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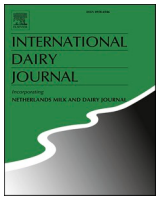

Research paper

Changes in microbiota during the cheese making process – from the raw milk to the final cheeses

Thomas M. Eliasson [a], Åse Lundh [a], Annika Höjer [b], Karin Hallin Saedén [b], Mårten Hetta [c,*], Li Sun [a,b,d]

[a] Department of Molecular Sciences, P.O. Box 7015, Swedish University of Agricultural Sciences, SE-750 07, Uppsala, Sweden
[b] Norrmejerier Ek. Förening, SE-906 22, Umeå, Sweden
[c] Department of Applied Animal Science and Welfare, Swedish University of Agricultural Sciences, SE-901 83, Umeå, Sweden
[d] Quantivisory Data Analysis, SE-756 59, Uppsala, Sweden

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ABSTRACT

The objectives were to evaluate effects of feeding different types of silages on the microbiota of the raw milk and the resulting cheeses. Different silages were produced without additives, acid treated and inoculated with a LAB starter culture, and each silage was fed to dairy cows during a three-week period. A brine-salted long ripened type of hard cheeses was produced and samples of milk, cheese curd and cheese during ripening were collected, and their microbiota characterised using 16S rRNA sequencing. Raw milk microbiota consisted of many different genera, the top 20 genera explaining 75 % of the relative abundance. In cheese *Lactococcus*, followed by *Leuconostoc* and *Lactobacillus,* were the dominant genus throughout the ripening, indicating a strong influence from the starter culture. Feeding the different silages did not result in differences in the microbiota in the raw milk or ripened cheeses. Results at amplicon sequence variant level showed that the relative abundance of LAB present both in the raw milk and in cheeses, decreased along the value chain.

1. Introduction

There is a growing research interest in understanding the development of non-starter lactic acid bacteria (NSLAB), which could grow under the selective pressure of ripening cheeses. The origin of NSLAB is still debated, but they are believed to enter the cheese vat with the raw milk (Martley & Crow, 1993). Cheese NSLAB often belong to the genus *Lactobacillus*, especially the species *Lactobacillus paracasei* (Antonsson et al., 2001). They begin to grow from very low numbers in the fresh cheese to 6–7 $\log_{10}$ CFU/g after a few weeks of cheese ripening, a level that may persist for several months during the ripening process (Barzideh et al., 2022). In a recent study, the milking system on-farm was identified as one major factor in the variation in raw milk microbiota (Sun et al., 2022). This is probably explained by differences in pre-treatment of the teats before milking, with some studies reporting that teat skin is an important vector of the milk microbiota (Verdier-Metz et al., 2012).

Improvements in hygiene on modern dairy farms and strict hygiene in the dairy facilities have resulted in total numbers of bacteria in raw milk that are often below 4 $\log_{10}$ CFU/mL (Glantz et al., 2020). The Swedish cheese in question in this study is reliant on NSLAB for development of its characteristic aroma, indicating that numbers of living NSLAB in the raw milk are now insufficient to develop and become dominant in the ripening cheeses in the same way as in the past (Rehn et al., 2010).

Previous studies have shown that farm management influences raw milk microbiota (Skeie et al., 2019; Sun et al., 2022; Verdier-Metz et al., 2009). However, many factors in those field studies were confounded, making it difficult to draw conclusions regarding the effects of individual components of variation. Feeding cows with silages preserved with different treatments, keeping all other factors constant, was evaluated as major factor behind any variation in bulk milk microbiota (Eliasson et al., 2024). The objective of the present study was to evaluate the microbiota in cheeses made from raw milk originating from that feeding experiment. The microbiota in milk samples collected from the tanker truck arriving at the cheese making facility and in samples taken from cheeses matured up to 22 months were characterised using the 16S rRNA (V4 region) gene sequencing approach. It was hypothesised that

* Corresponding author.
E-mail address: marten.hetta@slu.se (M. Hetta).

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the abundance of aroma producing NSLAB in raw milk, can be manipulated by the type of silage fed to the dairy cows that produce the milk for cheese production.

2. Materials and methods

The milk in this cheese making experiment comes from a feeding experiment (Eliasson et al., 2024), were different ensiling methods used to produce silages with and without conserving additives. Silage production took place in June and July 2020, and experimental diets based on the silages were fed to cows from January to April 2021. The Cheese production took place at a dairy facility in Burträsk (64°52′N, 20°66′E) during January to April 2021, and cheese ripening proceeded until February 2023. The cheeses produced were of the similar regional type as described by Rehn et al. (2010), a brine-salted, long ripened hard cheese.

2.1. Raw milk

The milk for the experiment was produced by approximately 67 Swedish Red dairy cows at Röbäcksdalen Research Farm, Swedish University of Agricultural Sciences (SLU), Umeå, Sweden (63°81′N, 20°23′E). The farm is part of the Swedish Infrastructure for Ecosystem Science (SITES) within SLU. All use of animals in the study and the experimental protocol were approved by the Swedish Ethics Committee on Animal Research (Permit A 6–2021), represented by the Court of Appeal for Northern Norrland in Umeå, in line with Swedish laws and regulations implementing EU Directive 2010/63/EU on animal research. The cows were kept in a free-stall barn, fed four experimental silages (each for a three-week experimental period) and milked in a milking parlour twice daily. The bulk milk was collected three times (every second day) in the last week of each three-week feeding period. The different silages fed to the cows during the 12-week experiment were produced with the following treatments: without additive (T1-UNTR), with inoculation by starter culture (T2-INOC and T4-INOC) and with acid treatment (T3-ACID), as described by Eliasson et al. (2024). The feeding of inoculated silage was repeated a second period to evaluate if an achieved effect on raw milk microbiota could be repeated by feeding the same silage a second time. Approximately 3500 L of milk were collected at the farm at each event and delivered within 2 h to the dairy facility in Burträsk. Upon arrival at the plant, the milk was pumped into a clean, empty dairy silo.

2.2. Cheese making

The milk was pre-heated to 55 °C and standardised by cream removal to a fat content of approximately 3.65 g/100 g milk. The standardised milk was then pasteurised at 72 °C for 15 s, followed by cooling and pumping to the cheese production facility. During pumping, 700–750 L was directed to a pilot cheese vat (SKH 800 HW Premium, Plevnik, Slovenia) for final cooling to 30 °C. A mesophilic direct vat set (DVS) starter culture was added at a rate of 0.135 units/L and the cheese milk was left for pre-ripening. According to the manufacturer's specification, the culture comprised *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* and unspecified *Leuconostoc*. After pre-ripening, bovine rennet (liquid rennet 75/25 180 IMCU, Caglificio Cerici, Italy) was added to the milk together with $CaCl_2$ and $NaNO_3$. Cheese making comprised long cooking periods (several hours) at temperatures above 40 °C. The cheese grains were transferred to moulds and pressed (Prepress PRP-R 120, Plevnik, Slovenia). Each pilot vat batch resulted in four round cheese wheels with a weight of approximately 18 kg each. The cheeses were brine-salted to a salt content of approximately 1.2 %, paraffined and ripened at specific temperatures between 10 °C and 13 °C in a dedicated cheese ripening facility.

2.3. Sampling

Raw milk were sampled from the balance tank just before pasteurisation. Fermented milk were sampled from the cheese vat after allowing the milk to pre-ripen with the mesophilic starter culture. Cheese curd was sampled after whey had been drained from the cheese vat, *i.e.* just before pressing. The raw milk, fermented milk, and cheese curd samples were all collected using a sterile metal scoop to fill 50-mL Falcon tubes. Fresh cheeses were sampled after 24 h of pressing cheesemaking, before they were transferred to the salt brine. During cheese ripening, cheese samples were taken at selected intervals over 22 months. Sampling of the cheeses was carried out using a sterile cheese drill, drilling out drill cores approximately 10 cm into the cheeses and discarding the outer 1–2 cm section. Each cheese wheel (in total four per test batch) was only sampled twice (on opposite sides), to avoid microbial contamination and excessive disturbance of the ripening process.

2.4. Sample preparation and analysis

For determination of chemical composition and bacterial enumeration, milk and cheese samples were analysed directly after sampling in the dairy laboratory facilities. Samples for microbiota analysis were frozen at −80 °C and transported to SLU, Uppsala, for further processing.

Gross chemical composition of milk samples were analysed using MilkoScan (FOSS Electric, Hillerød, Denmark) without further preparation. The cheese samples were grated before compositional analysis using FoodScan (FOSS Electric, Hillerød, Denmark). pH of milk and cheese samples was measured using a pH meter (Metler-Toledo 1120).

2.4.1. Milk samples

The number of cultivable bacteria in raw milk were estimated by plating undiluted or diluted (with peptone water) samples on different media. Total aerobic bacteria count were estimated by incubation on milk plate count agar (MPCA) at 30 °C for 72 h. Thermoresistant bacteria count were estimated by heating the milk to 63 °C for 38 min, followed by incubation on plate count agar (PCA) at 30 °C for 72 h. Number of psychrotrophic bacteria were estimated by incubation on PCA at 21 °C for 25 h. Number of *Enterobacteriaceae* were estimated by incubation on violet red bile agar with glucose (VRBG) at 37 °C for 24 h.

2.4.2. Cheese samples

The number of cultivable bacteria in cheese were estimated by running 10 g of cheese drill core in a stomacher with 90 g K_2HPO_4 buffer solution (2.7 %, pH 6.7). Dilution series of the emulsion from the stomacher were made in 0.1 % peptone water. Number of total aerobic bacteria were estimated by incubation on PCA at 30 °C for 72 h. Number of lactobacilli were estimated by anaerobic incubation on de Man, Rogosa, and Sharpe (MRS) agar (54.6 g/L MRS agar, Merck) without vancomycin at 30 °C for 72 h. The number of citrate-fermenting LAB in fresh cheese were determined by incubation on calcium citrate agar (KCA, ref. ISO17792/IDF180) at 25 °C for 72 h. At enumeration, the colonies were divided into three groups; i) acid-forming bacteria, ii) *Lactococcus lactis* sp. *diacetylactis* and iii) *Leuconostoc*. Colonies enumerated as *Leuconostoc* could also have been *Lactobacillus* or *Pediococcus*, but in fresh cheese those were expected to occur in negligible numbers. Yeast and moulds in fresh cheese were evaluated by incubation on yeast glucose chloramphenicol agar (YGC) at 25 °C for 120 h.

The sensory properties of the cheeses were evaluated at 12, 14, 18, 20 and 22 months of age. The cheeses were graded for sensorial properties using their defined criteria for "Västerbottensost" using a 9-graded scale according to a standardized method (Svenska ostklassiker). The evaluation was made by 2–3 trained sensory panellists from the dairy company.

2.5. Microbiota analysis

Frozen Falcon tubes (50 mL) with milk samples were thawed in a water bath at 25 °C for 1 h. The thawed milk was carefully mixed by inverting the tubes by hand a few times and then a 1.8 mL subsample was pipetted into 2-mL Collection tubes provided with the PowerFood DNA isolation kit (Qiagen AB, Sollentuna, Sweden). The frozen cheese drill cores were thawed at 21 °C for 1 h and 25 g portions were weighed into stomacher bags, followed by addition of 100 g Phosphate Buffered Saline solution. After running the stomacher for 2 min, 1.8 mL aliquots were transferred to lysis tubes provided with the PowerFood DNA isolation kit.

All tubes were centrifuged at 13 000 G at 4 °C for 15 min, followed by incubation on ice. The serum was carefully removed without disturbing the pellet or the fat layer. The DNA extraction proceeded from the above-described steps, using the customised protocol in Sun et al. (2023). The extracted bacterial DNA were then stored at −80 °C before being sent for amplification and sequencing. Additional samples of cheese at 22 months of age were treated with propidium monoazide (PMA) during the DNA extraction process to remove DNA derived from dead bacteria, as described in Nocker et al. (2007).

2.5.1. Library construction and sequencing

The bacterial DNA were sent to Novogene (Cambridge, UK) for library construction and sequencing. An initial quality control of the DNA was performed by agarose gel electrophoresis. The V4 region of the 16S rRNA gene was amplified using the primers 515f (GTGBCAGCMGCCGCGGTAA) and 805r (GACTACHVGGGTATCTAATCC), and a library was constructed. The library was checked with Qubit and real-time PCR for quantification and bioanalyzer for size distribution detection. Sequencing was performed on the Illumina NovaSeq PE250 platform (50k tags per sample). The raw reads were demultiplexed before delivery. The raw sequencing data were deposited in the Sequence Read Archive at the National Center for Biotechnology Information database, under accession number PRJNAXXXXXX.

2.5.2. Bioinformatics

Bioinformatic data processing were performed using QIIME 2 2022.11 (Bolyen et al., 2019). The raw de-multiplexed reads were trimmed with Cutadapt to remove primer sequences (Martin, 2011), and all reads containing non-identified bases or missing primer sequences were removed. Further trimming, de-nosing, de-replication, read merging, and removal of chimeras were performed with DADA2 (Callahan et al., 2016). Truncation length was set to 142 bp for forward reads and 161 bp for reverse reads, as it gave the best-read recovery after testing different levels of truncation. Phylogenetic trees were built using FastTree and MAFFT (Katoh & Standley, 2013; Price et al., 2010). Alpha- and β-diversity were estimated, and principal coordinate analysis (PCoA) was performed using the q2-diversity plugin. Faith's phylogenetic diversity index (FPDI; Faith, 1992) was used to compare diversity, while weighted UniFrac distance matrix (Lozupone et al., 2007) and PCoA results were used to compare microbiota composition between and within the sampling points. Taxonomy was assigned to ASVs with q2-feature-classifier (Bokulich et al., 2018), using release 138 from the Silva database (Quast et al., 2012) as reference. For ASVs with higher relative abundance (RA) not passing species annotation by QIIME2, selected ASVs were elaborated further using Nucleotide BLAST and the 16S ribosomal RNA sequences database as reference (accessed 2024-08-01), where only hits with 100 % query cover and identity were considered (Zhang et al., 2000).

2.6. Statistical evaluation

The raw output files (.qza) from QIIME 2 were imported to R (R Core Team, 2024) with the *qiime2R* package (Bisanz, 2018), together with all other data. Tables and figures were produced with R 4.4.0 using the *Tidyverse* package (Wickham et al., 2019). The statistical evaluations were performed with the additional packages *car* (Fox & Weisberg, 2019) and *emmeans* (Lenth, 2024). Pairwise comparisons of alpha-diversity for groups of samples or treatments, was performed according to Kruskal and Wallis (1952). Significant differences in beta-diversity were evaluated with PERMANOVA (Anderson, 2017). *p*-values were adjusted to avoid falsely rejected hypotheses (Benjamini & Hochberg, 1995), where $p \leq 0.05$ was considered significant. For evaluation of total bacteria count, arithmetic means were calculated by sampling group, sampling point, treatment or batch. Bacterial composition were evaluated by pooling the reads by technical replicates ($n = 2$), followed by rarefication at the lowest sampling depth found in the dataset (32033 reads/sample). Arithmetic means were calculated to the levels described in diagrams, and data were evaluated descriptively. Taxa found at below 0.1 % relative abundance (RA), comprising 32 rarefied reads, were considered as detected, but not as clear findings. For evaluation of treatment effects, Quasi-Poisson regression and pairwise comparisons with Tukey adjustment were performed per genus or ASV.

3. Results

3.1. Microbiota in the different materials

The samples were first categorised into four groups: i) raw milk, ii) starter culture, iii) cheese making (fermented milk, cheese curd, fresh cheese), and iv) cheese ripening (cheese at 4–22 months of age). The highest average alpha-diversity value, measured as FPDI was found in raw milk (24.2), followed by cheese ripening (18.6), cheese making (17.5) and starter culture (8.7) samples. Pairwise comparisons showed significant differences ($p \leq 0.05$) between all groups except for cheese ripening vs. cheese making ($p > 0.05$). Principal coordinate analysis (PCoA) of the weighted UniFrac distance matrix showed that the microbiota in raw milk diffed from that in the other sample groups, while the microbiota associated with starter culture, cheese making and cheese during ripening partly overlapped with each other (Fig. 1).

Further analysis of the weighted UniFrac distance matrix with PERMANOVA showed that the microbiota in all sample groups differed significantly ($p < 0.05$) except for starter culture vs. cheese ripening ($p > 0.05$). The microbiota of all sampling points is summarised on genus level in Fig. 2, which shows the average top 20 genera across all sampling points. The raw milk microbiota were explained by many different genera, where the top 20 genera explained ~75 % of RA. For the other groups, *Lactococcus* was the dominant and contributed most to RA, followed by *Leuconostoc* and *Lactobacillus* at varying RA.

3.2. Chemical composition, bacteria counts and microbiota in raw milk

The results obtained in the bacterial enumeration on different media and in analysis of chemical composition and pH in raw milk are summarised on batch level in Table 1. In general, the variation was more associated with individual batches than with the different silage treatments. The raw milk from treatments T2-INOC (B1) and T4-INOC (B2) stood out, with higher total bacteria counts on PCA and higher count of *Enterobacteriaceae*, while the latter also had exceptionally high counts of psychrotrophic bacteria. As regards chemical composition, a general tendency of higher dry matter content in treatment T4-INOC was observed.

The alpha-diversity (FPDI) of raw milk was numerically lower in treatments T2-INOC (23.5) and T3-ACID (23.9) than in T1-UNTR (24.6) and T4-INOC (24.9), although pairwise comparisons showed that only T2-INOC was significantly different from T1-UNTR and T4-INOC ($p < 0.05$). Further analysis of the weighted UniFrac distance matrix using PERMANOVA showed no significant difference between the silage treatments ($p > 0.05$). The raw milk microbiota is summarised on genus level in Fig. 3, which shows the average top 28 genera across all raw milk samples, together with the individual batches in each period.

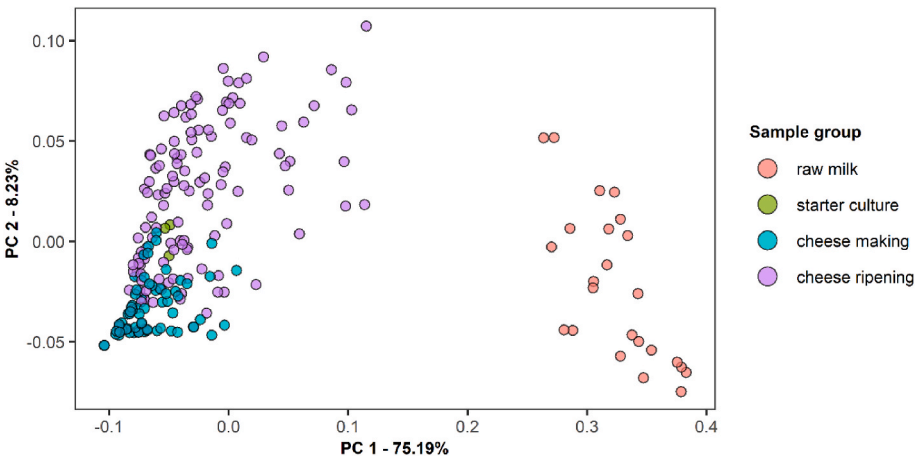

Fig. 1. Principal coordinate analysis plot (PCoA) of the weighted UniFrac distance matrix of microbiota in the different sample groups (type).

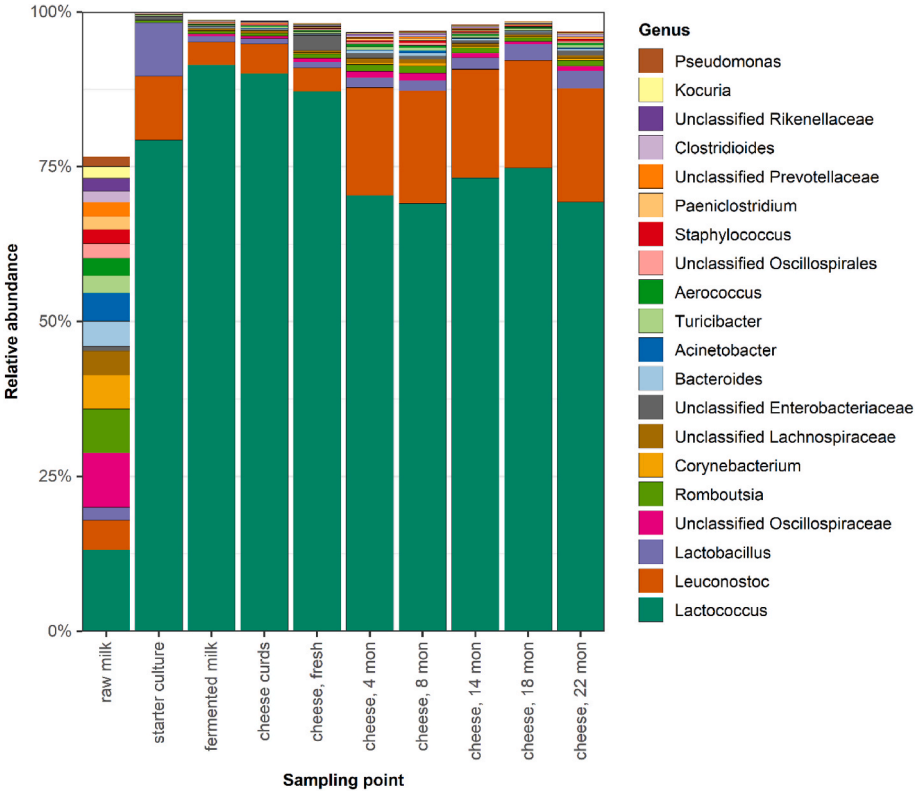

Fig. 2. Average top 20 genera across all sampling points along the value chain from raw milk to mature cheese including starters culture.

In the raw milk samples, there was more variation within treatments, *i.e.* between batches, than between treatments. Raw milk from the treatments T2-INOC (B1) and T4-INOC (B2) had high RA of *Acinetobacter* and *Pseudomonas*. In addition, raw milk from T4-INOC (B2) had high RA of *Staphylococcus*.

3.3. Bacterial enumeration and chemical gross composition of cheese grains and fresh cheese

The results from bacterial enumeration of cheese grains and cheese on various agar media and from analysis of chemical composition and pH in fresh cheese are summarised in Table 2. For most treatments and batches, only minor variations were observed. However, the results for *Enterobacteriaceae* varied between batches and in many dilutions the agar plates were overgrown (>3.7 $\log_{10}$ CFU/g). After addition of the mesophilic starter culture, the microbiota of the milk changed dramatically. Total bacteria count increased from 3.0 to 4.2 $\log_{10}$ CFU/mL in the raw milk to 6.1–6.6 $\log_{10}$ CFU/g in the cheese curds and 5.8–7.5 $\log_{10}$ CFU/g in fresh cheese. The numbers of *Leuconostoc* were below the detection limit in the fresh cheese samples, but the number of acid-producing bacteria in fresh cheese ranged from 6.5 to 6.8 $\log_{10}$ CFU/g on calcium citrate agar.

3.4. Microbiota of samples collected during cheese making

The microbiota of the samples obtained during cheese making, *i.e.* fermented milk, cheese curd and fresh cheese, changed dramatically from that in the raw milk, as seen in Figs. 1 and 2. Pairwise comparisons of alpha-diversity (FPDI) showed no significant change during cheese making, as there was no difference between fermented milk (17.2),

Table 1
Bacteria count (cfu/mL), chemical composition (g/100 g) and pH of raw milk by treatment (T1-T4) and batch (B1-B3) within treatments.

Treatment	T1-UNTR			T2-INOC			T3-ACID			T4-INOC		
Batch	B1	B2	B3	B1	B2	B3	B1	B2	B3	B1	B2	B3
Total bacteria count[a]	2000	3000	3000	9000	3000	5000	3000	6000	1000	3000	15000	<1000
Enterobacteriaceae	6	5	4	25	<1	1	3	4	5	2	32	2
Psychrotrophic bacteria	700	100	200	<100	400	<100	200	200	270	280	>5000	280
Thermoresistant bacteria	1	<1	2	<1	<1	<1	<1	5	1	1	<1	<1
Fat	4.15	4.20	3.92	4.24	4.02	4.12	4.17	4.24	4.18	4.36	4.31	4.29
Protein	3.34	3.32	3.62	3.49	3.37	3.38	3.36	3.40	3.32	3.57	3.53	3.58
Lactose	–	4.38	4.79	4.63	4.40	4.41	4.36	4.44	4.39	4.71	4.57	4.59
Dry matter	12.45	12.49	13.02	13.02	12.35	12.48	12.55	12.66	12.50	13.27	13.63	12.89
pH	6.74	6.75	6.72	6.67	6.72	6.70	6.72	6.74	6.72	6.76	6.71	6.73

Treatments: Feeding silages without additive (T1-UNTR), with inoculation by starter culture (T2-INOC and T4-INOC) and with acid treatment (T3-ACID), as described by Eliasson et al. (2024).
[a] Plate count on MPCA.

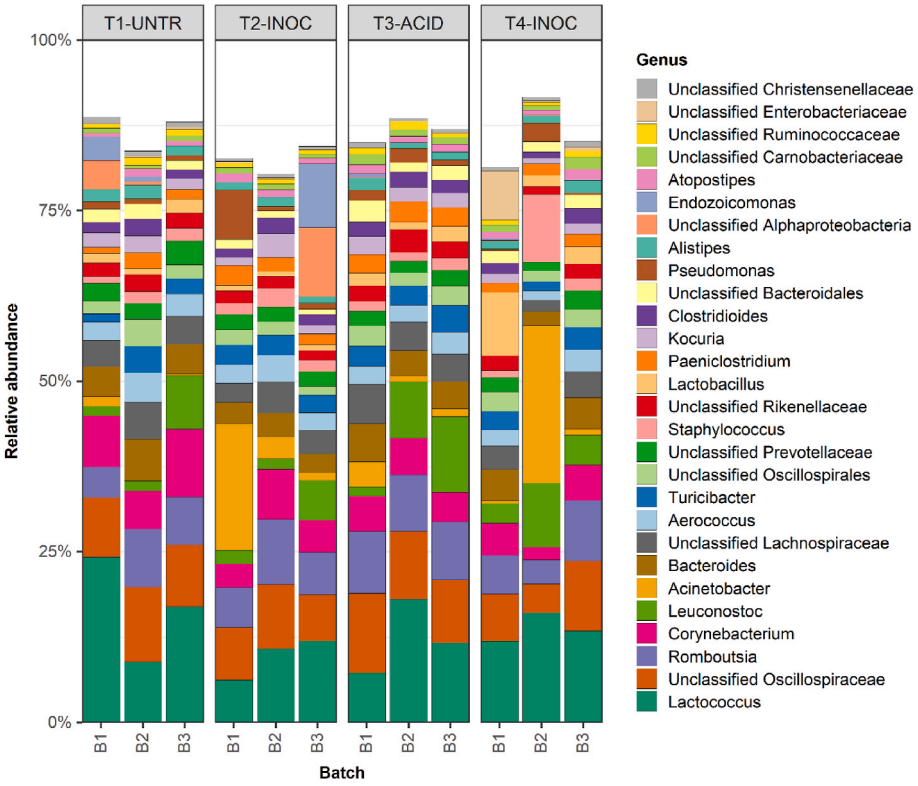

Fig. 3. Average top 28 genera in raw milk, across all samples by treatments (T1-T4) and production batch (B1-B3) within treatment. Treatments: Feeding silages without additive (T1-UNTR), with inoculation by starter culture (T2-INOC and T4-INOC) and with acid treatment (T3-ACID), as described by Eliasson et al. (2024).

cheese grains (17.1) and fresh cheese (17.9). However, in the pairwise comparisons of treatments, a significant difference ($p < 0.05$) was observed between samples from treatments T1-UNTR (20.5) and T2-INOC (15.2).

A PCoA plot of the weighted UniFrac distance matrix was created for the three sample types, to further evaluate the effect of the treatments (Fig. 4). A tendency for clustering between the treatments, independent of sampling point, was observed. Further analysis with PERMANOVA showed significant differences ($p < 0.05$) between T4-INOC and all other treatments and between T1-UNTR and T2-INOC.

For further evaluation of treatment differences, the top 25 amplicon sequence variants (ASVs) were selected based on their average RA across the three sampling points (Fig. 5). For most treatments and batches, dominance of *Lactococcus* (dd41a) was observed, followed by *Leuconostoc* (4b97e) at varying RA. *Lactobacillus* (5f86a) was observed in most of the treatments and batches, with a tendency for higher RA in treatment T4-INOC (B3), while *Lactobacillus* (4f10a and 7a5bc) were mainly observed in T4-INOC (B1). Unclassified *Enterobacteriaceae* (54780 and 05c1e) were observed at higher abundance in some batches of fresh cheese, especially those based on milk from treatments T1-UNTR (B3) and T4-INOC (B2), while *Enterobacteriaceae* (54780) were also more abundant in T4-INOC (B1).

3.5. Microbiota and quality attributes of cheese during ripening

Quality attributes of the cheese during ripening from 4 to 22 months of age are summarised by treatment and batch in Table 3. The microbiota of the ripening cheeses were first evaluated with PCoA on the weighted UniFrac distance matrix for each stage, from fresh cheese to cheese sampled at 22 months of ripening, and the results are summarised in Fig. 6 (panels A to F).

The microbiota in cheeses resulting from the same treatment varied

Table 2
Bacterial enumerations (log_{10} CFU/g), chemical gross composition (g/100 g) and pH of mainly fresh cheese samples by treatments (T1-T4) and batch (B1-B3) within treatments[a].

Treatment	T1-UNTR			T2-INOC			T3-ACID			T4-INOC		
Batch	B1	B2	B3	B1	B2	B3	B1	B2	B3	B1	B2	B3
Cheese grains												
Total bacteria	6.4	6.5	6.2	6.2	6.1	6.3	-[b]	6.6	6.4	6.6	6.4	6.7
Fresh cheese												
Total bacteria	6.3	6.0	6.2	6.1	5.7	6.3	5.7	7.5	5.8	6.6	6.2	6.4
Enterobacteriaceae	>3.7	>3.7	>3.7	>3.7	<1.0	>3.7	3.2	<1.0	1.7	1.9	>3.7	>3.7
Acid-producing	6.8	6.6	6.7	–	6.7	–	6.7	6.7	6.5	6.7	6.6	–
Leuconostoc	<3.0	<3.0	<3.0	–	<3.0	–	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0
Diacetyl[c]	5.2	5.1	4.5	–	5.0	–	4.8	4.5	4.8	5.0	4.9	–
Fat	30.0	31.3	31.2	31.4	31.5	31.2	31.4	31.4	31.8	31.1	31.3	31.1
Protein	26.2	25.3	25.2	25.6	25.7	26.0	26.2	26.1	25.6	26.2	26.0	25.7
Water	39.1	38.5	38.6	38.1	37.7	38.2	37.9	37.9	37.9	37.8	37.9	38.1
Fat in DM	49.3	50.9	50.8	50.8	50.6	50.5	50.6	50.6	51.3	50.0	50.4	50.2
Mositure non-fat solids	55.9	56.0	56.1	55.5	55.1	55.6	55.2	55.2	55.6	–	55.2	55.2
pH	5.29	–	5.44	5.39	5.38	5.37	5.42	–	–	5.35	5.40	5.40

[a] Treatments: feeding silages without additive (T1-UNTR), with inoculation by starter culture (T2-INOC and T4-INOC) and with acid treatment (T3-ACID), as described by Eliasson et al. (2024).
[b] Due to misunderstandings, some samples were not taken/analysed, and values denoted (-) are missing.
[c] *Lactococcus lactis* subsp. *diacetylactis*.

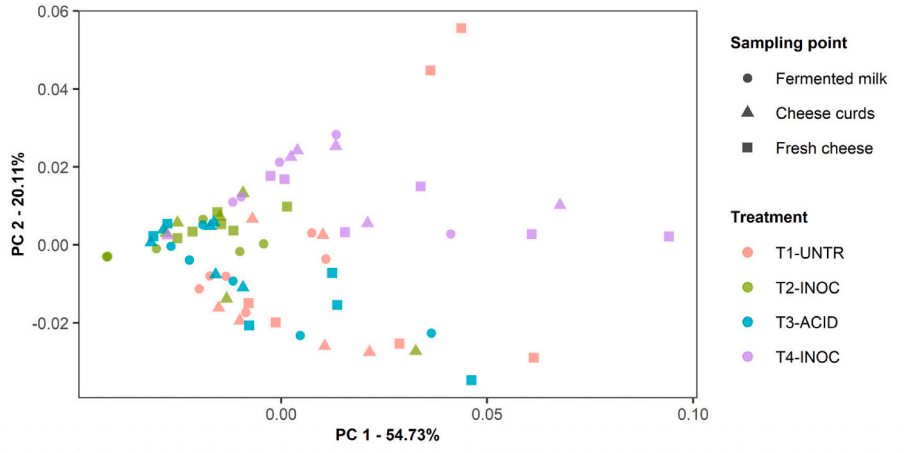

Fig. 4. Principal coordinate analysis plot of the weighted UniFrac distance matrix of the microbiota in relation to treatments and sampling points in the cheese making process. Treatments: Feeding silages without additive (T1-UNTR), with inoculation by starter culture (T2-INOC and T4-INOC) and with acid treatment (T3-ACID), as described by Eliasson et al. (2024).

throughout the ripening process, with a tendency for the microbiota in T2-INOC cheeses to vary less in the early stages of ripening (up to 14 months). In general, the microbiota in cheeses associated to the different silage treatments overlapped in most ripening stages. The differences between microbiota in the cheese batches within treatments were often larger than those between treatments, i.e., larger variation in the cheese microbiota due to effect of batch rather than to treatment. Multiple PERMANOVA analyses were performed for each ripening stage, to check for significant differences in cheese microbiota ($p < 0.05$) between the treatments. In fresh the cheese, treatment T2-INOC was found to differ from T1-UNTR and T4-INOC. In the cheese sampled at 4 months of ripening, all treatments differed except T3-ACID vs. T4-INOC. In the cheeses sampled at 8 and 14 months of ripening, T2-INOC differed from the other treatments, while in the cheeses sampled at 18 and 22 months of ripening, no differences were found. For further evaluation of differences between treatments and batches, the top 10 ASVs in the starter culture and in all cheeses from fresh to 22 months cheese were compared, as summarised in Fig. 7.

The most abundant ASVs at all ripening stages, for most of the batches, belonged to *Lactococcus* (dd41a) and *Leuconostoc* (4b97e). The cheeses from T1-UNTR (B3) and T4-INOC (B3) showed increasing RA of *Lactobacillus* (5f86a), while this ASV was also found in the other batches but at rather similar RA throughout ripening. *Leuconostoc* (46006) and *Romboutsia* (fa247) were found at varying levels in most batches and were present at higher levels in some batches. Unclassified *Enterobacteriaceae* (54780) was found at all stages of ripening in the cheeses from T1-UNTR (B3), in other batches only randomly at higher RA.

The microbiota in the cheeses at 22 months were also determined following PMA treatment of bacterial cells before amplification, to only amplify the DNA from intact cells, and the results were compared (Fig. 8). This showed that *Lactococcus* (dd41a) was clearly over-represented, while *Leuconostoc* (4b97e) and *Lactobacillus* (5f86a) were under-represented, when DNA from dead cells was included in DNA amplification.

The sensory grades of the cheese were in general low but increased with maturity (see grades for months 14, 18 and 22 in Table 3). The smell and taste of the cheeses had low intensity of the typical Västerbotten-cheese aroma but few off-flavors. In the evaluation of the cheese texture (mouth feel), there were comments related to a sandy cheese structure.

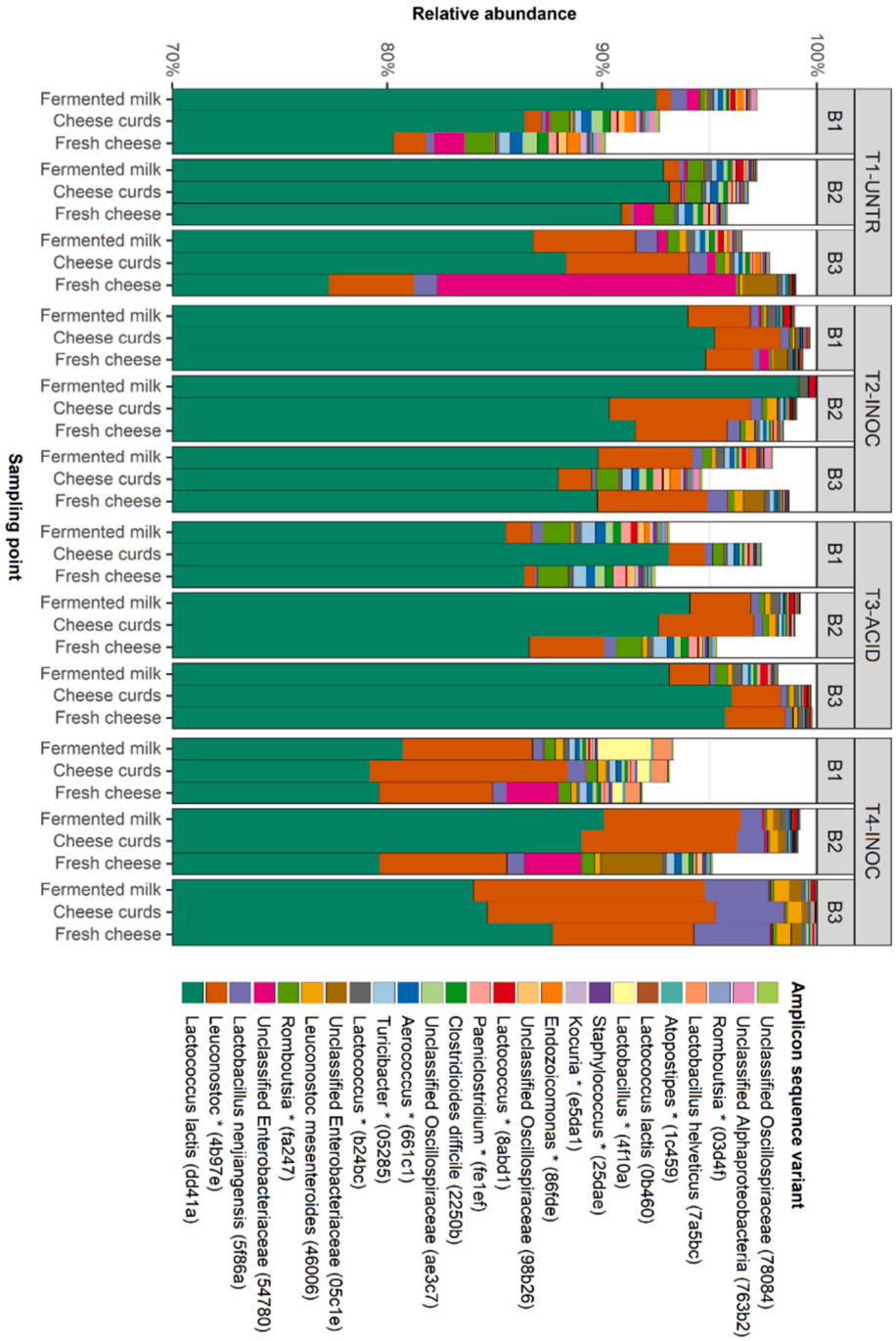

Fig. 5. Top 25 amplicon sequence variants (ASVs) in fermented milk, cheese curds and fresh cheese by treatment (T1-T4) and batch (B1-B3) within treatment. Y-axis scale cut at 70 % relative abundance for higher resolution. Treatments: Feeding silages without additive (T1-UNTR), with inoculation by starter culture (T2-INOC and T4-INOC) and with acid treatment (T3-ACID), as described by Eliasson et al. (2024).

3.6. Transfer of lactic acid bacteria from milk to cheese

The full ASV table was filtered to only include lactic acid bacteria (LAB, order *Lactobacillales*). With these ASVs, a final filtering was conducted batch-wise to select ASVs that were present in both replicates of both the raw milk and the cheese at 22 months. This resulted in a heatmap of unique ASVs that were clearly found at both sampling points (Fig. 9).

For the LAB ASVs present at both sampling points, there was a general trend for a decrease in RA from raw milk to the final cheeses. The only ASVs showing increased RA in cheese were *Lactobacillus* (5f86a), *Lactococcus* (dd41a) and *Leuconostoc* (4b97e), *i.e.* the ASVs making up the starter culture, and this was the case in all the batches. Furthermore, *Lactobacillus* (8a306) showed increased RA in treatment T4-INOC and *Leuconostoc* (46006) showed increased RA in all batches except B1 and B2 from T1-UNTR and B1 from T2-INOC.

4. Discussion

4.1. Microbiota in the different materials

In agreement with expectations, the raw milk had the highest alpha-diversity, and its microbiota were clearly different from that in the other sample groups (Fig. 1). The high diversity of the raw milk microbiota

Table 3
Cheese quality attributes evaluated at different stages during ripening summarised by treatments (T1-T4) and batch (B1-B3) within treatments. Sensory grades (smell and taste, texture) were from 1 to 9, with 9 corresponding to excellent sensory attributes of the cheese.

Treatment	T1-UNTR			T2-INOC			T3-ACID			T4-INOC		
Batch	B1	B2	B3	B1	B2	B3	B1	B2	B3	B1	B2	B3
pH measurements												
4 mon	–	–	–	5.44	5.42	5.42	–	–	–	5.37	5.43	5.42
8 mon	5.59	5.58	5.58	5.43	5.42	5.43	–	–	–	5.43	5.42	5.43
14 mon	–	–	–	–	–	–	5.44	5.43	5.43	–	–	–
18 mon	5.46	5.43	5.51	5.48	5.49	5.49	5.37	5.36	5.36	5.42	5.45	5.44
22 mon	5.47	5.46	5.50	5.44	5.46	5.46	5.43	5.41	5.40	5.41	5.40	5.41
Plate count agar, log_{10} CFU/g												
4 mon	5.6	5.0	4.8	5.5	5.7	5.7	–	–	–	6.2	5.3	5.3
8 mon	3.9	4.0	4.8	–	–	–	4.1	4.1	4.1	4.2	4.3	4.3
14 mon	3.0	2.8	3.3	3.0	3.0	3.5	2.9	3.7	2.0	3.8	<2.0	3.3
18 mon	2.9	2.8	2.7	4.2	2.8	4.9	2.7	3.4	2.8	3.0	2.7	5.1
22 mon	2.7	1.5	2.8	2.4	2.6	3.4	2.8	3.1	2.4	3.0	3.0	2.1
MRS agar, log_{10} CFU/g												
4 mon	–	–	–	–	–	–	–	–	–	7.3	6.7	6.9
8 mon	5.9	5.8	6.2	–	–	–	5.5	5.5	5.4	5.2	5.3	5.5
14 mon	3.0	4.0	3.9	4.2	4.7	5.0	4.2	5.2	4.7	5.0	4.3	–
18 mon	<2.0	<2.0	<2.0	4.5	4.0	<2.0	5.0	5.1	4.8	3.6	<2.0	4.0
22 mon	<2.0	<2.0	<2.0	<2.0	<2.0	3.5	<2.0	<2.0	3.3	2.7	2.6	3.3
Smell and taste, grade												
14 mon	4.25	4.25	4.75	4.00	3.75	4.25	4.75	4.25	3.75	4.75	4.50	4.75
18 mon	4.50	4.75	4.50	4.50	4.50	4.25	4.25	5.00	4.00	4.75	5.00	5.00
22 mon	5.50	5.25	5.75	5.00	5.25	4.75	4.75	4.50	5.00	5.75	5.00	4.75
Texture, grade												
14 mon	4.50	4.50	4.50	5.00	5.00	5.00	5.00	5.00	4.25	5.00	5.25	5.50
18 mon	5.00	5.25	5.50	5.50	4.75	4.00	4.25	4.25	4.00	4.50	4.75	4.50
22 mon	5.00	4.50	5.00	5.00	4.50	4.50	4.50	4.50	4.50	4.50	5.00	4.50

Treatments: Feeding silages without additive (T1-UNTR), with inoculation by starter culture (T2-INOC and T4-INOC) and with acid treatment (T3-ACID), as described by Eliasson et al. (2024).

were evident from the findings that the average top 20 genera made up less than 75 % of the bacteria in the raw milk, in contrast to almost 100 % in the starter culture, cheese making (fermented milk, cheese curds and fresh cheese) and cheese ripening samples (Fig. 2). Despite a high number of genera in the raw milk samples, the total bacteria counts were in general low, and typically below 5000 per ml of milk. The combination of a low bacteria count and high alpha diversity indicates that numbers of bacteria belonging to each specific genera are low and thus results may become uncertain. For this reason, it was decided not to include more than the top 20 genera in the evaluation. The microbiota in cheese-making and cheese ripening samples were equally diverse (alpha-diversity), but beta-diversity analysis showed that the microbiota of the two sample groups differed. This is likely explained by the shift in RA of the dominant genera during cheese ripening process. The RA of *Lactobacillus*, and particularly *Leuconostoc* increased during ripening, while the RA of *Lactococcus* decreased (Fig. 2). However, beta-diversity analysis showed that there was no difference in microbiota between the starter culture and cheese ripening. In comparison with starter culture and samples of ripening cheeses, *Lactococcus* was very dominant in the cheese making samples, while the RA of *Leuconostoc* increased during the early stages of cheese ripening. Explanations for the rapid dominance of *Lactococcus* over *Leuconostoc* include the initial conditions in the starter culture, with *Lactococcus* making up 80–95 % of the bacteria. Also, *Lactococcus* thrive better at lower pH while *Leuconostoc* develops at a later stage, when pH has increased slightly. Finally, the fast-growing *Lactococcus* may suppress growth of *Leuconostoc* in the competition for amino acids (Bellengier et al., 1997).

The PMA treatment was not used for the raw milk, starter culture or fresh cheese making samples, since it was assumed that most of the bacteria in these samples consisted of living cells. The results after PMA treatment of bacterial cells in cheese at 22 months showed a low RA of by *Lactococcus* in comparison with the results for the cheese samples without PMA treatment, illustrating that not all DNA from this genus stemmed from live bacteria and that some was also derived from dead bacterial cells. However, irrespective of PMA treatment or not, at 22 months of ripening the genera *Lactococcus* and *Leuconostoc* were still the dominant in the cheeses, although the RA of *Lactobacillus* was higher in the PMA-treated cheese samples than when not using the PMA treatment.

4.2. Milk microbiota varied to a minor extent

Considering that the treatments in the feeding experiment only resulted in minor effects on the microbiota in silage and bedding material (Eliasson et al., 2024), it was not surprising that the microbiota of the milk associated with the different treatments showed little variation (Fig. 3). Total bacteria count in the raw milk were generally low in this study, which is in line with findings in other studies investigating raw milk from Swedish farms (Glantz et al., 2020; Sun et al., 2024). The high RA of *Lactococcus* and *Leuconostoc* in the raw milk sampled in the dairy balance tank in this study an unexpected observation, since the milk were identical to the on-farm bulk milk described in our recent publication (Eliasson et al., 2024). One possible explanation for the differences between microbiota in milk sampled from the bulk tank on the farm, and milk sampled from the balance tank at the dairy, is contamination and enrichment of certain bacteria during truck transportation, as suggested by Kable et al. (2016). The same authors investigated viable and total bacterial content in milk samples collected in large-scale dairy manufacturing equipment prior to cheese manufacture (Kable et al., 2019). Using cleaning-in-place (CIP) as a reference point, they found that at later sampling points, e.g. more than 19 h after CIP, 90 % of the dairy silos contained elevated viable cell numbers enriched in *Acinetobacter* and/or *Lactococcus*.

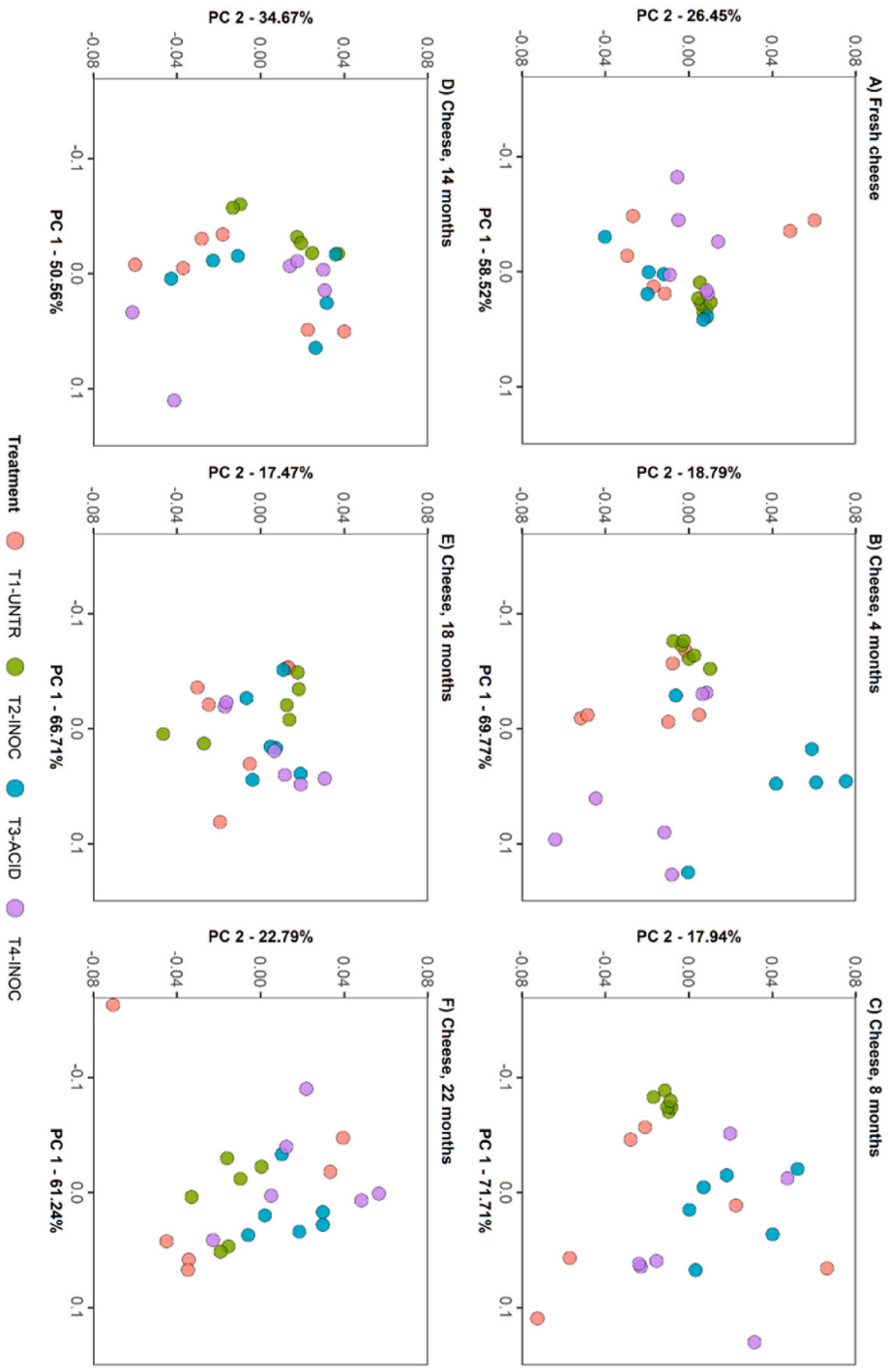

Fig. 6. Multiple principal coordinate analysis plots of the weighted UniFrac distance matrix of microbiota in the cheeses at different ripening ages in months by treatment and batch. Treatments: Feeding silages without additive (T1-UNTR), with inoculation by starter culture (T2-INOC and T4-INOC) and with acid treatment (T3-ACID), as described by Eliasson et al. (2024).

4.3. Bacteria counts and microbiota of samples collected during cheese making

The PERMANOVA analyses showed that the microbiota in cheese making samples associated with treatment T4-INOC differed from that in samples from all other treatments and that the microbiota in samples associated with T1-UNTR differed from that in T2-INOC samples. The T4-INOC batches of cheese clearly had less dominance of *Lactococcus* (dd41a) and higher RA of *Leuconostoc* (4b97e) and *Lactobacillus* (5f86a) than batches associated with the other treatments (Fig. 5). However, since there were no differences in raw milk microbiota between the four treatments, the differences in microbiota of the cheese making samples between treatments were most likely not associated with the silage treatments but explained by process-related factors. As discussed above in relation to work by Kable et al. (2019), routines for cleaning of the equipment could play a role. In addition, the dairy environment itself could potentially contribute some bacteria, as discussed in a study on NSLAB by Somers et al. (2001). The two different unclassified *Enterobacteriaceae* (54780 and 05c1e) were observed in some of the batches, and often in the fresh cheese samples, but were not among the top 10

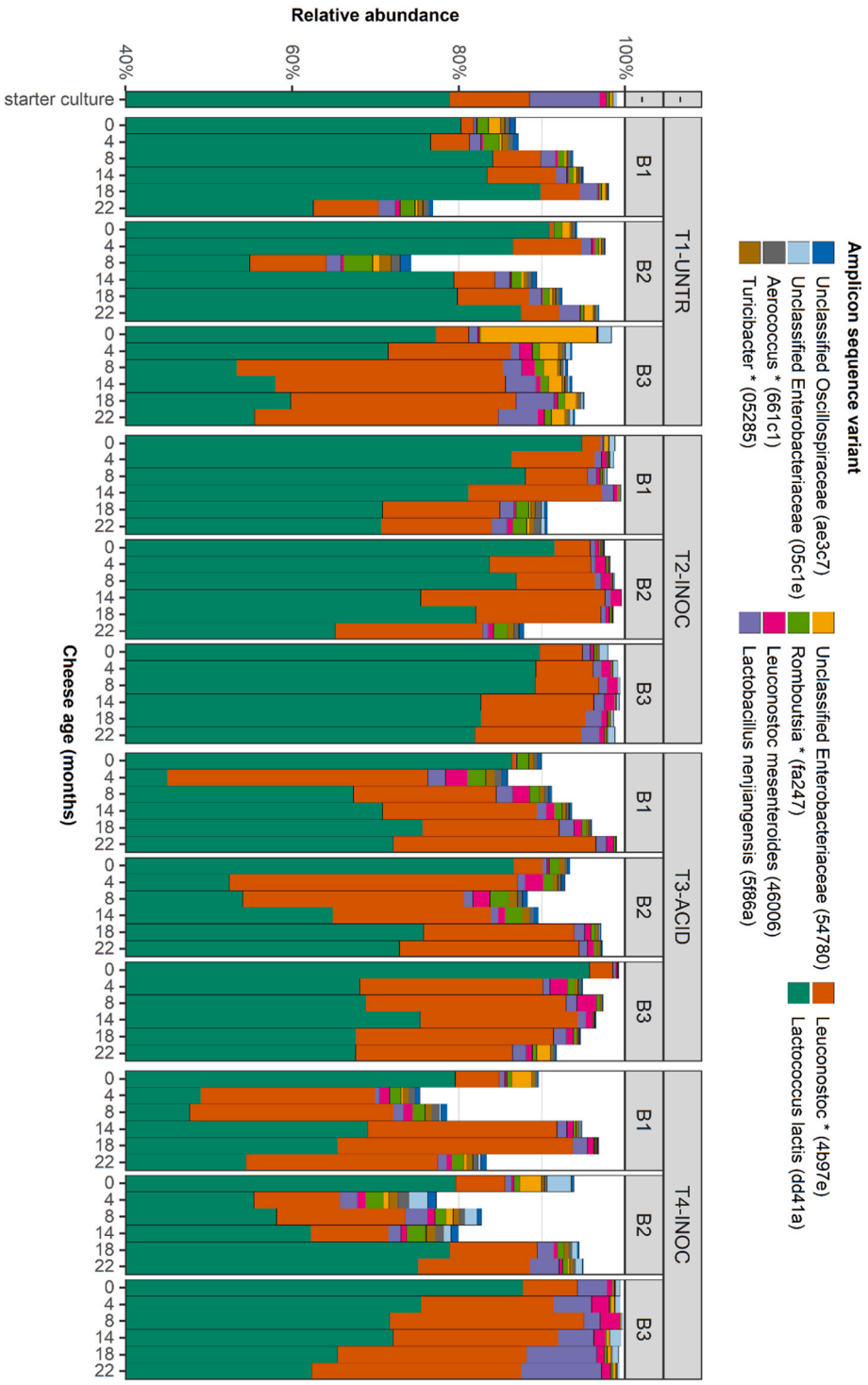

Fig. 7. Top 10 amplicon sequence variants (ASVs) in starter culture and cheeses during the ripening process, by treatment (T1-T4) and batch (B1-B3) within treatment at different maturation stages. Y-axis scale cut at 40 % relative abundance for higher resolution. Treatments: Feeding silages without additive (T1-UNTR), with inoculation by starter culture (T2-INOC and T4-INOC) and with acid treatment (T3-ACID), as described by Eliasson et al. (2024).

ASVs in cheese ripening samples (Fig. 7). However, in all T1-UNTR batches, *Enterobacteriaceae* (54780) was found in cheeses also at 22 months of ripening, both with and without PMA treatment of bacterial cells (Fig. 8). Evaluation of numbers of *Enterobacteriaceae* in the fresh cheese often resulted in over-grown plates, *i.e.* numbers >3.7 $\log_{10}$ CFU/g. This was the case for all T1-UNTR batches, but also for many other batches, indicating that the dilutions of the samples before plating were not sufficient. The *Enterobacteriaceae* most probably originated from insufficient cleaning of the equipment, as this type of bacteria tends to form a biofilm on various surfaces in the dairy environment (Cherif-Antar et al., 2016) but could also simply have originated from contamination by the cheese makers. In the T2-INOC batches, *Lactococcus lactis* was more dominant compared to batches associated with the other treatments, and in batches B1 and B2 its RA exceeded 90 % irrespective of sample type. The between-batch variation in samples associated with T2-INOC was also smaller than in samples associated

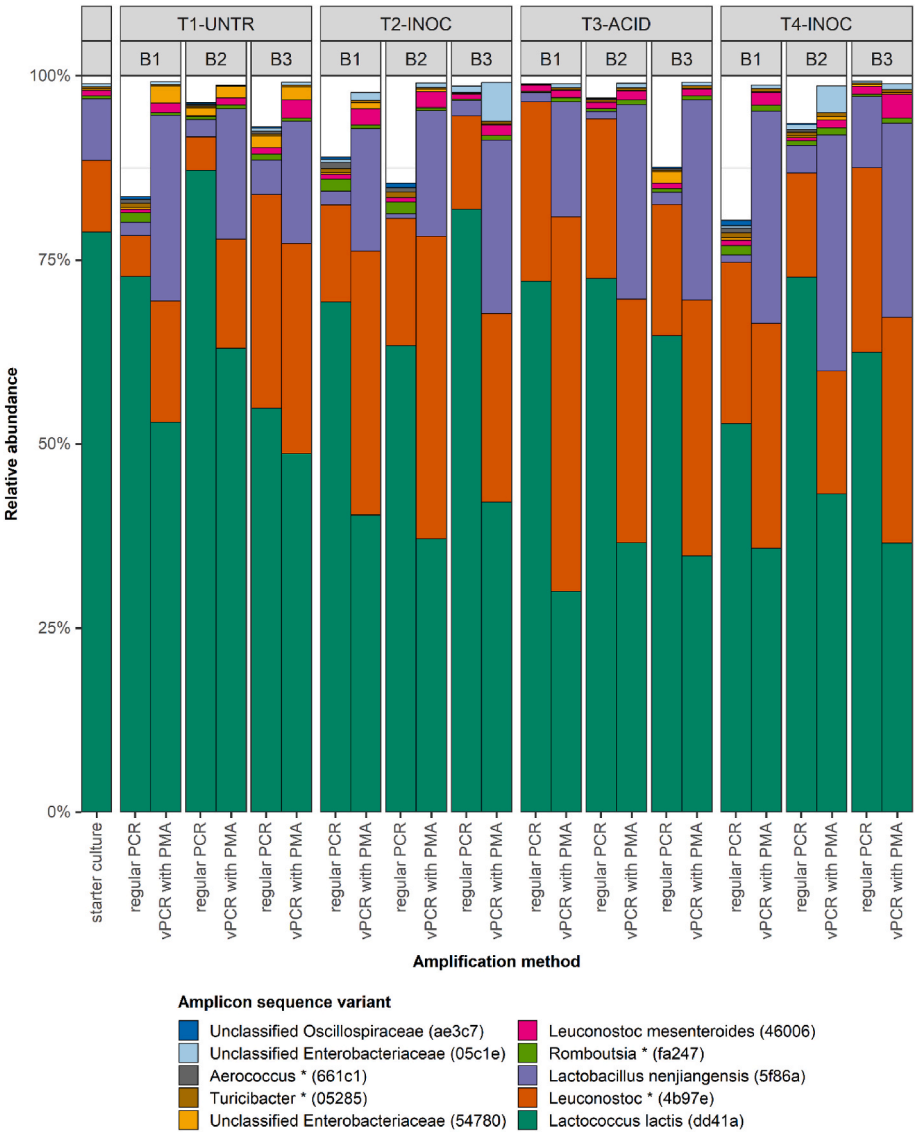

Fig. 8. Comparison of amplification methods between the 10 most abundant amplicon sequence variants in cheese at 22 months of age by treatment (T1-T4) and batch (B1-B3) within treatment. Regular polymerase chain reaction (PCR) vs. viability PCR (vPCR) with propidium monoazide (PMA). Starter culture (regular PCR) included for reference. Treatments Feeding silages without additive (T1-UNTR), with inoculation by starter culture (T2-INOC and T4-INOC) and with acid treatment (T3-ACID), as described by Eliasson et al. (2024).

with the other treatments, as can be seen in Fig. 5.

The starter culture used in this study was a defined mesophilic DVS culture which according to the manufacturer consisted of *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* and unspecified *Leuconostoc*. Characterising the composition of the starter culture, however, in addition to *Leuconostoc* and *Lactococcus* we also observed *Lactobacillus* at ~10 % of RA.

At the early stages of fermentation, the acid-producing *Lactococcus* dominated, in agreement with our previous findings for the same type of cheese (Sun et al., 2023). *Leuconostoc* was observed already in the fermented milk (Fig. 5), often at the same RA in cheese curd or fresh cheese and in the starter culture, while in our previous study *Leuconostoc* appeared first in the fresh cheese and at very low RA (Sun et al., 2023). The use of DVS starter culture in this study, in contrast to the bulk starter used in Sun et al. (2023), may explain this difference. In addition, a different kind of starter culture was used in Sun et al. (2023), although the core microbiota remained the same according to the manufacturer. Frantzen et al. (2017) investigated the diversity of *Leuconostoc* in five undefined DL-type starter cultures commonly used by the dairy industry, using both traditional cultivation and sequencing methods. Bacterial counts for the starter cultures on the two media used, MRS and MPCA, revealed large differences in the RA of *Leuconostoc*, with most of the *Leuconostoc* in two of the starter cultures being unable to grow on MRS. Comparative genomic analysis revealed great differences between the *Leuconostoc* species and subspecies, and the composition of the *Leuconostoc* population was significantly different between the starter cultures. Three of the cultures were dominated by *Leuconostoc mesenteroides* subsp. *cremoris*, *Leuconostoc pseudomesenteroides* dominated in two of the cultures and *Lactococcus lactis*, reported to be a major constituent in fermented dairy products, was only present in low amounts in one of the cultures (Frantzen et al., 2017). Without further documentation on the genomic diversity of *Leuconostoc* in the starter culture used in the present study, it is not possible to draw any conclusions regarding *Leuconostoc* species present.

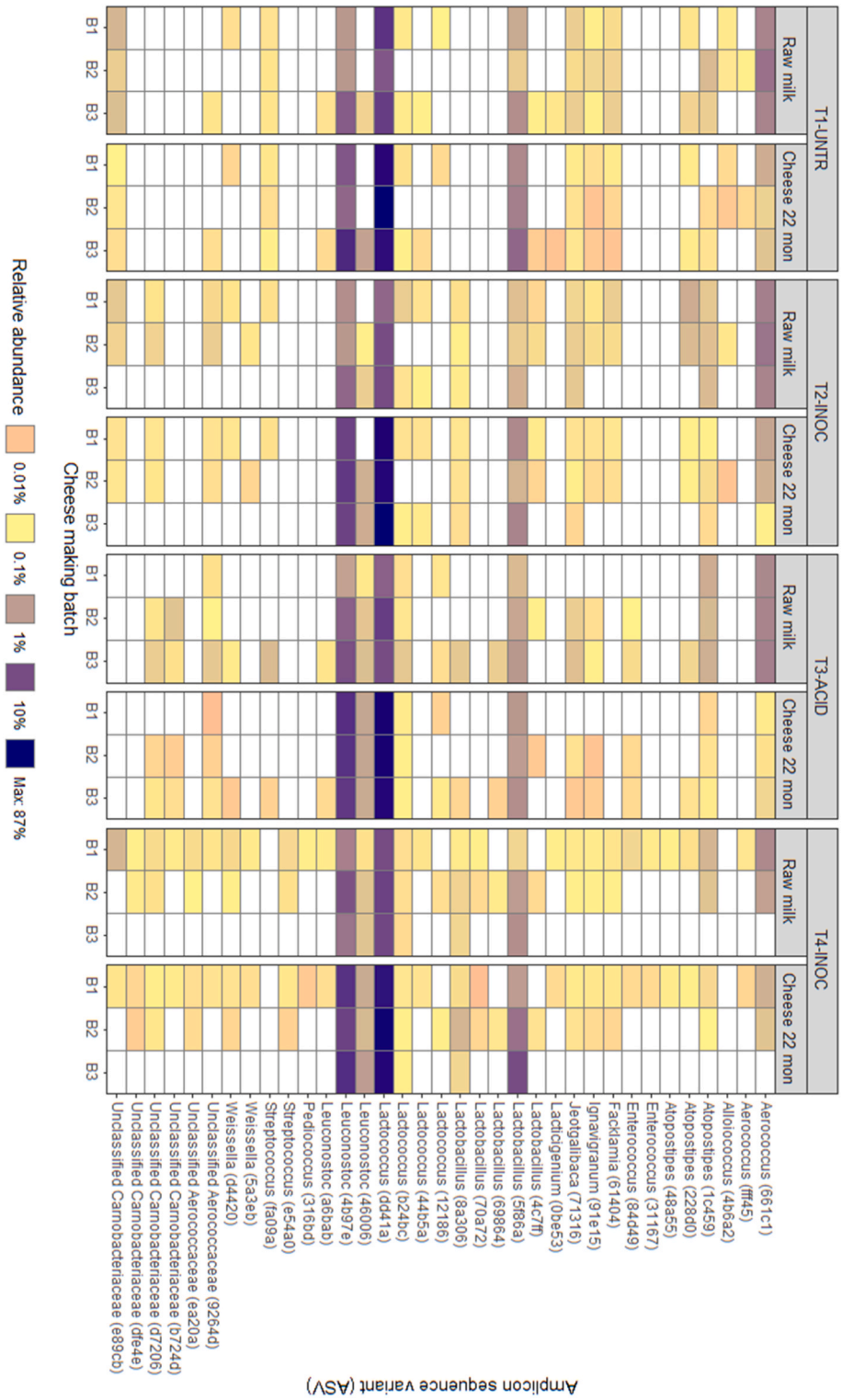

Fig. 9. Amplicon sequence variants (ASVs) belonging to lactic acid bacteria, i.e., order Lactobacillales, present in both raw milk and in cheeses at 22 months of ripening. The filtering included only ASVs present in both replicates at both sampling points, batchwise. Treatments feeding silages without additive (T1-UNTR), with inoculation by starter culture (T2-INOC and T4-INOC) and with acid treatment (T3-ACID), as described by Eliasson et al. (2024).

4.4. Microbiota and quality attributes of ripening cheese

In agreement with results in our recent study (Sun et al., 2023), there was clear dominance by the starter culture also at the later stages of the cheese ripening (Fig. 7). All the cheeses in the present study were produced at the same time of day throughout the whole study. Until 14 months of ripening, the cheese microbiota in batches associated with silage treatment T2-INOC differed from the other treatments, developing with little variation between the bathes. This is illustrated in the PCoA plot in Fig. 6, where the microbiota of T2-INOC cheeses is less dispersed than that associated with samples from the other treatments. This pattern changed at 14 months of ripening, after which there was

less variation between the silage treatments.

During ripening of the type of Swedish hard cheese analysed in this study, NSLAB e.g. lactobacilli, are reported to be dominant already after a few weeks, contributing to the characteristic flavour of the long-ripened cheese (Rehn et al., 2010). Since *Lactobacillus* was only observed at later stages of ripening in our previous study, and only in a few batches, it was hypothesised that the low RA of *Lactobacillus* was due to amplification of excessive amounts of DNA from dead starter culture bacteria, *i.e.*, underestimation of the number of lactobacilli (Sun et al., 2023). To evaluate how much of the sequenced DNA that represented live bacteria, bacterial cells in cheese at 22 months were treated with PMA before DNA extraction, to prevent amplification of DNA from dead cells by the PCR procedure (Porcellato et al., 2015). After PMA treatment the RA of *Lactococcus* (dd41a) was still ~30–35 % irrespective of treatment, and in T1-UNTR (B1 and B2) it exceeded 50 %. The RA of *Leuconostoc* (4b97e) varied, but in some batches, it reached the same level as that of *Lactococcus* (dd41a). One major difference between results with and without PMA treatment was that *Lactobacillus* (5f86a), identified at 10 % RA in the starter culture, became rather dominant in the cheese microbiota following PMA treatment, with RA at 22 months of ~15–30 % in different batches.

In addition to the LAB, there were also fewer dominant taxa among the top 10 ASVs making up the cheese microbiota in this study. Unclassified *Enterobacteriaceae* (05c1e and/or 54780) were found in most cheeses at 22 months of ripening, also after PMA treatment, and in general the same ASVs were identified also in the fresh cheeses. This was clear for samples from treatment T1-UNTR (B3), in which unclassified *Enterobacteriaceae* (54780) was found at ~20 % RA in the fresh cheeses and at ~2–3 % RA in cheeses aged for 22 months. It is likely that this ASV found its way into the cheeses via contamination of the fresh cheese, since it was not observed in the milk.

4.5. Lactic acid bacteria present in both raw milk and cheese at 22 months

Filtration for ASVs belonging to the order *Lactobacillales*, present in both raw milk and cheese at 22 months, mainly identified the three ASVs that also made up the starter culture, *i.e. Lactococcus* (dd41a), *Leuconostoc* (4b97e) and *Lactobacillus* (5f86a). While only a small fraction of the 16S rRNA gene is amplified with the method used, it cannot be guaranteed that the strain/species in raw milk and cheeses were identical. Considering that the raw milk were sampled from the balance tank, *i.e.,* after reception at the dairy facility, contamination of the raw milk with bacteria from the local dairy environment could be a potential explanation. Other *Lactobacillus* ASVs were observed more occasionally, *i.e. Lactobacillus* (4c7ff, 69864 and 70a72), whereas *Lactobacillus* (8a306) was observed in raw milk and cheeses in all batches associated with treatments T2-INOC and T4-INOC. Some *Lactococcus* ASVs appeared in a few batches only, *i.e. Lactococcus* (44b5a), while other ASVs were common in most of the batches, *i.e. Lactococcus* (b24bc). The strain *Aerococcus* (661c1) was identified in both raw milk and in cheeses in all batches except T4-INOC (B3), although its abundance was always higher in the raw milk than in the cheese.

4.6. General discussion

The bacteria found in the final cheeses were mostly present in the starter culture used in the cheese making process. Factors in the dairy plant environment seemed to affect the microbiota of the final cheeses more than the contribution of the flora of the raw milk on the research farm. The slow maturation of the cheeses could be a consequence of lack of the important NSLAB believed to stem from the raw milk, responsible for the formation of the characteristic cheese flavour. In contrast, the lactobacilli observed in the aged cheese stemmed from the starter culture. A sandy texture in this type of cheese is often experienced in younger unmature cheese, in this study indicating slow protein degradation during maturation. It is suggested that the hygienic practices implemented in both modern dairy farms and the dairy facility during recent decades, possibly in combination with changes in the properties of starter cultures, have likely had a negative effect on the presence of NSLAB milk microbiota of importance for long-ripening cheeses.

5. Conclusions

The expected effects of the composition of microbiota on raw milk from a feeding trail with dairy cows fed different silages were absent. In contrast, the main variation in microbiota during cheese making and cheese ripening were related to individual cheese batches, not microbiota of the raw milk caused by feeding. The cheese microbiota was dominated by the bacteria in the starter culture also at late stages of cheese ripening (22 months).

CRediT authorship contribution statement

Thomas M. Eliasson: Writing – original draft, Investigation, Formal analysis, Data curation. **Åse Lundh:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization. **Annika Höjer:** Writing – review & editing, Writing – original draft, Validation, Resources, Investigation, Funding acquisition, Data curation, Conceptualization. **Karin Hallin Saedén:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Funding acquisition, Conceptualization. **Mårten Hetta:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Investigation, Funding acquisition. **Li Sun:** Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology, Formal analysis, Data curation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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