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Farm-to-fork profiling of bacterial communities associated with an artisan cheese production facility

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ABSTRACT

The various stages of the cheese-making process harbor distinct bacterial communities which may influence the sensory characteristics of artisanal cheeses. The objective of this study was to investigate the microbiota from dairy farm to final cheeses along an artisanal cheese-making continuum. Environmental and food samples were collected from 21 sites, including the dairy farm, milk, cheese plant, and finished cheeses. The microbiota of these samples were analyzed using 16S rRNA amplicon sequencing, with sequences grouped into operational taxonomic units (OTUs) by phylotype at the genus level. Alpha diversity decreased from dairy farm to finished cheese. *Firmicutes* was the dominant phylum, ranging from 31% to 92% between the dairy farm and finished cheeses, respectively, with *Proteobacteria*, *Actinobacteria*, and *Bacteroides* also present (25%, 11%, and 9% overall relative abundance, respectively). Of the 37 core OTUs (> 5 reads in > 80% of site replicates) observed in cheese, 32 were shared with the dairy farm. Starter-related genera (*i.e.*, *Lactococcus*, *Lactobacillus*, *Streptococcus*, and *Leuconostoc*) represented between 69% and 98% relative abundance in final cheeses depending on style, with the remainder likely acquired from various environmental sources on the farm and during the cheese-making process.

1. Introduction

Cheese-making 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 harbors 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 and 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 and 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 (HTS) technologies has allowed deeper probing of the microbiota involved at various stages of dairy processing. Studies using 16S rRNA 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 and 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 present study used 16S rRNA 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.

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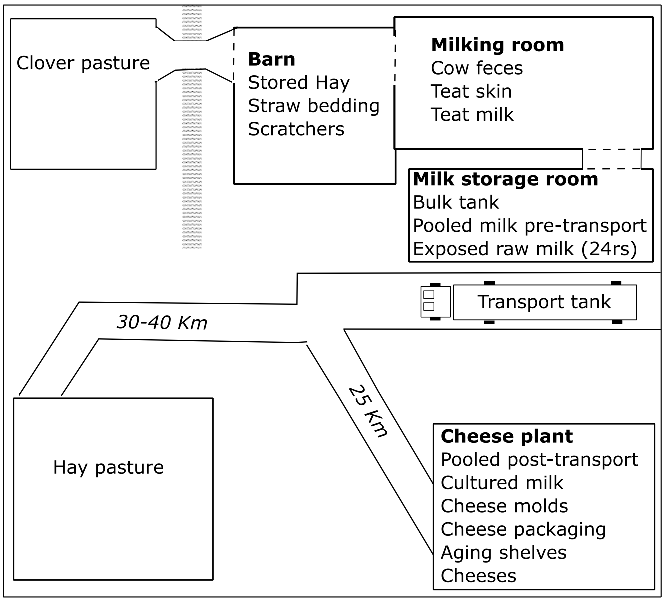

Fig. 1. Spatial layout of sampling sites along the farm-to-cheese continuum used in this study. The cows have open access to the clover pasture, which was separated from the barn by a small stream. Hay was transported in from a pasture 30–40 Km away. The cows were led into a separate room for milking, and the milk was pooled in a separate chilled room. Milk was transported 25 Km to the cheese plant where all cheeses were produced.

Table 1
Production characteristics of cheese samples evaluated in this study.

Style[a]	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[b]	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.

[a] The style of the cheese was that defined by the cheesemaker and carried on the packaging label.

[b] The curd was cooked at 52 °C for 30 min.

2. Materials and methods

2.1. Cheese production site

The site consisted of a cheese-making plant and a dairy farm that are approximately 25 Km apart in British Columbia, Canada (Fig. 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 h at 0–4 °C before being transferred into a transport tank and transported approximately 20 min down the road to the cheese plant. The milk at the cheese plant was sourced solely from the diary 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 1). All cultures are added directly into the cheese milk prior to coagulation.

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.) Different seasons were not sampled at this time to increase statistical power of the biological replicates and to ensure data reproducibility. Up to 20 independent (*i.e.*, separately collected) samples from 21 sample types were collected across four sample domains as outlined in Table S1: 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 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 h 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.

Samples types were obtained directly by aseptic collection or swabbed as described below. Environmental samples were collected from hay and clover pasture, ensuring each sample was 100 m apart, included soil and root clumps, and fresh sterile gloves were used for each sampling. 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 (~50 mg) 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 of four teats, including orifices, were sampled at the farm by dipping swabs in sterile water and then rubbing each teat individually for 15 s 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 (teat dipping and wiping) and then milk samples were collected by milking with the industrial milking equipment; 5 ml 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 h before being capped and frozen; flies were removed with aseptic technique after the 24hr period.

For swabbed samples, cotton-tipped swabs (Puritan Medical, Guilford, ME, USA) were dipped in sterile buffer (0.15 M NaCl and 0.1% Tween-20) and then rubbed vigorously in a zig zag pattern against a 17 × 17-cm surface for 60 s with the exception of packaging materials, which were swabbed for 2 min instead. Because of size limitations, the entire surface of the cheese molds were sampled for 60 s in a zig zag pattern. In addition, the scratcher was swabbed laterally and horizontally in an approximate 5 cm^3 region for 60 s. Aging shelves were

Table 2
Alpha diversity of samples 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, ACE) and evenness (*i.e.*, Shannon, Inv. Simpson, Fisher's alpha).

Sample Domain	Sample Type	N[a]	Observed OTUs[b]	Chao1	ACE	Shannon	Inv. Simpson	Fisher's alpha
Dairy Farm								
	Clover pasture	19	193 ± 23^{a}	218 ± 29^{ab}	217 ± 29^{ab}	3.82 ± 0.21^{a}	24.3 ± 5.6^{a}	34.0 ± 4.8^{a}
	Hay pasture	14	187 ± 16^{a}	209 ± 26^{a}	205 ± 19^{a}	3.81 ± 0.31^{a}	22.7 ± 9.0^{ab}	32.6 ± 3.4^{a}
	Hay	20	185 ± 38^{a}	223 ± 34^{ab}	225 ± 34^{ab}	2.77 ± 0.86^{bc}	9.67 ± 7.31^{cd}	32.5 ± 8.0^{a}
	Straw bedding	19	185 ± 27^{a}	221 ± 28^{ab}	217 ± 27^{ab}	3.58 ± 0.29^{ad}	19.3 ± 6.9^{ae}	32.4 ± 5.6^{a}
	Cow scratchers	19	207 ± 8^{a}	247 ± 23^{b}	247 ± 14^{b}	3.39 ± 0.08^{bd}	13.1 ± 1.4^{bce}	37.0 ± 1.7^{a}
	Cow feces	20	90 ± 11^{b}	115 ± 21^{c}	117 ± 23^{c}	2.55 ± 0.08^{c}	7.62 ± 0.76^{d}	13.6 ± 2.0^{b}
	Teat skin	17	178 ± 27^{a}	221 ± 35^{ab}	217 ± 28^{ab}	3.16 ± 0.35^{bcd}	11.6 ± 3.8^{cde}	30.8 ± 5.6^{a}
	Bulk tank[c]	12	187 ± 36^{a}	214 ± 27^{ab}	211 ± 29^{ab}	3.26 ± 0.70^{abd}	14.5 ± 11.8^{bcde}	32.8 ± 7.5^{a}
	Transport tank[c]	19	191 ± 26^{a}	218 ± 31^{ab}	214 ± 28^{a}	3.59 ± 0.31^{ad}	18.2 ± 6.1^{abe}	33.7 ± 5.3^{a}
Milk[d]								
	Teat milk[e]	14	139 ± 52^{a}	166 ± 54^{bc}	168 ± 48^{a}	2.17 ± 0.92^{a}	5.37 ± 5.16^{a}	23.3 ± 10.1^{a}
	Pooled pre-transport	16	144 ± 19^{a}	175 ± 21^{ab}	179 ± 21^{ab}	2.69 ± 0.25^{a}	6.63 ± 1.86^{ab}	23.9 ± 3.8^{a}
	Pooled post- transport	17	201 ± 42^{a}	231 ± 35^{a}	228 ± 38^{b}	3.34 ± 0.84^{a}	14.4 ± 5.7^{b}	36.0 ± 8.1^{a}
	Exposed raw milk[f]	18	70 ± 16^{b}	113 ± 39^{c}	113 ± 31^{c}	0.445 ± 0.109^{b}	1.16 ± 0.06^{c}	10.2 ± 2.7^{b}
	Cultured milk	19	76 ± 29^{b}	118 ± 51^{c}	119 ± 38^{c}	0.658 ± 0.396^{b}	1.57 ± 0.53^{c}	11.3 ± 4.9^{b}
Cheese Plant								
	Cheese molds	19	165 ± 46^{a}	195 ± 40^{a}	196 ± 41^{a}	2.82 ± 0.84^{a}	10.8 ± 8.5^{a}	28.4 ± 9.4^{a}
	Aging shelves[g]	20	97 ± 22^{b}	143 ± 26^{b}	144 ± 18^{b}	1.41 ± 0.25^{b}	2.64 ± 0.57^{b}	15.0 ± 4.0^{b}
	Cheese packaging	18	199 ± 41^{a}	222 ± 39^{a}	223 ± 38^{a}	3.29 ± 0.87^{a}	16.3 ± 11.8^{a}	35.4 ± 8.8^{a}
Cheese[h]								
	Cheddar	11	42 ± 6^{ac}	75 ± 33^{ab}	78 ± 25^{ab}	0.272 ± 0.146^{a}	1.12 ± 0.12^{a}	5.59 ± 0.87^{ac}
	Gruyere	9	65 ± 17^{b}	95 ± 24^{b}	104 ± 26^{b}	0.985 ± 0.244^{b}	2.11 ± 0.58^{b}	9.36 ± 2.97^{b}
	Jarlsberg	8	57 ± 17^{ab}	98 ± 20^{b}	104 ± 16^{b}	0.746 ± 0.329^{b}	1.69 ± 0.60^{b}	8.10 ± 2.71^{ab}
	Brie	15	38 ± 6^{c}	58 ± 25^{a}	56 ± 11^{a}	0.920 ± 0.485^{b}	2.13 ± 0.95^{b}	5.05 ± 0.95^{c}

ACE = Abundance-based coverage estimator.

[a] Number of independently collected and sequenced samples.

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

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

[d] All milk samples were unpasteurized.

[e] Teat milk was collected directly from the teat before pooling.

[f] Milk was exposed to the air in the chilled milk storage room for 24 h.

[g] Aging shelves only came into direct contact with Gruyere cheese.

[h] Cheddar, Gruyere, and Jarlsberg cheeses were made using unpasteurized milk; Brie was produced using pasteurized milk.

swabbed in distinct locations between aging rounds of Gruyère cheese.

For cheese samples, sale-ready wedges/blocks of aged cheeses, produced from milk separately collected from the same cows included in this study, were collected from the retail store at the cheese plant. 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 10 mm of the outer surface of the cheese was aseptically removed and not included.

2.3. DNA extraction, 16s rRNA gene amplicon library preparation and sequencing

Sample bags were opened under aseptic conditions after thawing. Approximately 5 g of hay, clover, and pasture samples were re-suspended in 25 mL of sterile HyClone DPBS +/+ in TEMPO® filter bags (biomerieux SA; 80015) and processed in a Stomacher 400 circulator at 230 rpm for 2 min, before being filtered and spun at 2500 × *g* for 30 min to isolate microbiota. For all samples, prior to DNA extraction, cells were mechanically lysed with a 10 min 65 °C incubation prior to physical disruption using a FastPrep instrument (MP Biomedicals, Solon, OH) for two 60 s 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. 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.

2.4. Data processing and sequence analysis

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, 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).

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; Springer-Verlag, New York [http://ggplot2.org]) except for the Venn diagram, which was produced using the VennDiagram package (version 1.6.20; Chen and Boutros, 2011; [https://CRAN.R-project.org/package=VennDiagram]). Dunn's tests were conducted using the DescTools package with $\alpha = 0.05$ (version 0.99.23; Signorell, 2017 [http://CRAN.R-project.org/package=DescTools]).

We analyzed α-diversity using a dataset rarefied to 10,000 reads per sample, resulting in a loss of 33 samples (~9%) (Table 2). Compositional and β-diversity, on the other hand, 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.

3. Results and discussion

3.1. Sequencing results and α-diversity

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. This method, however, reduces the likelihood of inflated richness due to random sequencing errors.

The α-diversity was measured as the richness (total OTUs, Chao1, abundance-based coverage estimator) and diversity (Shannon diversity index, inverse Simpson index, Fisher's Alpha) of and within sample domains. Both richness and diversity differed significantly between sample domains across all measures (Kruskal-Wallace test; $p < 0.001$; Fig. 2). The α-diversity was the lowest overall in cheese and highest in the dairy farm environment across all measures (Dunn's 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 already been determined (Frétin et al., 2018). This finding suggests that the cheese making process favours only a small subset of non-starter microbiota, most being crowded out by starter-related taxa.

Table 2 shows that α-diversity also differed within each of the sample domains. Within the dairy farm domain, both richness and evenness were, in general, lower in cow feces than in the other sample types, but this was only significant for observed OTUs, Chao1, ACE, and Fisher's alpha measurements (Dunn's tests with Holm-Bonferroni correction; $p < 0.05$). This observation may be driven by the high proportion of "unclassified" OTUs in cow feces (~85%), which would make the lower diversity only an artifact of our analysis and not a true difference. Cultured milk and raw milk exposed to air for 24 h were significantly less rich and even compared with other raw milk samples by all measures accept for Chao1 (Dunn's tests with Holm-Bonferroni correction: $p < 0.05$); probably driven by the fact that cultured and raw milk samples were overwhelmingly populated by *Lactococcus* (83% relative abundance) and *Pseudomonas* (92% relative abundance), respectively (Fig. 4). Richness was also higher in teat milk than pooled raw milk after transport but only significantly for Chao1 and ACE measures (Dunn's 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). Diversity was significantly lower on the aging shelves than on the packaging materials or the cheese mold surface within the cheese plant (Dunn's 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's 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 measures (Dunn's 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) compared with the other three cheeses.

3.2. Taxonomic characterization

Table 3 and Figs. 3 and 4 respectively show the taxonomic profile of the sample domains and types. 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% relative abundance) to the final cheese (92.1% relative abundance). 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).

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 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 and Wells, 2016; Wong et al., 2016), with *Bacteroides* as the second largest genus (Kim and 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

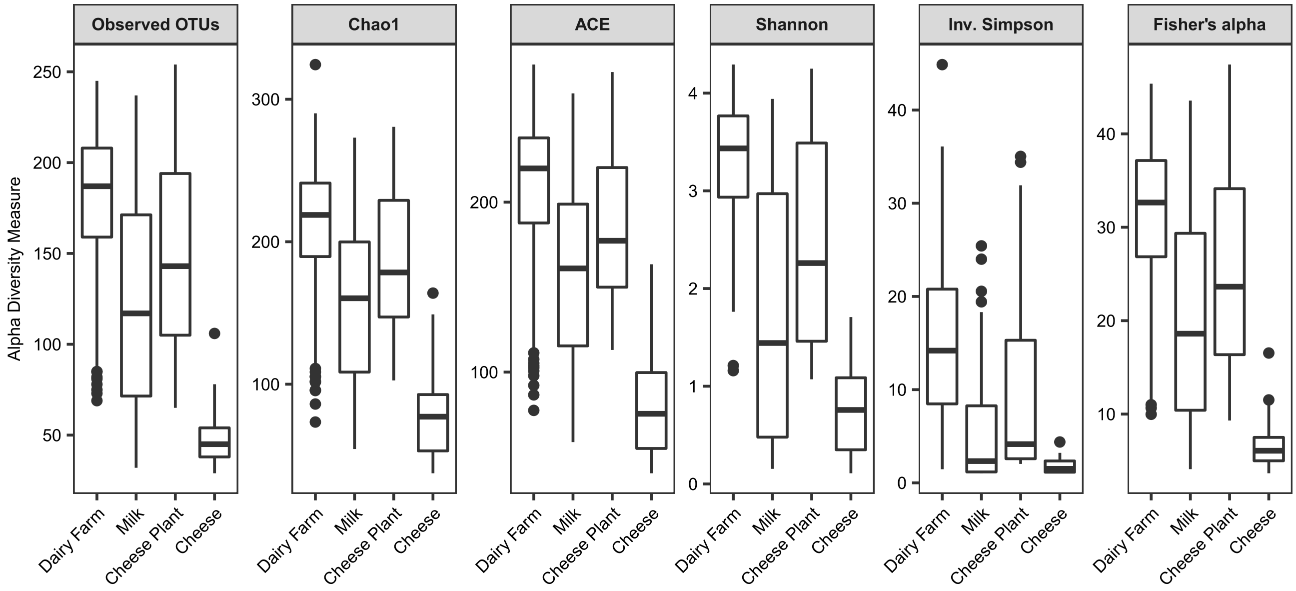

Fig. 2. Alpha diversity of sample domains after rarefying to an even depth of 10,000 reads per sample. Alpha diversity is measured as the richness (*i.e.*, Observed OTUs, Chao1, Abundance-based coverage estimator [ACE]) and evenness (*i.e.*, Shannon, Inv. Simpson, Fisher's alpha).

Table 3
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
Bacteroidetes	8.94	15.5	7.38	2.97	0.21
Unclassified	7.32	14.9	1.60	2.53	0.26

Bacteroidetes phylum, and *Actinomycetales* (7%–10% relative abundance) from the *Actinomycetes* phylum were also represented, more in pasture samples than from stored hay. The microbial composition of the hay and 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 and Stavrinides, 2015), and might capitalize on reduced plant immunity after harvest, leading to an increase in growth.

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* (51%-53% relative abundance), 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,

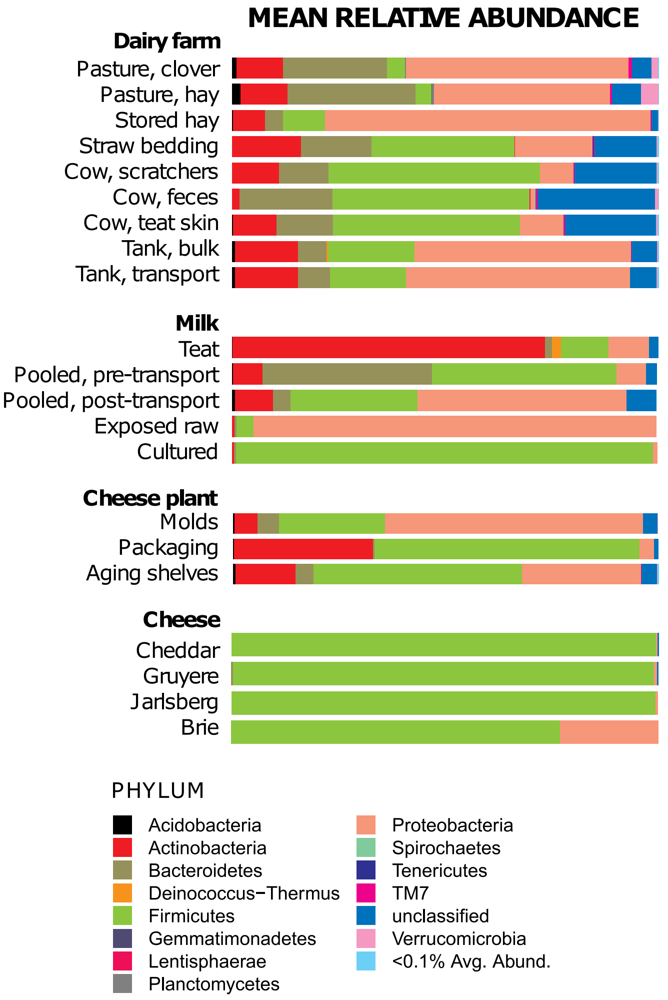

Fig. 3. Relative abundances of the most common phyla in the dairy farm environment, milk samples, cheese plant, and finished cheeses. Common phyla were defined as any phylum representing greater than 0.1% average relative abundance across sample replicates.

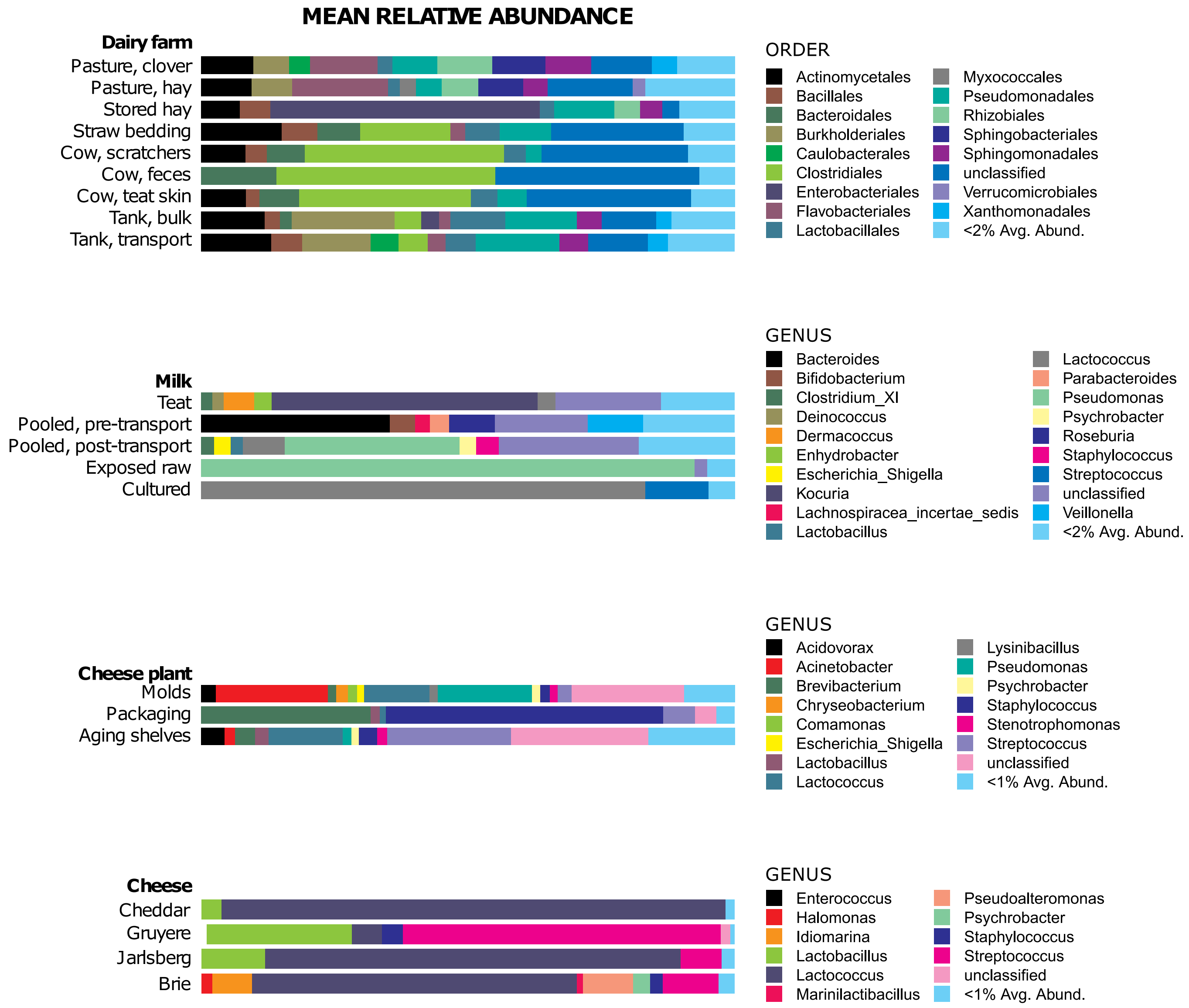

Fig. 4. Relative abundances of the most common orders or genera in the dairy farm environment, milk samples, cheese plant, and finished cheeses.

the sanitation step of the transport tanks might result in the added milk defining the shared microbiota.

Firmicutes was the most abundant phylum overall in the milk samples (43% relative abundance), followed by *Proteobacteria* (33% relative abundance), as previously observed (Quigley et al., 2013), but the abundance differed considerably among individual sample types. The *Actinobacteria* phylum notably dominated the teat milk samples (73% relative abundance), primarily from the genus *Kocuria*, but also *Dermacoccus* and *Dietzia*. The *Firmicutes* (*Lactococcus* and *Clostridium* XI) and *Proteobacteria* (*Enhydrobacter* and *Psychrobacter*) phyla were represented at low levels in the teat milk samples (11% and 9.5% relative abundance 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* (43% and 40% relative abundance, 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, 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* (33%, 3%, and 1% relative abundance, 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 h 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). 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.

Once at the cheese-making facility, the milk was 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%.

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.

The microbiota of the cheese molds and the cheese packaging differed, with the cheese molds showing higher proportions of the genera *Acinetobacter*, *Comamonas*, *Pseudomonas*, *Lysinibacillus*, *Escherichia/Shigella*, and *Chryseobacterium*; whereas the cheese packaging showed a higher relative abundance of *Streptococcus*. This difference may result from the direct contact that the cheese molds have with the raw cheese curds, with a selection against these observed genera through the cheese production/aging. Differences in microbiota of various locations of a cheese processing plant have previously been described (Bokulich and Mills, 2013).

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 and 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; 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.

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%) that were not part of the starter culture of Cheddar. *Lactobacillus* was also omitted as a starter in the Cheddar cheese. This finding indicates that the starter genera cannot be attributed solely to the starter culture, and NSLAB strains may influence 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; 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 bacteria 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 a higher 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 higher relative abundance in Gruyère which is cooked at 52 °C for 30 min.

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 higher 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 a lower 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 and 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).

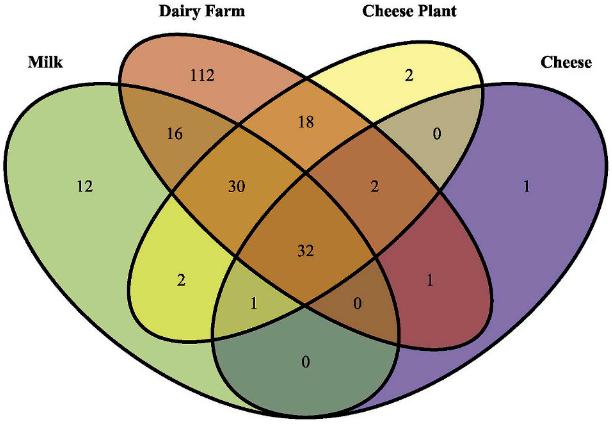

Fig. 5. Venn diagram of core OTUs within each sample domain. Core OTUs were defined as any OTU that showed at least 5 reads in > 80% of replicates of at least on sample type within that domain.

3.3. Shared- and co-occurrence of core OTUs across sample domains

Core OTUs were assessed to determine common genera across all sample domains. Fig. 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.

Of the 102 shared core OTUs, 59 of them were identifiable at the genus level and are shown in Table 4. 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.

3.4. β-Diversity and background microbiota

We analyzed β-diversity to determine similarities between the microbiota of the various sample types and domains and conducted principle coordinate analyses using Jaccard and Bray-Curtis methods. 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. S1). Normal confidence ellipses around each sample domain showed considerable overlap, except 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.

We generated a dendrogram based on the Jaccard distance between samples to further investigate compositional relationships between the sample types (Fig. 6A). 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. The third main cluster contained samples from hay and pasture, teat milk, raw milk after exposure to air for 24 h, bulk and transport tanks, the cheese plant samples, and Gruyère cheese.

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), while all but one of the core OTUs of the cheese domain are shared with other domains (Fig. 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. 5), and since the other farm samples clustered closely with other domains, many of those unique 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).

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, therefore affecting the surface microbial composition 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 differs 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 targeted amplicon sequencing, it is possible that the milk coming in to the cheese

Table 4
Taxonomic classification of core OTUs[a] shared between at least two domains and identified at the genus level.

Phylum	Family	Genus	DF[b]	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	

[a] Core OTUs were defined as any OTU that showed at least 5 reads in > 80% of replicates of at least one sample type within that domain.
[b] DF = dairy farm, M = milk, CP = cheese plant, Ch = 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 5 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 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.

Fig. 6Bshows a reassessment of sample relatedness based only on background microbiota. 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

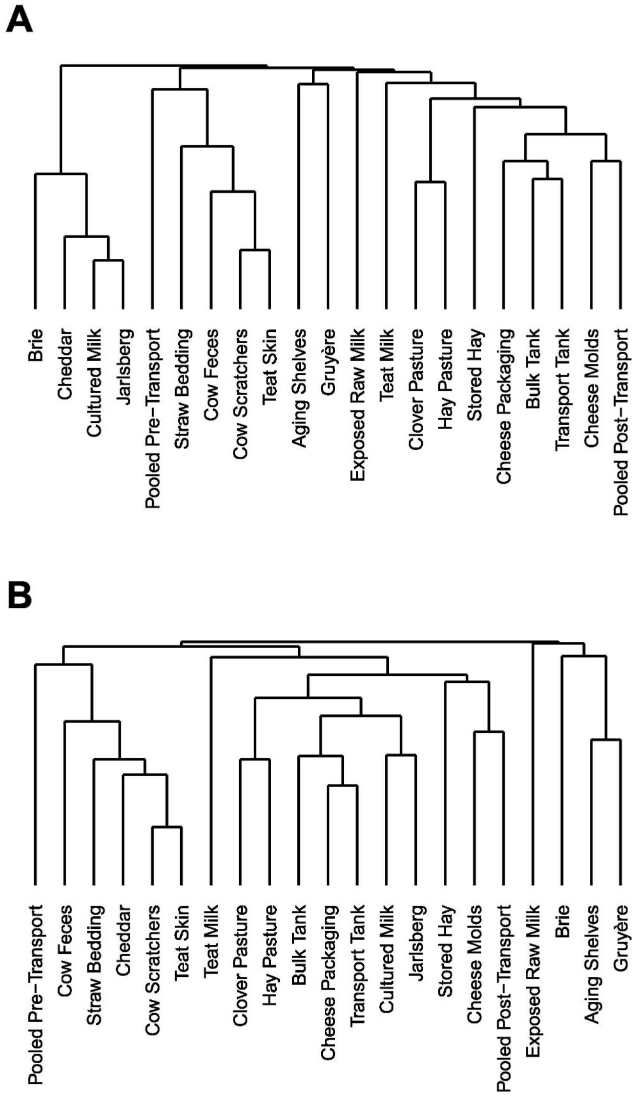

Fig. 6. Dendrograms showing the relatedness between sample types with (A) and without (B) the presence of starter-associated genera, (*Lactococcus*, *Lactobacillus*, *Streptococcus*, and *Leuconostoc*), using the Jaccard dissimilarity method. Dendrograms were produced using data that had been rarefied 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.

Table 5
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}
Gruyère	No	0.0878 ± 0.0720^{a}
Jarlsburg	No	0.0155 ± 0.0064^{a}
Brie	Yes	0.313 ± 0.242^{b}

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

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 micro-organisms 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 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 and 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.

4. Conclusions

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, processing environment, and diary farm can be fully elucidated.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2019.04.002.

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References

Bartram, A.K., Lynch, M.D.J., Stearns, J.C., Moreno-Hagelsieb, G., Neufeld, J.D., 2011. Generation of multimillion-sequence 16S rRNA gene libraries from complex microbial communities by assembling paired-end Illumina reads. Appl. Environ. Microbiol. 77, 3846–3852. https://doi.org/10.1128/AEM.02772-10.

Bokulich, N.A., Lewis, Z.T., Boundy-Mills, K., Mills, D.A., 2016. A new perspective on microbial landscapes within food production. Curr. Opin. Biotechnol. 37, 182–189.

https://doi.org/10.1016/j.copbio.2015.12.008.

Bokulich, N.A., Mills, D.A., 2013. Facility-specific "house" microbiome drives microbial landscapes of artisan cheesemaking plants. Appl. Environ. Microbiol. 79, 5214–5223. https://doi.org/10.1128/AEM.00934-13.

Bokulich, N.A., Subramanian, S., Faith, J.J., Gevers, D., Gordon, J.I., Knight, R., Mills, D.A., Caporaso, J.G., 2013. Quality-filtering vastly improves diversity estimates from Illumina amplicon sequencing. Nat. Methods 10, 57–59. https://doi.org/10.1038/nmeth.2276.

Bulgarelli, D., Schlaeppi, K., Spaepen, S., van Themaat, E.V.L., Schulze-Lefert, P., 2013. Structure and functions of the bacterial microbiota of plants. Annu. Rev. Plant Biol. 64, 807–838. https://doi.org/10.1146/annurev-arplant-050312-120106.

Chen, H., Boutros, P.C., 2011. VennDiagram: a package for the generation of highly-customizable Venn and Euler diagrams in R. BMC Bioinf. 12, 35. https://doi.org/10.1186/1471-2105-12-35.

Coton, M., Delbés-Paus, C., Irlinger, F., Desmasures, N., Le Fleche, A., Stahl, V., Montel, M.C., Coton, E., 2012. Diversity and assessment of potential risk factors of Gram-negative isolates associated with French cheeses. Food Microbiol. 29, 88–98. https://doi.org/10.1016/j.fm.2011.08.020.

Delcenserie, V., Taminiau, B., Delhalle, L., Nezer, C., Doyen, P., Crevecoeur, S., Roussey, D., Korsak, N., Daube, G., 2014. Microbiota characterization of a Belgian protected designation of origin cheese, Herve cheese, using metagenomic analysis. J. Dairy Sci. 97, 6046–6056. https://doi.org/10.3168/jds.2014-8225.

Donnelly, C.W., 2014. From Pasteur to probiotics: a historical overview of cheese and microbes. In: Donnelly, C.W. (Ed.), Cheese and Microbes. ASM Press, Washington D.C., pp. 1–15.

Doyle, C.J., Gleeson, D., O'Toole, P.W., Cotter, P.D., 2017. Impacts of seasonal housing and teat preparation on raw milk microbiota: a high-throughput sequencing study. Appl. Environ. Microbiol. 83, 1–12. https://doi.org/10.1128/AEM.02694-16.

Dugat-Bony, E., Straub, C., Teissandier, A., Onésime, D., Loux, V., Monnet, C., Irlinger, F., Landaud, S., Leclercq-Perlat, M.N., Bento, P., Fraud, S., Gibrat, J.F., Aubert, J., Fer, F., Guédon, E., Pons, N., Kennedy, S., Beckerich, J.M., Swennen, D., Bonnarme, P., 2015. Overview of a surface-ripened cheese community functioning by meta-omics analyses. PLoS One 10, 1–25. https://doi.org/10.1371/journal.pone.0124360.

Dungan, R.S., 2012. Use of a culture-independent approach to characterize aerosolized bacteria near an open-freestall dairy operation. Environ. Int. 41, 8–14. https://doi.org/10.1016/j.envint.2011.12.004.

Ercolini, D., Russo, F., Ferrocino, I., Villani, F., 2009. Molecular identification of mesophilic and psychrotrophic bacteria from raw cow's milk. Food Microbiol. 26, 228–231. https://doi.org/10.1016/j.fm.2008.09.005.

Estendorfer, J., Stempfhuber, B., Haury, P., Vestergaard, G., Rillig, M.C., Joshi, J., Schröder, P., Schloter, M., 2017. The influence of land use intensity on the plant-associated microbiome of dactylis glomerata L. Front. Plant Sci. 8, 1–10. https://doi.org/10.3389/fpls.2017.00930.

Fierer, N., Jackson, R.B., 2006. The diversity and biogeography of soil bacterial communities. Proc. Natl. Acad. Sci. U.S.A. 103, 626–631. https://doi.org/10.1073/pnas.0507535103.

Frétin, M., Martin, B., Rifa, E., Isabelle, V.-M., Pomiès, D., Ferlay, A., Montel, M.-C., Delbès, C., 2018. Bacterial community assembly from cow teat skin to ripened cheeses is influenced by grazing systems. Sci. Rep. 8, 200. https://doi.org/10.1038/s41598-017-18447-y.

Fuka, M.M., Engel, M., Skelin, A., Redžepović, S., Schloter, M., 2010. Bacterial communities associated with the production of artisanal Istrian cheese. Int. J. Food Microbiol. 142, 19–24. https://doi.org/10.1016/j.ijfoodmicro.2010.05.008.

Gill, J.J., Sabour, P.M., Gong, J., Yu, H., Leslie, K., Griffiths, M.W., 2006. Characterization of bacterial populations recovered from the teat canals of lactating dairy and beef cattle by 16S rRNA gene sequence analysis. FEMS Microbiol. Ecol. 56, 471–481. https://doi.org/10.1111/j.1574-6941.2006.00091.x.

Goerges, S., Mounier, J., Rea, M.C., Gelsomino, R., Heise, V., Beduhn, R., Cogan, T.M., Vancanneyt, M., Scherer, S., 2008. Commercial ripening starter microorganisms inoculated into cheese milk do not successfully establish themselves in the resident microbial ripening consortia of a south German red smear cheese. Appl. Environ. Microbiol. 74, 2210–2217. https://doi.org/10.1128/AEM.01663-07.

Kable, M.E., Srisengfa, Y., Laird, M., Zaragoza, J., McLeod, J., Heidenreich, J., Marco, M.L., 2016. The core and seasonal microbiota of raw bovine milk in tanker trucks and the impact of transfer to a milk processing facility. mBio 7, 1–13. https://doi.org/10.1128/mBio.00836-16.

Kim, M., Wells, J.E., 2016. A meta-analysis of bacterial diversity in the feces of cattle. Curr. Microbiol. 72, 145–151. https://doi.org/10.1007/s00284-015-0931-6.

Laursen, M.F., Dalgaard, M.D., Bahl, M.I., 2017. Genomic GC-content affects the accuracy of 16S rRNA gene sequencing bsed microbial profiling due to PCR bias. Front. Microbiol. 8, 1–8. https://doi.org/10.3389/fmicb.2017.01934.

Mallet, A., Guéguen, M., Kauffmann, F., Chesneau, C., Sesboué, A., Desmasures, N., 2012. Quantitative and qualitative microbial analysis of raw milk reveals substantial diversity influenced by herd management practices. Int. Dairy J. 27, 13–21. https://doi.org/10.1016/j.idairyj.2012.07.009.

Marino, M., Innocente, N., Maifreni, M., Mounier, J., Walsh, A.M., 2017. Diversity within Italian Cheesemaking Brine-Associated Bacterial Communities Evidenced by Massive Parallel 16S rRNA Gene Tag Sequencing, vol. 8. pp. 1–13. https://doi.org/10.3389/fmicb.2017.02119.

Masoud, W., Vogensen, F.K., Lillevang, S., Abu Al-Soud, W., Sørensen, S.J., Jakobsen, M., 2012. The fate of indigenous microbiota, starter cultures, *Escherichia coli, Listeria innocua* and *Staphylococcus aureus* in Danish raw milk and cheeses determined by pyrosequencing and quantitative real time (qRT)-PCR. Int. J. Food Microbiol. 153, 192–202. https://doi.org/10.1016/j.ijfoodmicro.2011.11.014.

McMurdie, P.J., Holmes, S., 2013. Phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. PLoS One 8. https://doi.org/10.1371/journal.pone.0061217.

Montel, M.C., Buchin, S., Mallet, A., Delbes-Paus, C., Vuitton, D.A., Desmasures, N., Berthier, F., 2014. Traditional cheeses: rich and diverse microbiota with associated benefits. Int. J. Food Microbiol. 177, 136–154. https://doi.org/10.1016/j.ijfoodmicro.2014.02.019.

Mounier, J., Goerges, S., Gelsomino, R., Vancanneyt, M., Vandemeulebroecke, K., Hoste, B., Brennan, N.M., Scherer, S., Swings, J., Fitzgerald, G.F., Cogan, T.M., 2006. Sources of the adventitious microflora of a smear-ripened cheese. J. Appl. Microbiol. 101, 668–681. https://doi.org/10.1111/j.1365-2672.2006.02922.x.

Mounier, J., Monnet, C., Vallaeys, T., Arditi, R., Sarthou, A.S., Hélias, A., Irlinger, F., 2008. Microbial interactions within a cheese microbial community. Appl. Environ. Microbiol. 74, 172–181. https://doi.org/10.1128/AEM.01338-07.

Pangallo, D., Šaková, N., Koreňová, J., Puškárová, A., Kraková, L., Valík, L., Kuchta, T., 2014. Microbial diversity and dynamics during the production of May bryndza cheese. Int. J. Food Microbiol. 170, 38–43. https://doi.org/10.1016/j.ijfoodmicro.2013.10.015.

Poretsky, R., Rodriguez-R, L.M., Luo, C., Tsementzi, D., Konstantinidis, K.T., 2014. Strengths and limitations of 16S rRNA gene amplicon sequencing in revealing temporal microbial community dynamics. PLoS One 9. https://doi.org/10.1371/journal.pone.0093827.

Quigley, L., McCarthy, R., O'Sullivan, O., Beresford, T.P., Fitzgerald, G.F., Ross, R.P., Stanton, C., Cotter, P.D., 2013. The microbial content of raw and pasteurized cow milk as determined by molecular approaches. J. Dairy Sci. 96, 4928–4937. https://doi.org/10.3168/jds.2013-6688.

Quigley, L., O'Sullivan, O., Beresford, T.P., Ross, R.P., Fitzgerald, G.F., Cotter, P.D., 2012. High-throughput sequencing for detection of subpopulations of bacteria not previously associated with artisanal cheeses. Appl. Environ. Microbiol. 78, 5717–5723. https://doi.org/10.1128/AEM.00918-12.

Raats, D., Offek, M., Minz, D., Halpern, M., 2011. Molecular analysis of bacterial communities in raw cow milk and the impact of refrigeration on its structure and dynamics. Food Microbiol. 28, 465–471. https://doi.org/10.1016/j.fm.2010.10.009.

Rainard, P., 2017. Mammary microbiota of dairy ruminants: fact or fiction? Vet. Res. 48, 1. https://doi.org/10.1186/s13567-017-0429-2.

Rodrigues, M.X., Lima, S.F., Canniatti-Brazaca, S.G., Bicalho, R.C., 2017. The microbiome of bulk tank milk: characterization and associations with somatic cell count and bacterial count. J. Dairy Sci. 100, 2536–2552. https://doi.org/10.3168/jds.2016-11540.

Schloss, P.D., Westcott, S.L., Ryabin, T., Hall, J.R., Hartmann, M., Hollister, E.B., Lesniewski, R.A., Oakley, B.B., Parks, D.H., Robinson, C.J., Sahl, J.W., Stres, B., Thallinger, G.G., Van Horn, D.J., Weber, C.F., 2009. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl. Environ. Microbiol. 75, 7537–7541. https://doi.org/10.1128/AEM.01541-09.

Scott, R., Robinson, R.K., Wilbey, R.A., 1998. Cheesemakeing Practice, third ed. Springer Science + Business Media, New York, New York.

Signorell, A., 2017. DescTools: Tools for Descriptive Statistics. R package version 0.99.20. CRANhttps://doi.org/10.1016/j.foreco.2014.09.033.

Vacheyrou, M., Normand, A.C., Guyot, P., Cassagne, C., Piarroux, R., Bouton, Y., 2011. Cultivable microbial communities in raw cow milk and potential transfers from stables of sixteen French farms. Int. J. Food Microbiol. 146, 253–262. https://doi.org/10.1016/j.ijfoodmicro.2011.02.033.

Verdier-Metz, I., Gagne, G., Bornes, S., Monsallier, F., Veisseire, P., Delbès-Paus, C., Montel, M.C., 2012. Cow teat skin, a potential source of diverse microbial populations for cheese production. Appl. Environ. Microbiol. 78, 326–333. https://doi.org/10.1128/AEM.06229-11.

Větrovský, T., Baldrian, P., 2013. The variability of the 16S rRNA gene in bacterial genomes and its consequences for bacterial community analyses. PLoS One 8, 1–10. https://doi.org/10.1371/journal.pone.0057923.

von Neubeck, M., Baur, C., Krewinkel, M., Stoeckel, M., Kranz, B., Stressler, T., Fischer, L., Hinrichs, J., Scherer, S., Wenning, M., 2015. Biodiversity of refrigerated raw milk microbiota and their enzymatic spoilage potential. Int. J. Food Microbiol. 211, 57–65. https://doi.org/10.1016/j.ijfoodmicro.2015.07.001.

Walterson, A.M., Stavrinides, J., 2015. Pantoea: insights into a highly versatile and diverse genus within the *Enterobacteriaceae*. FEMS Microbiol. Rev. 39, 968–984. https://doi.org/10.1093/femsre/fuv027.

Wickham, H., 2009. ggplot2, Media. Springer New York, New York, NY. https://doi.org/10.1007/978-0-387-98141-3.

Wolfe, B.E., Button, J.E., Santarelli, M., Dutton, R.J., 2014. Cheese rind communities provide tractable systems for in situ and in vitro studies of microbial diversity. Cell 158, 422–433. https://doi.org/10.1016/j.cell.2014.05.041.

Wong, K., Shaw, T.I., Oladeinde, A., Glenn, T.C., Oakley, B., Molina, M., 2016. Rapid microbiome changes in freshly deposited cow feces under field conditions. Front. Microbiol. 7, 1–12. https://doi.org/10.3389/fmicb.2016.00500.

Yoon, J.-H., 2005. *Psychrobacter celer* sp. nov., isolated from sea water of the South Sea in Korea. Int. J. Syst. Evol. Microbiol. 55, 1885–1890. https://doi.org/10.1099/ijs.0.63682-0.