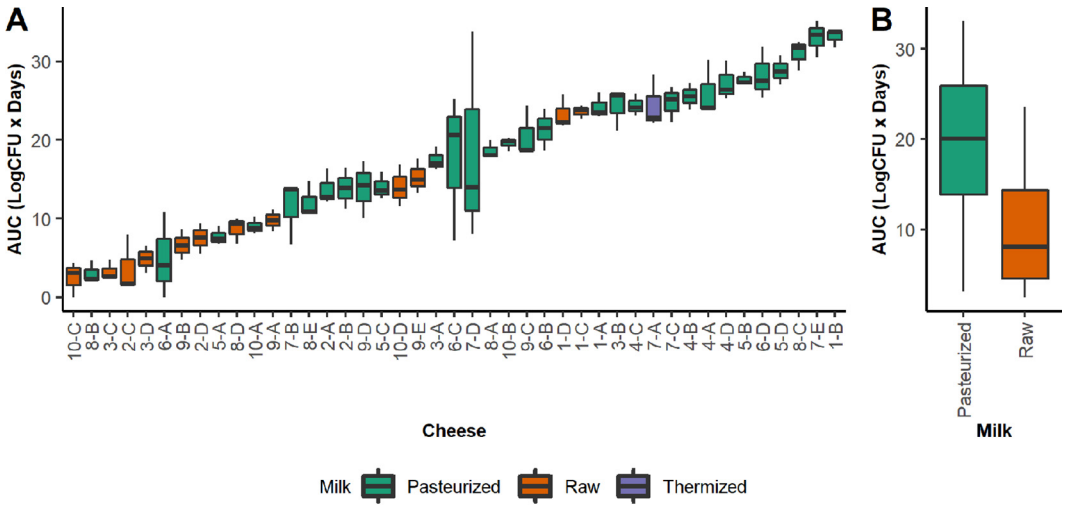


**FIG 1** Box plots summarizing of the area under the curve (AUC) of *Listeria monocytogenes* growth across the individual cheeses (A) and treatment of the milk used for cheesemaking (B). Each box is colored based on milk treatment. Since only one cheese was produced using thermized milk, it was not included in the comparison between milk treatments.

Furthermore, no raw milk cheeses allowed more than a 3.5 log CFU increase in *L. monocytogenes* concentration over the incubation period (Table S1). It should be noted, however, that *L. monocytogenes* growth in some individual pasteurized-milk cheeses was lower than that in some raw milk cheeses (Fig. 1A). No significant difference in levels of *L. monocytogenes* growth was observed between washed- and bloomy-rind cheeses (*t* test; $P = 0.70$).

**High-throughput sequencing of the microbial communities of SRCs.** 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 S1). 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 operational taxonomic units (OTUs). After rarefaction to 3,000 reads per sample, 135 samples and 222 OTUs remained. An average of 30 ± 15 OTUs was observed for each cheese.

Metagenomic sequencing was used to conduct species- and strain-level taxonomic analysis on a subset of 15 cheeses used in this study (Table S1). A total of 137,937,980 paired-end reads from 58 samples remained after quality filtering. Not including two sample replicates that 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 an 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.

**Physicochemical, microbial, and $\alpha$-diversity analysis of SRC with respect to *L. monocytogenes* growth.** The pH and $a_w$ of cheeses are summarized in Table 1. The average pH across the tested cheeses was 6.9 ± 0.5 and ranged from 5.5 to 7.7. The $a_w$ of cheeses ranged from 0.95 to 0.99, with a mean value of 0.97 ± 0.01. Raw milk cheeses showed a significantly lower average water activity than cheeses made with pasteurized milk (Wilcoxon test; $n = 42$; $P = 0.03$), and $a_w$ was significantly correlated with the growth of *L. monocytogenes* in cheeses (Spearman's rank correlation; $\rho = 0.417$; $P = 0.005$). No significant difference in pH was observed between milk treatments, and no significant correlation was observed between pH and the growth of *L. monocytogenes* in the cheeses.

Of the organic acids assessed, only lactic and citric acids were consistently detected across the tested cheeses, with mean concentrations of 2.42 ± 1.28 $\mu$g/mg and 3.86 ± 1.80 $\mu$g/mg for lactic and citric acids, respectively (Table 1). As shown in Table 1,

**TABLE 1** Summary of physicochemical and microbial characteristics of cheeses, and their correlation with the growth of *L. monocytogenes* in the respective cheeses

| Characteristic | *n* | Milk treatment[a] | | All cheeses | Range | Corr[b] | *P* value |
|---|---|---|---|---|---|---|---|
| | | Pasteurized | Raw | | | | |
| Physicochemical | | | | | | | |
| pH | 39 | 6.8 ± 0.6 A | 7.1 ± 0.3 B | 6.9 ± 0.5 | 5.5–7.7 | −0.057 | 0.732 |
| $a_w$ | 43 | 0.97 ± 0.01 A | 0.96 ± 0.01 B | 0.97 ± 0.01 | 0.95–0.99 | 0.417 | 0.005 |
| Organic acid concn ($\mu$g/mg) | | | | | | | |
| Lactic acid | 8 | —[c] | — | 2.42 ± 1.28 | 1.11–4.73 | 0.167 | 0.703 |
| Citric acid | 9 | — | — | 3.86 ± 1.80 | 0.734–5.93 | −0.267 | 0.493 |
| Viable plate counts (log CFU/g) | | | | | | | |
| TSA | 43 | 8.2 ± 1.1 A | 9.0 ± 0.5 B | 8.4 ± 1.0 | 4.8–9.6 | −0.430 | 0.004 |
| M17 | 42 | 8.0 ± 1.2 | 8.5 ± 0.5 | 8.1 ± 1.1 | 4.1–9.5 | 0.103 | 0.514 |
| MRS-5.4 | 43 | 6.7 ± 1.6 | 7.2 ± 0.9 | 6.8 ± 1.4 | 3.0–8.9[d] | −0.128 | 0.413 |
| $\alpha$ diversity | | | | | | | |
| Observed OTUs | 39 | 30 ± 15 A | 45 ± 8 B | 34 ± 15 | 5–65 | −0.394 | 0.013 |
| Shannon diversity | 39 | 1.08 ± 0.61 A | 1.81 ± 0.35 B | 1.26 ± 0.63 | 0.12–2.23 | −0.292 | 0.072 |

[a]Values in the same row followed by different letters were significantly different between milk treatments (Wilcoxon test; $P < 0.05$).
[b]Corr, Spearman's rank correlation ($\rho$).
[c]—, insufficient samples for each individual milk treatment group.
[d]The starting point of the range, 3.0, is below the reliable limit of enumeration (~3.4 log CFU/g).

no significant correlation was observed between either of these two organic acids and the growth of *L. monocytogenes* (Spearman's rank correlation; $P > 0.5$). Malic acid was detected in only two cheeses: one each from the low- and no-growth groups.

The total aerobic microbial count (TAMC) was enumerated for all cheeses by spread plating on tryptic soy agar (TSA) and incubating at 30°C (Table 1). 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 TAMCs of 9.0 ± 0.5 log CFU/g and 8.2 ± 1.1 log CFU/g for each milk treatment, respectively (Wilcoxon test; $n = 42$; $P = 0.003$). Furthermore, a significant negative correlation was observed between the TAMC and the growth of *L. monocytogenes* in the cheeses (Spearman's rank correlation; $\rho = -0.43$, $P = 0.004$).

The viable plate count was also used to enumerate total lactic acid bacteria (TLAB) on both M17 (aerobic) and MRS-5.4 (anaerobic) agars (Table 1). The average viable plate count 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 it ranged from below the limit of enumeration (~3.4 log CFU/g) to 8.9 log CFU/g. No significant difference in TLAB on either medium was observed between cheeses made with raw milk and those made with pasteurized milk (Wilcoxon test; $n = 42$; $P > 0.4$), nor were there any significant correlations observed between TLAB and the growth of *L. monocytogenes* (Table 1).

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 (Table 1; see Fig. S1 in the supplemental material). The number of observed OTUs (richness) in each cheese ranged from 5 to 65, with an average of 34 ± 15, and was significantly negatively correlated with *L. monocytogenes* growth in the individual cheeses (Spearman's rank correlation; $\rho = -0.394$; $P = 0.013$). On average, cheeses made with raw milk had a greater number of observed OTUs than cheeses made with pasteurized milk (Wilcoxon test; $P < 0.004$), but the cheese with the highest richness in the study was made with pasteurized milk (Fig. S1A). Of note, there was no significant correlation observed between microbial richness and TAMC ($r = 0.24$; $P = 0.13$). The Shannon diversity index of cheeses (evenness) was greater in raw milk cheeses than in pasteurized milk cheeses (Wilcoxon test; $P < 0.001$), but no

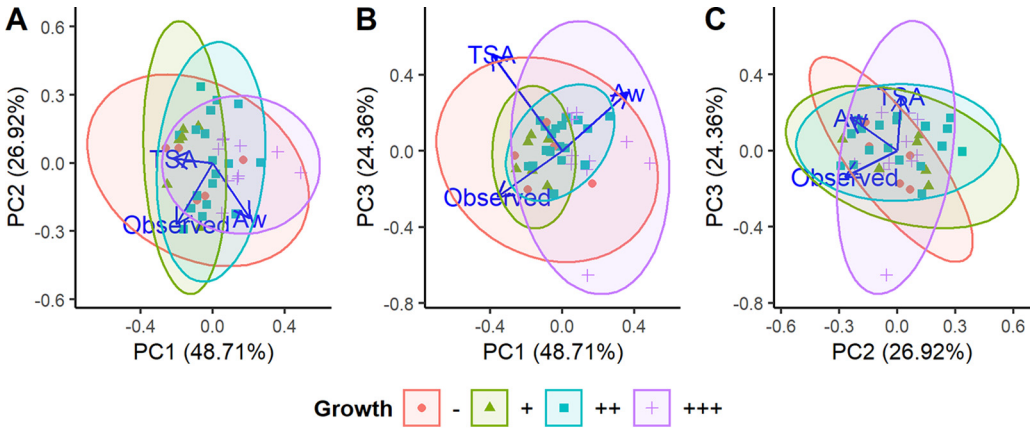


**FIG 2** Principal-component analysis of total aerobic microbial count (TSA), water activity ($a_w$), and total observed OTUs measured for each cheese. Graphs show the relationship between principal component 1 (PC1) and PC2 (A), PC1 and PC3 (B), and PC2 and PC3 (C). Cheeses are colored and surrounded by ellipses based on growth category.

significant correlation was observed between the Shannon diversity index of cheeses and the growth of *L. monocytogenes*.

Principle-component analysis was conducted to assess the combined predictive power of the three metrics that were significantly correlated with *L. monocytogenes* growth in cheese (i.e., $a_w$, TAMC, and observed richness) (Fig. 2). Despite the inclusion of all three correlated metrics, no significant separation was observed between the growth categories (permutational multivariate analysis of variance [PERMANOVA]; $P = 0.061$). Furthermore, multiple linear regression combining the three metrics explained only 26% of the observed variation ($R^2_{adj} = 0.264$; $n = 39$; $P = 0.003$).

**Taxonomic analysis of SRCs relative to *L. monocytogenes* growth.** Taxonomic analysis at the phylum level is summarized in Fig. 3A. *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.

After adjusting for the false-discovery rate (FDR), significant correlations were observed between *L. monocytogenes* growth and two of the most abundant phyla: *Firmicutes* and *Actinobacteria* (Table 2). The relative abundance of the *Firmicutes* phylum was positively correlated with the growth of *L. monocytogenes* in the cheeses (Spearman rank correlation; $\rho = 0.396$; $P = 0.013$; FDR [$q$ value] = 0.038), while the growth of *L. monocytogenes* was negatively correlated with the relative abundances of *Actinobacteria* (Spearman rank correlation; $\rho = -0.475$; $P = 0.0023$; $q$ value = 0.014). The *Proteobacteria* phylum was also negatively correlated with the growth of *L. monocytogenes* (Spearman rank correlation; $\rho = -0.351$; $P = 0.028$; $q$ value = 0.057), but this correlation was not significant after correcting for FDR.

*Streptococcus* and *Lactococcus* were the two most common genera observed, represented by 28.6% ± 35.1% and 29.1% ± 27.6% relative abundances in cheeses, respectively (Fig. 3B). At a finer taxonomic resolution, the overrepresentation of these two genera was shown to be the result of just one OTU each, with relative abundances in the cheeses of 28.5% ± 35.0% and 27.4% ± 26.7% for the dominant *Streptococcus* and *Lactococcus* OTUs, respectively (Fig. S2A and B). Other *Firmicutes* genera representing greater than 1% relative abundance included *Lactobacillus*, *Psychrobacter*, *Pseudoalteromonas*, *Staphylococcus*, *Brevibacterium*, *Carnobacterium*, and *Arthrobacter*. The overrepresentation of *Streptococcus* and *Lactococcus* notwithstanding, the most abundant genus in many of the cheeses was not either of those


**FIG 3** Mean relative abundance of phyla (A) and genera (B) representing greater than 1% of total reads across all cheeses as measured by 16S rRNA targeted amplicon sequencing. Cheeses are grouped by growth category.

two. The genera with the highest relative abundance in some cheeses were *Lactobacillus* (4 cheeses), *Psychrobacter* (2 cheeses), *Pseudoalteromonas* (2 cheeses), *Brevibacterium* (2 cheeses), and an unidentified member of the *Planococcaceae* family (1 cheese).

After FDR adjustment, significant correlations were observed between *L. monocytogenes* growth and the relative abundance of the genera *Streptococcus* and *Brevibacterium* as summarized in Table 2. The relative abundance of *Streptococcus* was positively correlated with *L. monocytogenes* growth in cheeses (Spearman rank correlation; $\rho = 0.606$; $q$ value = 0.001),

**TABLE 2** Spearman rank correlations between the growth of *L. monocytogenes* and the 6 most relatively abundant phyla and 20 most abundant genera in the cheeses used in this study

| Taxonomy | Corr[a] | *P* value | *q* value[b] |
|---|---|---|---|
| Phlya | | | |
| *Actinobacteria* | −0.474 | 0.002 | 0.014 |
| *Firmicutes* | 0.396 | 0.013 | 0.038 |
| *Proteobacteria* | −0.351 | 0.028 | 0.057 |
| *Deinococcus-Thermus* | 0.269 | 0.098 | 0.147 |
| *Bacteroidetes* | −0.213 | 0.193 | 0.231 |
| *Fusobacteria* | −0.165 | 0.316 | 0.316 |
| Genera | | | |
| *Streptococcus* | 0.606 | <0.001 | 0.001 |
| *Brevibacterium* | −0.562 | <0.001 | 0.002 |
| *Psychrobacter* | −0.381 | 0.017 | 0.112 |
| *Brachybacterium* | −0.355 | 0.026 | 0.132 |
| *Lactococcus* | −0.310 | 0.055 | 0.220 |
| *Leuconostoc* | −0.293 | 0.070 | 0.234 |
| *Pseudoalteromonas* | −0.267 | 0.101 | 0.289 |
| *Corynebacterium* | −0.251 | 0.124 | 0.310 |
| *Halomonas* | −0.223 | 0.172 | 0.382 |
| *Arthrobacter* | −0.200 | 0.221 | 0.419 |
| *Brochothrix* | −0.195 | 0.234 | 0.419 |
| *Pseudomonas* | 0.188 | 0.251 | 0.419 |
| *Sporosarcina* | −0.154 | 0.350 | 0.539 |
| *Carnobacterium* | −0.136 | 0.408 | 0.566 |
| *Staphylococcus* | −0.132 | 0.424 | 0.566 |
| *Vagococcus* | −0.106 | 0.522 | 0.652 |
| *Vibrio* | −0.095 | 0.566 | 0.665 |
| *Alkalibacterium* | −0.044 | 0.790 | 0.878 |
| *Enterococcus* | 0.034 | 0.837 | 0.882 |
| *Lactobacillus* | 0.009 | 0.959 | 0.959 |

[a]Corr, Spearman's rank correlation.
[b]FDR-adjusted *P* value.

while the growth of *L. monocytogenes* was negatively correlated with the relative abundance of *Brevibacterium* (Spearman rank correlation; $\rho = -0.562$; *q* value = 0.002).

Shotgun metagenomic sequencing was used to conduct taxonomic analysis at the species level (Fig. 4). The two most abundant species were *Lactococcus cremoris* and *Lactococcus lactis*, which represented averages 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 averages 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*, *Lactilactobacillus 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.

***β* diversity and differential abundance.** The $\beta$ diversity of cheeses was measured using the weighted UniFrac distance method and is summarized in Fig. 5. A significant separation was observed between the *L. monocytogenes* growth categories (PERMANOVA; $P = 0.002$) (Fig. 5A). Specifically, the high-growth category was significantly different in microbial community structure from both the no- and low-growth

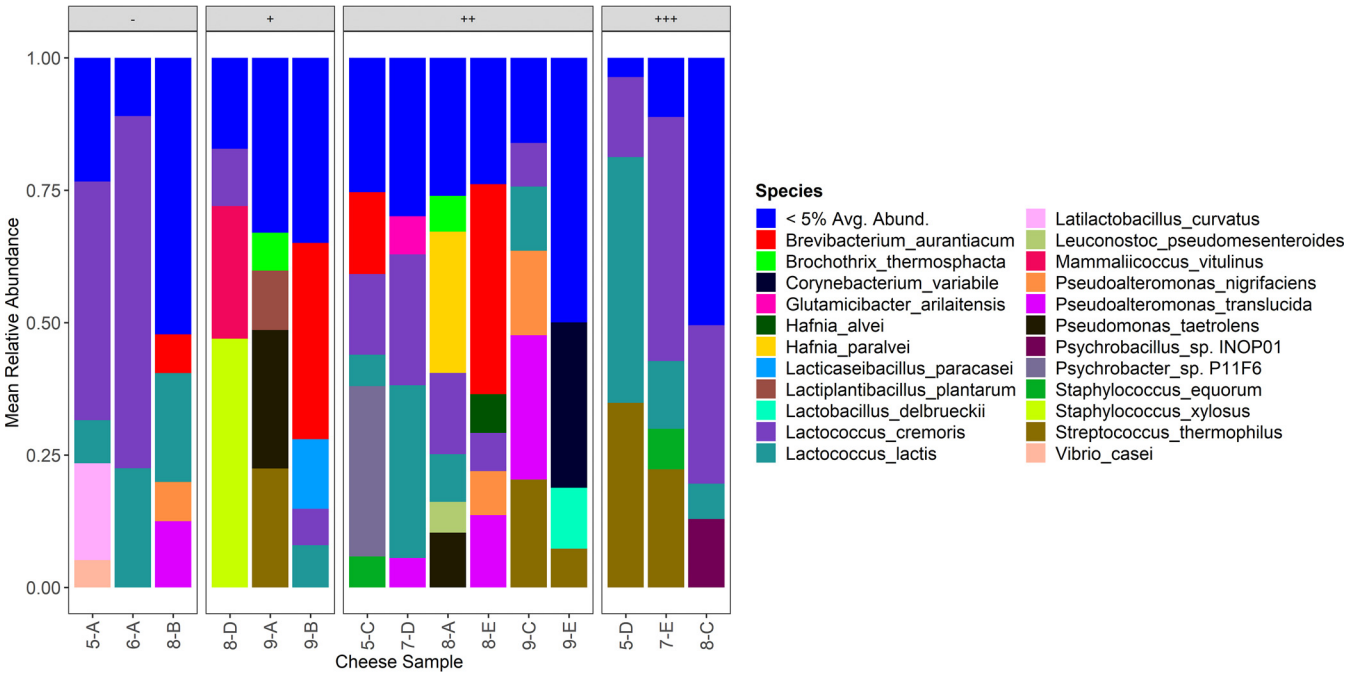


**FIG 4** Mean relative abundance of species representing greater than 5% of total reads across the cheeses using shotgun metagenomic sequencing. Cheeses are grouped by growth category.

categories (pairwise PERMANOVA; *q* value = 0.024). As shown in Fig. 5C, 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 versus pasteurized milk (PERMANOVA; $P = 0.001$) (Fig. 5B).

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 3). 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. Scatterplot analysis (Fig. S3) 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. S2A). The most abundant species of *Streptococcus* observed in the shotgun metagenomic sequencing data was *S. 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 was the most abundant OTU of the *Actinobacteria* phylum (Fig. S2D), 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. S2B). Therefore, it is unlikely that they represent *L. lactis* or *L. cremoris*, the most abundant species of *Lactococcus* observed in the shotgun metagenomic 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.

Of note, 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

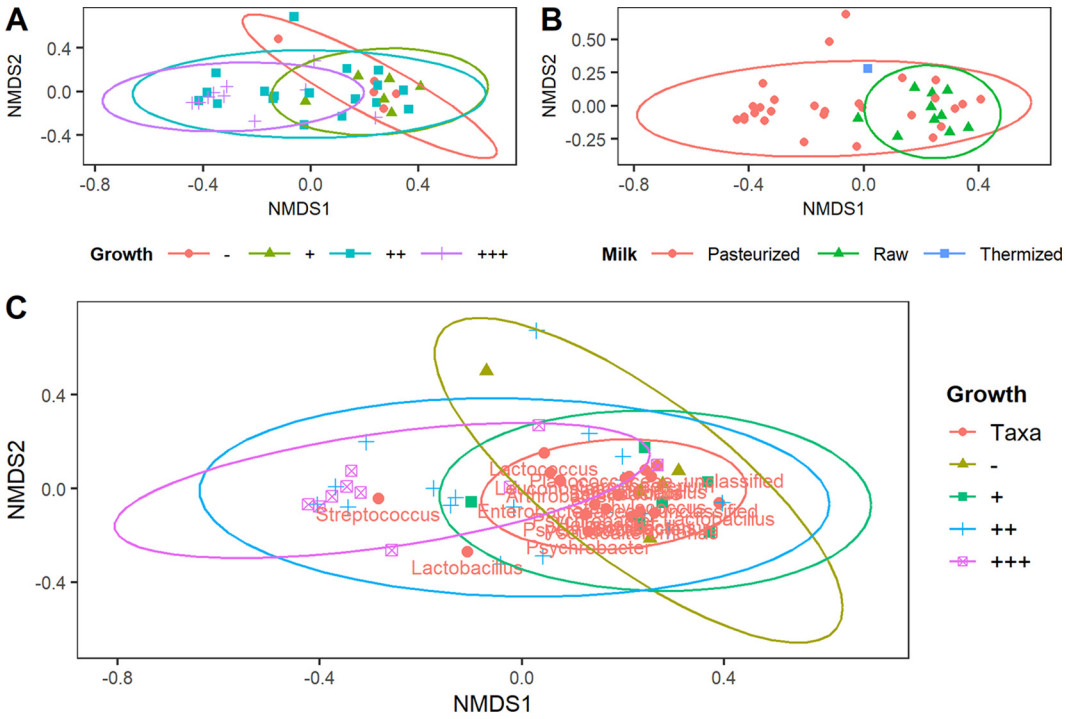


**FIG 5** Nonmetric multidimensional scaling ordination of cheeses based on weighted UniFrac distances calculated using all OTUs (A and B [stress = 0.15]) or the 20 most abundant OTUs (C [stress = 0.16]). Ellipses represent a multivariate *t* distribution for each growth category (A and C) or milk treatment (B). Ellipses are only presented for sample groups with ≥4 samples represented. Genera are labeled for the most common OTUs.

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.

In contrast, no prevalent species were identified as being differentially abundant relative to *L. monocytogenes* growth in cheese in the metagenomic 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.

**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 metagenomic assemblies. A summary of the identified bacteriocin-encoding genes in each cheese is shown in Fig. 6.

The two most common bacteriocin-encoding genes observed were those for lactococcin-A and enterocin X$\beta$, which were identified in 13 and 10 cheeses, respectively. Furthermore, both lactococcin-A and enterocin X$\beta$ were identified in cheeses from each growth category and milk treatment. Lactococcin-B and nisin U were also identified in each growth category and milk treatment, 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.

**TABLE 3** Spearman rank correlations of *Listeria monocytogenes* growth with the abundances of differentially abundant OTUs identified using the ANCOM-BC algorithm

| OTU | Genus | Spearman correlation | *q* value[a] |
|---|---|---|---|
| Otu00001 | *Streptococcus* | 0.606 | <0.0001 |
| Otu00003 | *Brevibacterium* | −0.561 | 0.0002 |
| Otu00046 | Unclassified member of *Brevibacteriaceae* | −0.614 | <0.0001 |
| Otu00058 | *Lactococcus* | −0.407 | 0.010 |
| Otu00061 | *Lactococcus* | −0.452 | 0.004 |

[a]FDR-adjusted *P* value.

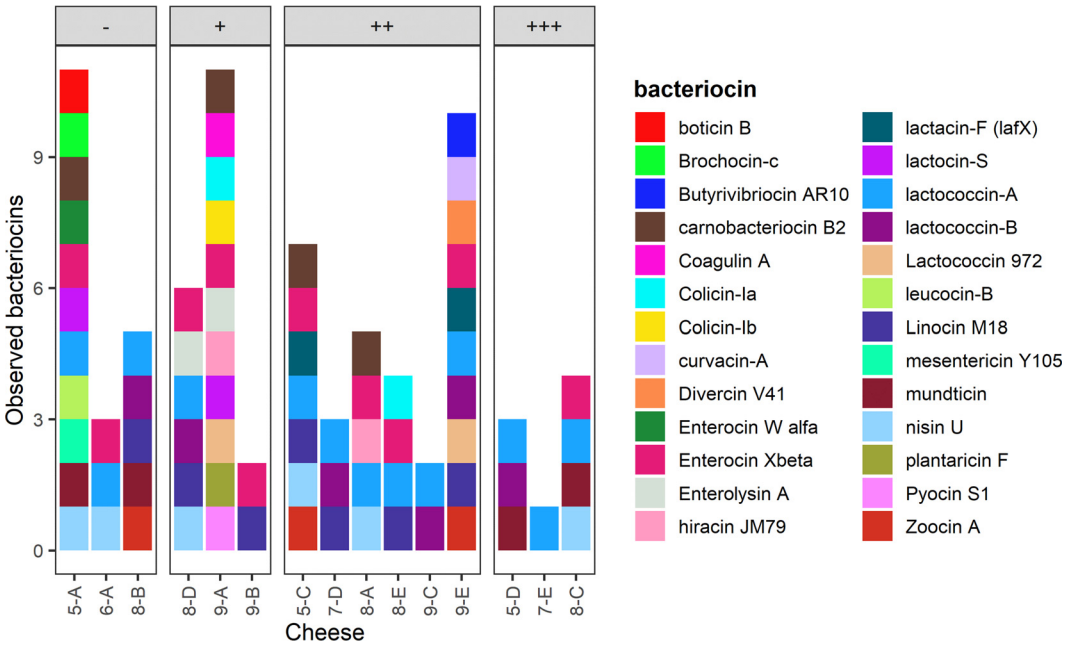


**FIG 6** Number of unique bacteriocin-encoding genes observed in each cheese metagenome according to the BACTIBASE database. Cheeses are grouped by growth category.

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 W$\alpha$, leucocin-B, and mesentericin Y105 were present only in cheeses from the no-growth category, while genes to produce coagulin A, colicin-Ib, enterolysin A, plantaricin F, and pyocin S1 were present only 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 present in only a single cheese: one from each growth category. The only exceptions were enterolysin A and lactocin-S, which were each observed in two cheeses. Furthermore, all these single-hit bacteriocin genes were from the same cheese within their respective growth category.

## DISCUSSION

**Variations across *L. monocytogenes* growth in retail SRCs are correlated with $a_w$ and milk treatment.** This experiment investigated the growth of *L. monocytogenes* in SRCs 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 SRCs at refrigerated temperatures during ripening (7), after ripening (9), and at retail establishments (8). Each of these previous studies showed levels of *L. monocytogenes* growth similar to or higher than the mean *L. monocytogenes* increase observed in the current study. Of interest to the 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 cheeses even showing less than a $<$0.5 log CFU increase of *L. monocytogenes* over the course of the experiment. This inhibition of *L. monocytogenes* growth is of note because SRCs, due to their favorable moisture content and near-neutral pH, are expected to promote the growth of *L. monocytogenes* (3). Previous studies have documented variations in *L. monocytogenes* growth in lab-prepared Camembert-style cheeses (10) and across a small sampling of washed-rind SRCs in Belgium (9), but to the best of our knowledge, this is the first study documenting considerable variations across a large sampling of SRCs from retail.

Two important physicochemical factors affecting *L. monocytogenes* growth in soft cheeses are pH and $a_w$ (3). In the current study, no correlation between *L. monocytogenes* and pH was observed. Since all cheese samples were 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 (3, 32). On the other hand, a significant positive correlation was observed between pathogen growth and $a_w$. Previous modeling data have suggested a stochastic effect on *L. monocytogenes* growth at $a_w$ values between 0.96 and 0.98 (33). At the population level, this stochastic effect would be extended to what proportion of viable cells will double at any point in time, thereby reducing the total rate of growth across the entirety of the *L. monocytogenes* population. These results confirm the importance of $a_w$ as the primary physicochemical factor associated with *L. monocytogenes* growth in SRC at retail establishments.

Despite the lack of correlation between *L. monocytogenes* growth and pH of SRC at retail establishments, the presence of weak organic acids (e.g., lactic acid) has been previously associated with *L. monocytogenes* inhibition in soft cheeses (16, 17). In the current study, however, no correlation was observed between the growth of *L. monocytogenes* and the concentration of lactic acid. During the ripening process, lactic acid is consumed by the ripening cultures (34), meaning that there may be insufficient concentrations of lactate present in ripened cheeses to have an inhibitory effect. Indeed, the concentration of lactic acid in the studies associating this acid with *L. monocytogenes* inhibition was 5 to 10 times higher than the levels observed in the cheeses from the current study (15, 17). Further studies investigating how lactate consumption during ripening affects *L. monocytogenes* growth in SRC are recommended.

Milk treatment was also associated with differences in the growth of *L. monocytogenes*, as pathogen growth, on average, was significantly lower in cheeses made with raw milk than in those produced with pasteurized or thermized milk, consistent with previous research (10, 33). The inhibition of *L. monocytogenes* in these previous studies was suggested to be an effect of increased microbial competition associated with the unpasteurized milk microbiota. In the current study, evidence for competition-based inhibition can be seen in the negative correlation between the growth of *L. monocytogenes* and both the TAMC and microbial richness in the cheeses. Interestingly, TAMC and the total number of observed OTUs in each cheese were not correlated, suggesting that each of these two factors may be inhibitory in its own way.

The 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 nonspecific competition for nutrients (27). The natural microbiotas of various food systems have previously been shown to inhibit the growth of *L. monocytogenes* (35), and microbial competition against *L. monocytogenes* has also been observed in other food systems, including apples (36), lettuce (37), and ready-to-eat meats (38).

***Streptococcus thermophilus* abundance positively correlates with *L. monocytogenes* growth in SRCs.** The relationship between microbial species richness and *L. monocytogenes* inhibition is less cut-and-dried, however, since the cheese with the highest microbial species richness was assigned to the medium-growth group, and 6 out of the 10 cheeses with the greatest species richness were assigned to either the medium- or high-growth groups. 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 (39), 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 (40). A plausible 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 than pasteurized milk cheeses, had a higher average species richness. Furthermore, 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 a greater predictor of *L. monocytogenes* growth. While the species richness may not play a direct role, it is reasonable to suggest that increased richness reduces the opportunity for *L. monocytogenes* to gain a foothold due to a more varied use of nutrients by the native population.

16S rRNA gene targeted amplicon sequencing identified an average of 35 OTUs per cheese sample, which is similar to previous studies using this method to investigate the microbiota of SRCs (19, 25). *Firmicutes* were identified as the dominant phylum in the cheeses, which is consistent with previous studies investigating the microbial community of cheese (20, 23, 25). The overabundance of *Firmicutes* may be attributed to the fact that most microbial species in defined starter cultures used in cheesemaking (e.g., *Lc. lactis*, *Lc. cremoris*, and *S. thermophilus*) are members of the *Firmicutes* phylum (41), and the *Streptococcus* and *Lactococcus* genera have been suggested to be especially suited to the cheese environment (42). Indeed, *Streptococcus* and *Lactococcus* were the two most abundant genera observed in the current study.

The presence of *Firmicutes* 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*. It should be noted that *Lactococcus* was negatively correlated with *L. monocytogenes* growth, implying that this observed trend is genus (or species) rather than phylum specific. 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 soft, mold-ripened cheeses (34). *Streptococcus* has been previously observed as a dominant genus in cheeses where it was not included as a starter culture (25), which may indicate it as part of the nonstarter lactic acid bacteria (NSLAB). However, recent interest in the production of "stabilized" or "solubilized" SRCs has led to the addition of *S. thermophilus* as a starter culture (34). 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 in mineral content, and a firmer resulting paste (43). 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 necessarily causative, but that the high relative abundance of *Streptococcus* is merely indicative of a favorable 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 by 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. Furthermore, 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.

***Lactococcus* abundance negatively correlates with *L. monocytogenes* growth in SRCs.** Despite the positive correlation between *Firmicutes* and *L. monocytogenes* growth, two OTUs of *Lactococcus* showed a significant negative correlation with growth of the pathogen ($P < 0.026$). 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 SRCs (25). Bacteriocins produced by *Lc. lactis* have regularly been shown to inhibit the growth of *L. monocytogenes* in soft cheeses (3), but based on the relative abundance of the potentially inhibitory OTUs observed in this study, they are unlikely to represent *Lc. lactis*. Furthermore, the presence of genes encoding these bacteriocins (i.e., nisin of lactococcin) was also not associated with *L. monocytogenes* inhibition in the studied cheeses. 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 been shown to have antimicrobial action against *L. monocytogenes*. Strains of *Lc. piscium* isolated from modified-atmosphere-packaged salmon were inhibitory against *L. monocytogenes in vitro* (44) as well as in cooked shrimp (45). Similarly, *Lc. garvieae* from raw milk was

previously credited with antilisterial activity observed in cheeses produced from the milk (14). A bacteriocin produced by select strains of *Lc. garvieae*, garvieacin Q, is known to inhibit *L. monocytogenes* (46) and has been identified in a strain collected from raw milk cheese (47). Further investigation into these two species for the inhibition of *L. monocytogenes* in soft cheeses is warranted.

***Brevibacterium* abundance negatively correlates with *L. monocytogenes* growth in SRCs.** 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 intercheese variation in the relative abundances of these two phyla, these bacteria have all been previously identified as part of the cheese rind community (21). They are also known to populate the house microbiota in cheese production facilities (22, 48).

*Actinobacteria* were 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* that was assumed to be *B. aurantiacum*. *B. aurantiacum* (previously *B. brevis* [49, 50]) has been shown to produce multiple antiliserial bacteriocins (51–53). One antilisterial bacteriocin associated with *B. aurantiacum*, linocin M18 (53), was identified in several cheeses from this study and was noticeably absent from any cheeses in the high-growth category. Additionally, linocin M18 was previous associated with *L. monocyotogenes* in SRCs (54).

Bacteriocins provide an attractive option for the control of *L. monocytogenes* since they are often found in bacteria already associated with the cheese microbiome (55). Therefore, it was relevant to assess the presence of bacteriocins in the cheese metagenomes relative to the growth of *L. monocytogenes*. 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 W$\alpha$ (56), leucocin-B (57), and mesentericin Y105 (58), 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 genes 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 shown only minimal inhibition against *L. monocytogenes in vitro* (59), which makes it an unlikely that it is driving the *L. monocytogenes* inhibition observed in these cheeses. To the best of our knowledge, no published work has been assessing the effect of lactocin-S against *L. monocytogenes*. Overall, there is little evidence to conclude that bacteriocins are having a meaningful effect on the growth of *L. monocytogenes* in the cheeses.

Care should be taken when analyzing the microbiome data presented, primarily due to the compositional nature of these 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 (60). 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. Another thing to consider with this piece of data is that the presence of *L. monocytogenes* in cheese can also affect the microbiome of the cheese itself (24), suggesting that the microbiomes observed in this study might not completely reflect the real-world scenario of *L. monocytogenes* contamination in the same cheeses. It is also important to acknowledge that the presence of a putative bacteriocin-encoding gene does not, in

and of itself, demonstrate that this gene is expressed. Additionally, *in vitro* confirmation of the inhibitory effects of any expressed antimicrobial peptides is necessary, since mutations within bacteriocin peptides have been shown to affect their antimicrobial efficacy (61). Since the bacteriocin-encoding genes identified in this study could share as little as 50% sequence homology with those of the BACTIBASE database, it is possible that they could differ in their abilities to inhibit *L. monocytogenes*. Similarly, the identification of antimicrobial peptides is biased by the database used for alignment (62), meaning novel genes with antilisterial effects may go unnoticed. Therefore, further studies should be conducted to confirm the findings of the current study and to investigate the possible mechanisms behind these effects on *L. monocytogenes* growth.

**Conclusion.** *L. monocytogenes* is capable of growing in SRCs at refrigerated temperatures, but the amount of growth varies across cheeses. Milk treatment, water activity, and microbial competition (i.e., TAMC and species richness) were predictive of *L. monocytogenes* growth but were not able to fully explain the total variation observed. Variation in the microbiome of SRCs may also affect, or at least predict, the growth of *L. monocytogenes* in the respective cheese. Of importance is a positive association between the relative abundance of *S. thermophilus* and *L. monocytogenes*, which may imply a higher food safety risk associated with industrialized cheese production processes. On the other hand, species of *Brevibacterium* and *Lactococcus* may provide a protective effect against the growth of *L. monocytogenes*. These results further our understanding of the effects of the cheese microbiome on *L. monocytogenes* growth and will lead to the development of safer SRCs by validating the efficacy of different microbiomes on specific *L. monocytogenes* strains in industrial applications.

## MATERIALS AND METHODS

**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* (63). For each biological replicate, an individual isolate of *L. monocytogenes* BCCDC-A3 was grown for 24 to 28 h 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 $\sim 10^9$ CFU/mL. An aliquot of this culture was then diluted 100-fold into fresh TSB-YE and incubated at 8°C without shaking until early stationary phase (optical density at 600 nm [$OD_{600}$] of ~0.900 [7 to 10 days]). These cold-adapted cultures were then washed three times with phosphate-buffered saline (PBS [pH 7.4]) (Alfa Aesar, Haverhill, MA) and resuspended in PBS to achieve a concentration of $10^5$ CFU/mL.

**Measuring the growth of *L. monocytogenes* in soft-ripened cheeses.** The growth of *L. monocytogenes* was monitored in 43 SRCs, comprised of 36 bloomy-rind and seven washed-rind cheeses (Table S1). Cheeses were purchased from local cheese retailers and selected to maximize the number of artisan-produced samples included. Information regarding style of production (i.e., milk treatment) was acquired from the label where possible or from the seller. Thirty cheeses 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 to 68°C for 10 to 20 s (64). Cheeses were produced in France ($n = 29$), Canada ($n = 12$), and Denmark ($n = 2$). Each cheese was aseptically aliquoted into 3.0 $\pm$ 0.1-g subportions and divided into sterile 118-mL Whirl-Pak sample bags (Whirl-Pak, Madison, WI). For each biological replicate ($n = 3$), up to 10 subportions were inoculated with 30 $\mu$L of cold-adapted *L. monocytogenes* culture to achieve a concentration of $\sim 10^3$ CFU/g. Negative controls were inoculated with 30 $\mu$L of sterile PBS. Inoculated cheeses and negative controls were all incubated at 8°C until enumeration.

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 10-fold in sterile PBS and homogenized in a stomacher for 2 min at 230 rpm. The homogenized samples were then diluted in sterile PBS before spread plating on PALCAM agar (Neogen Corp., Lansing, MI). The plated samples were enumerated after incubation at 37°C for 24 h. 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 modeled over 12 days using a logistic regression model. For each sample, the growth was measured as both the area under the modeled 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 component analysis), cheeses were grouped into four categories based on the total increase in *L. monocytogenes* modeled over the 12 days: no growth (<1 log CFU increase [−]), low growth (1 to 2 log CFU increase [+]), medium growth (2 to 3.5 log CFU increase [++]), and high growth (>3.5 log CFU increase [+++]).

**Physicochemical and viable microbial analysis of cheeses.** The pH of each cheese was measured in triplicate from three different areas of the cheese (i.e., edge, rind, and core) using an Oakton pHTestr 50S Spear-Tip waterproof pocket tester (Cole-Parmer Canada Company, Montreal, QC, Canada). The

water activity ($a_w$) of each cheese was measured on triplicate 3.0 ± 0.1-g aliquots using an Aqualab series 3 water activity meter (Decagon Devices, Inc., Pullman, WA). Measurements of pH and $a_w$ were taken within 48 h of time zero.

For organic acid analysis, 20 g of each cheese was homogenized in 80 mL of high-performance liquid chromatography (HPLC)-grade water by bending for 30 s. The resulting homogenates were then centrifuged at 6,000 × *g* for 10 min and filtered through 0.2-$\mu$m-pore cellulose acetate membranes (VWR International, Mississauga, ON, Canada) before being subjected to HPLC analysis. Chromatography was conducted using an Agilent 1100 HPLC system with Nucleogel Ion 300 OA column (300 mm by 7.8 mm inside diameter [i.d.]) and refractive index detector as previously described (65). The concentrations of lactic, citric, and malic acids in the cheeses were determined from calibration curves prepared using authentic standards.

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

**DNA extraction from cheeses.** Total microbial DNA was extracted from up to four uninoculated 3.0 ± 0.1-g portions of each cheese as follows. Each cheese portion was diluted 10-fold in sterile PBS and homogenized as described above for *L. monocytogenes* enumeration. DNA extraction was then conducted on 1.0-mL aliquots of the homogenates using the DNeasy PowerFood microbial kit (Qiagen, Inc., Toronto, ON, Canada) following the manufacturer's directions.

**Library preparation and sequencing.** For 16S rRNA gene targeted amplicon sequencing, dual-indexed sequencing libraries were prepared as described previously (66). Briefly, a one-step 10-$\mu$L PCR was performed on a LabCyte Access workstation using Quanta 5PRIME HotMasterMix with 1 ng input DNA. Amplification was conducted using complete "fusion primers," which included Illumina Nextera adaptors, indices, and sequences targeting the V4-V5 region of the 16S rRNA gene (515F, 5′-GTGYCAGCMGCCGCGGTAA-3′; 906R, 5′-CCGYCAATTYMTTTRAGTTT-3′) (67). The resulting amplicons were quantified using a Quant-iT PicoGreen double-stranded DNA (dsDNA) assay kit (Thermo Fisher Scientific, Ottawa, ON, Canada), before pooling 2 ng from each sample. The pooled library was then subjected to cleanup using the AmpureXP PCR cleanup protocol (Beckman Coulter Life Sciences; Mississauga, ON, Canada) and quantified using a PicoGreen assay. Sequencing of the pooled library was conducted using an Illumina MiSeq sequencer with reagent kit v.3 (600 cycles) 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.

**Taxonomic and functional profiling of cheese microbiomes.** For the 16S rRNA gene targeted amplicon sequencing, the resulting sequences were processed using mothur software (v.1.43.0) (68) 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 (v.132) (69) before removal of chimeric sequences or sequences from nonprokaryotic 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 (70). Finally, the samples were all rarefied to an even depth of 3,000 reads per sample.

For metagenomic sequence analysis, sequence data were processed using the READ_QC module of the MetaWRAP metagenomic wrapper suite (v.1.3.2) (71). Cleaned sequences were then classified using Kraken 2 (v.2.1.2) (72), and species-level abundance was estimated using Bracken (v.2.5) (73). Taxonomic analysis of the shotgun metagenomic sequence data was conducted using only species from the kingdom *Bacteria*.

The identification of putative bacteriocin-encoding genes in cheese metagenomes was assessed by aligning the metagenomic assemblies against the BACTIBASE database (74) using blastx (75). Metagenomic assemblies for each cheese were prepared from the quality-filtered reads using metaSPAdes (76) through the ASSEMBLY module of the MetaWRAP metagenomic wrapper suite (v.1.3.2) (71). Only hits to putative bacteriocin-encoding genes with an E value of $<10^{-5}$ and similarity of >50% were retained. In cases where multiple hits to the BACTIBASE database were observed on a single contig, only the hit with the highest bit-score was retained.

**Statistical analysis.** All data analysis was conducted using R software (v.4.1.0) R Foundation for Statistical Computing, Vienna, Austria [https://www.Rproject.org/]) with the assistance of the tidyverse collection of packages (v.1.3.1) (77). Growth modeling was achieved using the growthcurver package (v.0.3.1) (78). Taxonomic analysis was conducted using phyloseq (v.1.36.0) (79), except for permutational multivariate analysis of variance (PERMANOVA), which was conducted using the vegan software package (v.2.5–7) (80), and differential abundance analysis, which was conducted using the ANCOMBC package (v.1.2.2) (81). For $\beta$ diversity analysis, dissimilarity between the microbial communities of cheeses was measured using the weighted UniFrac distance method (82). For differential abundance analysis, only

OTUs with at least 0.1% relative abundance (~10 reads) in at least three different cheeses were included in order to prevent taxa from a single cheese from affecting the results. For all statistical analysis, including ANOVA, *t* test, Wilcoxon rank sum test, Spearman's rank correlation, and PERMANOVA, the significance level ($\alpha$) was set at 0.05. To correct for multiple comparisons where necessary, *P* values were adjusted using the false-discovery rate (FDR) method and are labeled as *q* values.

**Data availability.** Sequence data are available at the NCBI Sequence Read Archive under BioProject ID PRJNA863305.

## SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

**SUPPLEMENTAL FILE 1**, PDF file, 0.5 MB.

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