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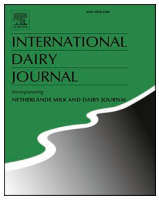

Research paper

How raw milk-based adjunct cultures influence microbial diversity in cheese

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A B S T R A C T

Natural milk starters, like Italy's lattoinnesto, are rarer than natural whey starters in traditional cheese production, representing a less studied niche. This study aimed to investigate how milk adjunct cultures could enhance microbial diversity, establish a link between terroir and cheese, stabilise production variations, and influence the aroma profile and safety of semi-hard cheeses. Natural milk adjunct cultures were produced from raw and thermised milk by spontaneous fermentation and utilised to produce Vacherin Fribourgeois model cheeses in combination with a commercial starter for acidification. Cheeses produced from raw milk exhibited more diverse flavour profiles and higher concentrations of several volatile compounds than those made from thermised milk. The increased proteolysis in cheeses with enriched lattoinnesto-like adjunct cultures correlated with increased levels of flavour-related compounds and relative abundance of *Lactobacillus helveticus*. The findings highlight significant differences in microbial composition and flavour profiles based on milk treatment and natural milk adjunct cultures.

1. Introduction

Microbes originating from raw milk play a significant role in shaping the aroma and overall quality of cheeses. These microbes, including bacteria, yeasts, and moulds, contribute to the complex biochemical processes that occur during cheese ripening, influencing texture, flavour, and aroma. Raw milk contains a diverse microbial community, including lactic acid bacteria (LAB) and non-starter lactic acid bacteria (NSLAB), contributing to the unique flavour profiles of cheeses by breaking down proteins, fats, and lactose during fermentation and ripening (Bettera, Levante, et al., 2023). Microbial activity during ripening, in particular from NSLAB, is crucial for the production of volatile compounds such as esters, ketones, alcohols, and sulphur-containing compounds from amino acids liberated by proteolysis, in particular the branched-chain amino acids, which then contribute to the characteristic aromas of cheeses (Andriot et al., 2024; Carbonell et al., 2002; McSweeney & McNamara, 2022, pp. 245–249; 79–90). As a consequence, the bacterial composition of the raw milk and the resulting cheese are both tied to the terroir (Bugaud et al., 2001; Irlinger et al., 2024).

In contrast, microbes of raw milk origin can also pose challenges for food safety and quality (Costanzo et al., 2020; N. H. Martin et al., 2021). Defects in cheese may include the production of gas, pigment formation, or the development of off-odours (N. H. Martin et al., 2021). Further, pathogenic microbes, such as *Listeria monocytogenes*, *Staphylococcus aureus*, *Escherichia coli*, and *Salmonella* spp., can contaminate raw milk and cause foodborne illness if not adequately managed (Bachmann & Spahr, 1995; Choi et al., 2016; Rangel-Ortega et al., 2023). Therefore, the quality of raw milk and the control of microbial populations during cheese production are crucial to ensuring both the safety and quality of the final product.

Traditionally, one way of fostering wanted microbes while suppressing unwanted ones was the use of traditional starter cultures. Natural whey cultures (NWCs) play a significant role in the production of Swiss, Italian, French, and Argentinian cheeses, as well as in other traditional cheese manufacturing processes (Demarigny et al., 2006; Gatti et al., 2014; Lucchini et al., 2018; Lutin et al., 2024; Moser et al., 2017; Reinheimer et al., 1996). They contain diverse microbial populations with LAB, such as *Lactobacillus* (*L.*) *helveticus* and *Streptococcus* (*S.*) *thermophilus* (Gatti et al., 2014; Moser et al., 2017). Other relevant species include *L. delbrueckii* subsp. *lactis* (Reinheimer et al., 1995), *Limosilactobacillus fermentum* (Cremonesi et al., 2011; Bertani et al., 2020) and *Levilactobacillus brevis*, particularly in starters prepared at temperatures below 40 °C (Mancini et al., 2021). Their complex microbial communities contribute significantly to the characteristics of cheese and the ripening processes (Gatti et al., 2014). In contrast, comparatively little is known about the composition of natural milk starters (Parente et al., 2016), presumably due to their more limited

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distribution, being mainly focused on northern Italy (Parente et al., 2016).

In a recent study, we proposed the concept of a natural adjunct culture using whey and raw milk, exploring the selective enrichment of the raw milk microbiota to produce an enriched raw milk whey culture for cheese production, offering an alternative to traditional adjunct culture formulation methods (Bettera, Dreier, et al., 2023).

Building on these results, we here produced natural milk adjunct cultures from raw milk and utilised them in the production of Vacherin Fribourgeois model cheeses, with the aim of exploring several critical aspects. In this study, our aims are threefold: (1) to investigate the potential of these cultures to increase and control microbial diversity in raw milk cheese and to strengthen the link between terroir and the final product; (2) to understand their influence on stabilising variations and mitigating defects in raw milk cheese production; and (3) to evaluate the safety and functionality of these adjunct cultures in the context of semi-hard cheeses.

2. Materials & methods

2.1. Natural milk adjunct cultures

The raw cow's milk for culture production was provided by the Agricultural Institute in Grangeneuve (Switzerland) within the framework of the Center of Excellence for Raw Milk Products. The milk was delivered to the laboratory on the same day in a refrigerated condition. A schematic representation of the preparation of traditional lattoinnesto cultures (Parente et al., 2017) and the natural raw milk adjunct cultures used in this study is shown in Fig. 1. Briefly, for the preparation of the lattoinnesto-like adjunct culture (LI), the raw milk was heated to 63 °C on a hotplate with constant stirring and cooled to approximately 40 °C on ice after 15 s. A pre-culture was obtained by incubation overnight at 38 °C, allowing for spontaneous fermentation. The next day, 10 % of this pre-culture was used to inoculate approximately 40 °C warm, thermally treated (63 °C, 15 s) raw milk. After incubation at 38 °C overnight, the pH value dropped from approximately 6.7 to below 4.5. The LI adjunct culture was then stored in the fridge at 4 °C until usage. For cheeses produced on production day 1, the adjunct cultures were prepared and stored at 4 °C overnight; for cheeses produced on production day 2, the cultures were stored for an additional 24 h before use. To produce an enriched lattoinnesto-like (eLI) adjunct culture, an LI culture was prepared as described above and stored at 10 °C in a refrigerator for 26 days. For the final culture, 10 % of the 26-day-old culture was used to inoculate approximately 40 °C warm, thermally treated (63 °C, 15 s) raw milk, incubated at 38 °C overnight, and stored in the fridge (4 °C) until usage. To produce the enriched lattoinnesto-like with salt (eLI.s) adjunct culture, an LI culture was prepared as described above, 2.5 % salt (NaCl) was added, and the culture was stored at 10 °C in a refrigerator for 26 days. For the final culture, 10 % of the 26-day-old culture was used to inoculate approximately 40 °C warm, thermally treated (63 °C, 15 s) raw milk. The culture was incubated at 38 °C overnight and stored in the fridge (4 °C) until usage.

2.2. Cheese production

Eight cheeses were produced following the Vacherin Fribourgeois PDO technology outlined by Bettera, Dreier, et al. (2023). The trial was conducted in duplicate with the same batch of adjunct culture in a separate flask stored at 4 °C overnight on two consecutive days in the pilot plant facility at Agroscope (Liebefeld, Switzerland). A starter culture commonly used in Vacherin Fribourgeois PDO production (*Lactococcus* (*Lc.*) *lactis*, *Lc. cremoris* and *Leuconostoc mesenteroides*; Liebefeld Kulturen AG) was added at a proportion of 0.12 % to the vat milk. The natural adjunct cultures were added at a proportion of 0.1 % to the vat

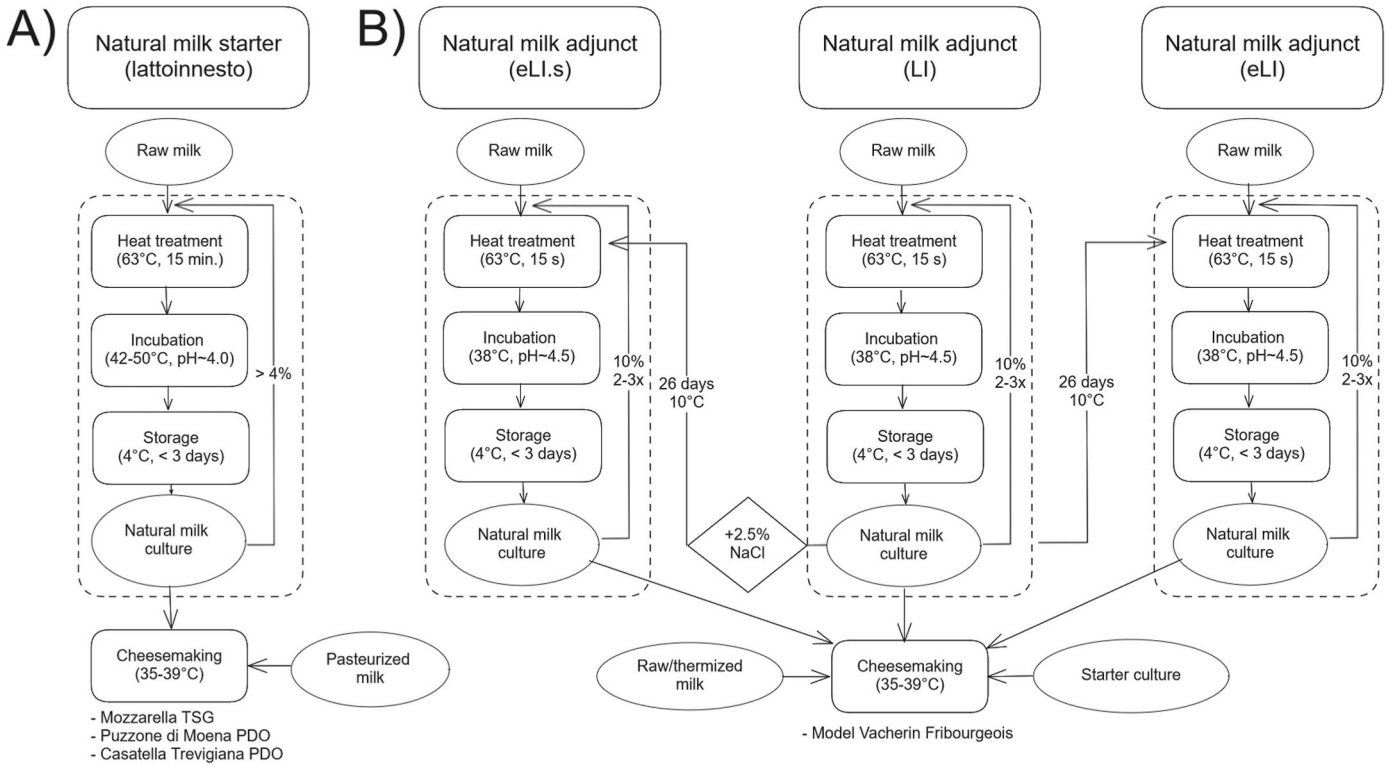

Fig. 1. Schematic representation of the preparation of the natural milk adjunct cultures used in the study. Panel A illustrates the conventional production process for traditional Italian cheese varieties designated with Protected Designation of Origin (PDO) and Traditional Specialties Guaranteed (TSG) labels. The scheme is adapted from the work of Parente et al. (2017). Panel B illustrates the production of the three natural milk adjunct cultures under investigation in this study: enriched lattoinnesto-like with salt (eLI.s), lattoinnesto-like (LI), and enriched lattoinnesto-like (eLI).

milk. The controls, one with raw milk and one with thermised milk, were produced using only the starter culture.

2.3. Microbiological analysis

The vat milk and cheese samples produced in the pilot plant (Agroscope, Liebefeld) were analysed by an external laboratory for agriculture and food analyses (LAAF) in Grangeneuve (Canton of Fribourg, Switzerland). The analysis consisted of plate counting on selective media for the following microbial groups for all samples: *Enterobacteriaceae*, Enterococci, Lactobacilli, Streptococci, facultative heterofermentative lactic acid bacteria (FH-LAB), aerobic mesophilic bacteria, propionic acid bacteria (PAB), salt-tolerant bacteria, coagulase-positive staphylococci, *Escherichia coli*, moulds, and yeasts. *Clostridium tyrobutyricum* spore counts (butyric spores) were estimated using the most probable number (MPN) method. Cheese samples after 60 days of ripening were additionally tested for the presence of *Listeria* and *Salmonella*. See Supplementary Table S1 for methods and references.

2.4. DNA extraction

2.4.1. Cheese samples

Bacterial pellets from cheese were obtained by adding 10 g of cheese to 90 mL modified peptone water (10 g L^{-1} peptone from casein, 5 g L^{-1} sodium chloride, 20 g L^{-1} trisodium citrate dihydrate, pH 7.0) and incubating for 10 min at 40 °C. The samples were homogenised for 3 min in a Stomacher (Masticator, IUL Instruments, Königswinter, Germany). 50 µL of 10 % (w/v) sodium dodecyl sulphate was added to 10 mL of the homogenate, and thoroughly mixed and centrifuged (4000×*g*, room temperature, 30 min).

Bacterial pellets were resuspended in 400 µL G2 buffer (EZ1 DNA Tissue kit, Qiagen, Hilden, Germany), transferred in 0.5 mL skirted tubes containing 100 mg of 0.1 mm low-binding zirconium beads (OPS Diagnostics, Lebanon, NJ, USA), and shaken for 60 s at medium speed in a bead ruptor (Omni International Inc., Kennesaw, GA, USA). After centrifugation, 200 µL of cell lysates and 10 µL proteinase K (Qiagen, Hilden, Germany) were incubated for 1 h at 56 °C and then processed by the BioRobot® EZ1 workstation (Qiagen, Hilden, Germany).

2.4.2. Liquid samples

For the extraction of DNA from raw milk, 10 mL was mixed with 2 mL NET buffer (50 mM NaCl, 125 mM EDTA, 50 mM Tris-HCl [pH 7.6]), heated at 80 °C for 10 min, and cooled on ice. After centrifugation (30 min 5000×*g*), about 1.5 mL containing the remaining pellet was transferred into 2 mL tubes and centrifuged again at 12000×*g* at 4 °C for 10 min. Bacterial pellets were processed like bacterial pellets from cheese samples.

For the extraction of DNA from culture samples, a pellet was obtained by centrifuging 5 mL of the sample at 16,000×*g* for 10 min and discarding the supernatant. One mL of 8 M of guanidinium chloride was added and centrifuged at 16,000×*g* for 10 min, and the supernatant was discarded. Following the removal of the supernatant, 400 µL of G2 buffer solution (EZ1 DNA Tissue Kit, Qiagen, Hilden, Germany) was added, and the entire sample was transferred to 0.5 mL skirted tubes containing 100 mg of 0.1 mm low-binding zirconium beads (OPS Diagnostics, Lebanon, NJ, United States) and shaken for 1 min in a bead ruptor (Omni International Inc., Kennesaw, GA, United States). The samples were centrifuged at 16,000×*g* for 10 min, after which 200 µL of the supernatant was transferred to a tube containing 10 µL of proteinase K (Qiagen) and incubated for 1 h at 56 °C. Cell lysates were then processed by the BioRobot® EZ1 workstation (Qiagen, Hilden, Germany).

2.5. RNA extraction

For RNA extraction, cheeses were kept at 14 °C before sampling, and 15 g was stored at −80 °C until processing. A sample of 15 g of cheese was homogenised in 135 g of peptone water at 4 °C in a stomacher for 3 min. After adding 250 µL of sodium dodecyl sulphate (10 %) to 50 mL of homogenate, the sample was centrifuged at 4600×*g*, 4 °C for 20 min. Approximatively 45 mL of supernatant was discarded, and the remaining pellet was dissolved in 5 mL of 8 M guanidinium chloride and centrifuged at 4600×*g*, 4 °C for 20 min. About 1.5 mL containing the remaining pellet was transferred into 2 mL tubes and centrifuged again at 10000×*g* at 4 °C for 5 min. Bacterial cells were lysed in 750 µL Qiazol Lysis Reagent. After the addition of 300 µL of chloroform, 350 µL of upper phase was used for RNA isolation using the EZ1 RNA Universal Tissue Kit (Qiagen), according to the supplier's protocol, without DNase digestion. The elution volume was 50 µL.

2.6. Reverse transcription and PCR

cDNA synthesis was performed using the ThermoScript RT-PCR System (Invitrogen) with 5 µL of RNA and the specific primer 350R_tail (5′-AGTGGTACACGCAGAGTACTTGCWGCCTCCCGTAGGAGT-3′), according to the supplier's instructions. The synthesis temperature was 53 °C. PCR was performed in 50 µL reactions, using 2 µL of cDNA, 0.1 µM primer NGS_ABCxF27 (5′- CCA TCT CAT CCC TGC GTG TCT CCG ACT CAG | Barcode X| AG AGT TTG ATC MTG GCT CAG – 3′), 0.1 µM primer tail (5′-AGTGGTACACGCAGAGTACTTG-3′), and the Platinum SuperFi PCR Master Mix (Invitrogen). The amplification was carried out under the following conditions: 94 °C for 2 min, followed by 18 and 28 cycles of 94 °C for 30 s, 55 °C for 30 s, and 68 °C for 30 s for raw milk and past milk cheeses, respectively.

2.7. 16S rRNA gene amplicon sequencing

We performed 16S rRNA gene amplicon sequencing and analysis according to the protocol outlined by Dreier et al. (2022). In brief, amplicon libraries were prepared using the unidirectional fusion method (Thermo Fisher Scientific, Waltham, MA, USA). The amplification was carried out as follows: The temperature was maintained at 98 °C for 30 s, followed by 18–35 cycles of 98 °C for 10 s, 55 °C for 20 s, 72 °C for 30 s, and a final elongation at 72 °C for 5 min. After quality control and quantification of the amplicon library, template preparation, chip loading, and sequencing were performed according to the manufacturer's instructions using Ion Chef™ System and Ion S5™ System and an Ion530 Chip (Thermo Fisher Scientific, Waltham, MA, USA). Sequencing was performed on an Ion Torrent Ion GeneStudio™ S5 System instrument (Thermo Fisher Scientific). The raw sequences were subjected to primer removal by cutadapt (M. Martin, 2011), and the raw reads were trimmed and quality-filtered with DADA2 (Callahan et al., 2016). Amplicon sequence variants (ASVs) were then obtained in DADA2 with the parameter POOL set to "pseudo". Taxonomic annotation was performed using DAIRYdb v3.0.0 (Meola et al., 2019) with IDTAXA (Murali et al., 2018). Basic biostatistical analyses and quality checks were performed using the PHYLOSEQ (McMurdie & Holmes, 2013) and vegan (Oksanen et al., 2024) packages in R v4.4.0 (R Core Team, 2023).

2.8. Volatile carboxylic acids

Volatile carboxylic acids were analysed in the 120-day-old ripened cheeses using a Hewlett Packard HP 6890 gas chromatograph (Agilent Technologies, Basel, Switzerland) as described by Fröhlich-Wyder et al. (2013).

2.9. Biogenic amines

Biogenic amines were analysed in the 120-day-old ripened cheeses as described by Ascone et al. (2017) using a UHPLC system (UltiMate 3000 RS; Thermo Fisher Scientific) equipped with a C18 column (Accucore C18: 2.6 µm, 4.6 mm × 150 mm; Thermo Fisher Scientific). All measurements were carried out in duplicate.

2.10. Free amino acids and di- and tripeptides

Total free amino acids and di- and tripeptides were analysed in the 120-day-old ripened cheeses using the ophthaldialdehyde (OPA) method (Egger et al., 2019). Briefly, the samples were diluted 10-fold prior to precipitation with perchloric acid (0.5 mol L^{-1}), and then derivatised with OPA in the presence of 2-mercapto-ethansulfonic acid. The produced 1-alkylthio-2-alkylisoindol compound was measured at 340 nm. To calculate the results, a standard curve based on glutamic acid was used.

2.11. Proteolysis

The extent of proteolysis in the 120-day-old ripened cheese was measured by analysing the following compounds: total nitrogen, water-soluble nitrogen, and non-protein nitrogen, according to the Kjeldahl method (Collomb et al., 1990).

2.12. Moisture and fat content

Cheese samples allowed to ripen for 24 h and 120 days were analysed for moisture, dry matter (IDF, 1982), fat (IDF, 1987), and fat in dry matter (FDM) using common standard methods.

2.13. Lactic acid, citric acid, and L-leucine aminopeptidase

Cheese samples at 1 day and 120 days of ripening were analysed for lactic acid and citric acid concentration and L-leucine aminopeptidase (LAP) activity. To determine lactate, 1.25 g of cheese was homogenised in 50 mL of water using an OmniPrep Multi-Sample Homogenizer (Omni International, Kennesaw, United States). For the determination of citrate, 5 g of cheese was used. The homogenates were then incubated at 2 °C for 20 min. Particles and fat were removed by filtration. The concentration of D- and L-lactate, and citrate in the filtrates was determined using commercial enzymatic assay kits (R-Biopharm AG, Murten, Switzerland). L-leucine-aminopeptidase (LAP) activity was determined using a colorimetric assay with L-leucine-4-nitroanilide as the substrate. For the assays, 60 µL cheese filtrate (1.25 g cheese sample homogenised in 50 mL water and filtered) and 250 µL of L-leucine-4-nitroanilide (final concentration: 0.995 mmol L^{-1}) in phosphate buffer containing 2 mmol/L Mg^{2+} (pH = 7.4) were mixed in a microtiter plate. Enzyme activity was calculated based on the micromolar extinction coefficient of 4-nitroaniline measured with a SpectraMax ABS plus plate reader (Molecular Devices) after 2 h of incubation using SoftMax Pro software (Molecular Devices).

2.14. Physicochemical analysis of cheese samples

The uniaxial compression test (ISO/TS 17996, IDF/RM 205, 2023) was performed on a Zwick universal machine (Zwick GmbH & Co., Ulm, Germany), as described by Guggisberg et al. (2017).

2.15. Cheese volatilome analysis

Untargeted volatile analysis was carried out using an Agilent 7890B gas chromatography (GC) system coupled with an Agilent 5977B mass selective detector (MSD) (Agilent Technology, Santa Clara, CA, USA). For volatile analysis, 500 mg of cheese pulverised with liquid nitrogen was placed in 20 mL headspace vials (Macherey-Nagel), hermetically sealed (blue silicone/Teflon septum (Macherey-Nagel)) and measured in a randomised order. After incubation of the samples for 10 min at 60 °C, the headspace was extracted for 10 min at 60 °C under vacuum (5 mbar), as described by Fuchsmann et al. (2019), using the vacuum transfer in trap extraction method. The trap used was a Tenax TA (2/3 bottom)/-Carbosieve S III (1/3 top) (BGB analytics). The temperature of the trap was fixed at 35 °C, and the temperature of the syringe was at 60 °C. The pipe and trap were dried for 2 min and 1 min, respectively, under a nitrogen stream of 220–250 mL min^{-1}. Desorption of the volatiles took place for 2 min at 300 °C under a nitrogen flow of 100 mL min^{-1}. For this purpose, the programmable temperature vaporisation injector (PTV) was cooled at 10 °C for 2 min, heated up to 250 °C at a rate of 12 °C sec^{-1}, and held for 20 min in solvent vent mode. After 2 min, the purge flow to the split vent was set to 100 mL $min^{-1.}$ The separation was carried out on a polar column OPTIMA FFAP fused silica capillary column 40 m × 0.20 mm × 0.3 µm (Macherey-Nagel) with hydrogen as the carrier gas at a flow rate of 1.5 mL min^{-1} (37 cm s^{-1}). The oven temperature was held for 6 min at 40 °C, followed by heating up to 240 °C at a rate of 9 °C min^{-1} with a total run time of 30 min. The trap was reconditioned after injection at a nitrogen flow of 100 mL min^{-1} for 15 min at 300 °C. The spectra were recorded in SCAN mode at a mass range between m/z 40 to m/z 350 with a gain at 1 with a solvent delay of 0.3 min. The samples were measured twice in random order.

A feature list of GC-MS analysis data was created using Profinder (Version 10.0, Agilent, Santa Clara, CA, USA) with the batch recursive feature extraction method (small molecules/peptides) and a minimal peak height of 2000 counts. The feature list, with the peak area as the response, was employed to perform multivariate statistics with SIMCA® (Version 15.0.2, Sartorius, Göttingen, Germany) to identify significant differences between the samples. The samples were divided into two categories: raw milk and thermised milk. Following a comprehensive evaluation of the model fits, an orthogonal partial least square discriminant analysis (OPLS-DA) was selected as the optimal model for data representation, exhibiting an R2X value of 0.651, an R2Y value of 0.938, and a Q2 value of 0.766. A variable importance in projection (VIP) analysis was conducted to identify compounds exhibiting a significant difference between the sample groups. Eight features exceeding a VIP value above 1 were identified in the chromatographic analysis data through spectral comparison with the NIST mass spectral search program (Version 2.3, National Institute of Standards and Technology, Gaithersburg, MD, USA) and their retention index. Subsequently, a quantification method was devised for these compounds to evaluate the peak areas in all samples.

2.16. Construction of phylogenetic trees from ASVs

ASVs assigned to *Streptococcus* species were filtered, and an ASV for *Lactobacillus helveticus* was also filtered as an outgroup for the phylogenetic tree. The ASVs of the V1–V2 region of the 16S rRNA gene were aligned using PRANK v.170427 (Löytynoja, 2014). Fasttree v2.1.11 (Price et al., 2010) was used to infer an approximate maximum likelihood phylogenetic tree from the multiple sequence alignment. The phylogenetic tree was visualised using the R package ggtree (G. Yu et al., 2017).

2.17. Statistical analysis

Standard statistical analysis for the two groups was performed using a Student's t-test when the variances were homogeneous; conversely, a Welch's *t*-test was applied when the variances were heterogeneous. The standard statistical analysis for more than two groups was performed using ANOVA when the variances were homogeneous. In the event that the ANOVA yielded significant differences, a multiple comparison Tukey's HSD test was subsequently applied. In instances where the variances were heterogeneous, non-parametric Kruskal–Wallis tests and Dunn's post hoc tests were employed. The standard statistical analysis was performed using the Python packages scipy v1.10.1 (Virtanen et al., 2020) and scikit-posthocs v0.9.0 (Terpilowski, 2019). Multivariate statistical methods (PERMANOVA, ANCOM, principal coordinate analysis [PCoA]) were performed using the scikit-bio v0.5.9 package (Rideout et al., 2023). Principal component analysis was performed using the scikit-learn v1.5.1 package (Pedregosa et al., 2011).

3. Results and discussion

3.1. Culture independent analysis of bacterial communities

3.1.1. Microbial composition of adjunct cultures

The lattoinnesto-like (LI) adjunct cultures consisted primarily of *Streptococcus* (*S.*) *thermophilus* (38.1 %), *Lactobacillus* (*L.*) *delbrueckii* (25.9 %), *Enterococcus* (*E.*) *durans* (3.7 %), *S. salivarius* (1.3 %), and an unidentified *Streptococcus* species (30.6 %), referred to as *S.* sp. VAR1 in this study (Fig. 2). A discussion of possible species classifications can be found below. However, the duplicate cultures of the two consecutive days differed in composition. The LI culture used on the first production day was primarily composed of *S. thermophilus* (60.8 %), with no *L. delbrueckii* detected. By contrast, the culture from the second production day was dominated by *L. delbrueckii*, with a relative abundance of 51.8 % (Supplementary Fig. S1D).

The enriched lattoinnesto-like (eLI) adjunct cultures were primarily composed of *L. helveticus*, *L. delbrueckii*, *E. durans*, *S. salivarius,* and *S. thermophilus*, with average abundances of 94.6 %, 4.6 %, 0.4 %, 0.3 %, and 0.1 %, respectively. The duplicate samples, derived from the same

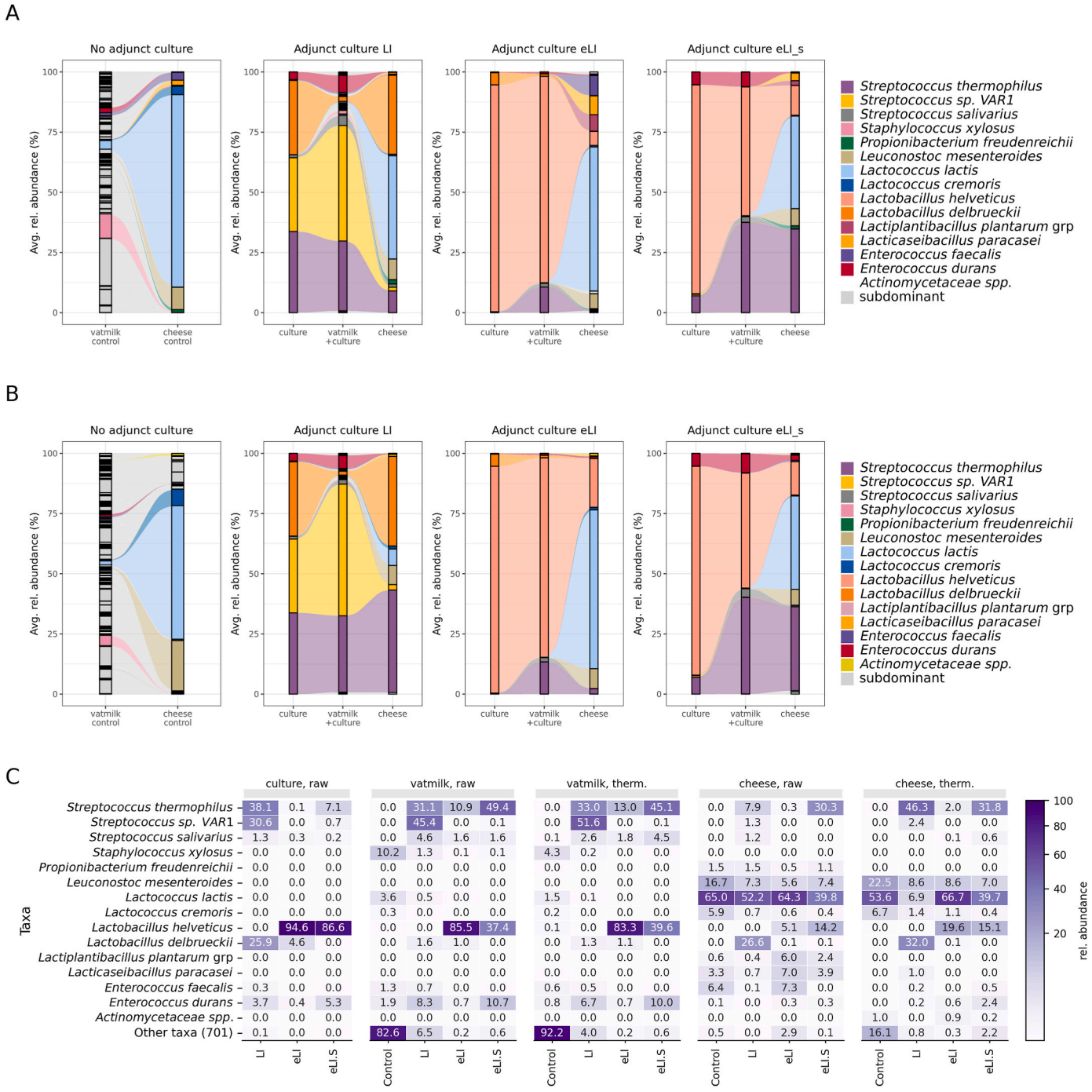

Fig. 2. Average relative abundance data for bacterial species from adjunct cultures to ripened cheese. The average relative abundance for the most abundant bacterial species is shown for the adjunct culture, vat milk with added adjunct culture and cheese samples after 4 months of ripening for cheeses made from raw milk in panel A and cheese made from thermised milk in panel B. In panel C, the average relative abundance is shown in a heat map, with samples ordered according to sample type and milk treatment.

batch of enriched adjunct culture, where one was used on the first day of cheese production after overnight storage and the other after an additional 24 h of storage on the second day, exhibited very similar microbial profiles with all the same species present, although the relative abundances of these species showed slight variations. The enriched lattoinnesto-like adjunct cultures with added salt (eLI.s) showed a similar composition to the eLI adjunct cultures but lacked the presence of *L. delbrueckii*. The cultures primarily consisted of *L. helveticus*, *S. thermophilus, E. durans*, *S.* sp. VAR1, and *S. salivarius*, with an average abundance of 86.6 %, 7.1 %, 5.3 %, 0.7 %, and 0.2 %, respectively.

In their investigation of the composition of undefined thermophilic starter cultures made from raw milk of nine pasta-filata cheesemaking plants, Parente et al. (2016) observed that the microbial communities in the starter cultures were dominated by either *S. thermophilus* or *L. delbrueckii* subsp. *lactis*, or a combination of the two. However, they also contained subdominant mesophilic species, such as *Lactococcus* and spoilage microorganisms. The composition observed was highly similar to that of the LI culture in the present study, although the milk was not pasteurised and the incubation temperature was slightly lower (38 °C instead of 42 °C). The combination of pasteurisation and incubation at high temperatures (42 °C) has been demonstrated to result in low viable counts of *Enterobacteriaceae* and Enterococci, even in milk with lower bacteriological quality. Parente et al. (1997) used culture-dependent methods to characterise the microbiota of natural milk starters and identified *L. helveticus* in lattoinnesto cultures, in addition to other Lactobacilli, Lactococci, Streptococci, and Enterococci. The cultivation-independent high-throughput sequencing approach in the present study did not identify any *L. helveticus* in the LI adjunct culture. However, *L. helveticus* was dominant in the eLI and eLI.s adjunct cultures.

3.1.2. Unidentified Streptococcus species

In this study, we found a *Streptococcus* species that we could not clearly identify. This made it difficult to rule out the possibility that it was a species of potential concern, warranting further investigation. *S.* sp. VAR1 was initially classified as *S. orisratti* by the classification pipeline using the dairyDB database (version 3.0). However, it is probable that a misassignment occurred in this case. To the best of our knowledge, *S. orisratti* has never been identified in or isolated from fermented foods. This species was originally identified in a study that focused on the isolation of oral *Streptococcus* strains from Sprague-Dawley rats (Zhu et al., 2000). In addition to *S. thermophilus*, no other *Streptococcus* species have so far been identified as part of the core microbiota of Vacherin Fribourgeois. However, *S. bovis, S. gallolyticus,* and *S. equinus* complex species have been detected in dairy products, particularly spontaneously fermented milk (Sanhoun et al., 2020).

A BLAST search was conducted against the 16S ribosomal RNA database (NCBI, August 2024), which yielded a nucleotide identity of 99.7 % for *S. parasuis* for the most abundant ASV for *S.* sp. VAR1 (ASV_f2HrNo0C). By contrast, another ASV assigned to *S.* sp. VAR1 (ASV_4DusmVIQ) had *S. thermophilus* as the best match, with a nucleotide identity of 93.8 %. The second case suggests that independent of the dairyDB used for the classification/assignment task, there may have been issues with the sequencing quality in this region. An additional potential explanation for the low identity is that a reference sequence for this *Streptococcus* species is currently absent from the 16S rRNA gene database.

S. parasuis has only recently been reported to be part of the microbiome of spontaneously fermented dairy products and artisanal cheeses in Northeast Asia (Elcheninov et al., 2023; Kochetkova et al., 2023; Z. Yu et al., 2021). There is no reference sequence of the 16S rRNA gene for *S. parasuis* in the current version of the dairyDB (Version 3.0). *S. parasuis* is known to be a zoonotic pathogen (Qi et al., 2023). Indeed, if a *S. parasuis* strain was in the produced LI culture, this would require adapting the production procedure more towards the traditional manufacturing method with a longer heat-treatment (or pasteurisation) of the raw milk prior to incubation, and maybe an incubation at higher temperatures (42 °C instead of 38 °C) as in original lattoinnesto starter cultures used in Italy.

In general, there are some limitations to accurate determination at the species level for the variable regions of the 16S rRNA gene. These include the selection and length of the variable regions, as well as the selected 16S rRNA gene database. These issues were discussed in the context of the *Lactiplantibacillus* (*L.*) *plantarum* group species in a previous study (Dreier et al., 2022). In this study, we also utilised the dairyDB database, which is a manually curated database of 16S rRNA sequences for bacteria in dairy products and primers for the variable regions V1–V2 of the 16S rRNA gene. It is noteworthy that in a recent study, primers for the V1–V2 variable region demonstrated improved resolution for *Streptococcus* spp. in clinical oral samples compared to the other investigated primers targeting different variable regions of the 16S rRNA gene (Na et al., 2023). Therefore, it is unlikely that there has been confusion with the commonly identified species *S. thermophilus* for the most abundant ASV of *S.* sp. VAR1. This is supported by the distance between the sequences in the phylogenetic tree and the sequence alignment of the ASVs of the most commonly identified *Streptococcus* spp. in the cheese samples (Supplementary Fig. S2).

3.1.3. Alpha diversity of vat milk samples

In (microbial) ecology, alpha diversity is a term used to describe "within-sample" diversity, usually considering the number of different species observed and sometimes their relative abundances or even phylogenetic relationships, depending on the index used. In this study, the control vat milk samples had a significantly higher alpha diversity compared to the vat milk samples with eLI (Shannon index, $p < 0.01$) and eLI.s (Shannon index, $p = 0.023$) adjunct cultures added (Supplementary Table S2). At the species level, 479 different bacterial species were identified in the raw control vat milk samples from both production days, while there were only 269, 65, and 83 species in the raw vat milk samples with the added cultures LI, eLI, and eLI.s, respectively (Supplementary Fig. S3). A similar pattern was also observed in the thermised vat milk samples, where 536 different bacterial species were observed in the control samples, while there were only 225, 59, and 85 species in the thermised vat milk samples with the adjunct cultures LI, eLI, and eLI.s, respectively. As expected, the species that were most abundant in the adjunct cultures were also observed in high abundance in the vat milk samples, apart from *L. delbrueckii*, which seems to be underrepresented in the vat milk samples with the adjunct culture LI; instead, in these samples, *S.* sp. VAR1 was the dominant species.

The presence of a greater number of unique species in thermised milk samples than in raw milk samples may appear counterintuitive. Nevertheless, it is likely that this observation is primarily attributable to methodological factors. The amplicon sequencing approach differs from the culture-dependent approach of measuring only viable cells. Instead, the total amount of DNA in the milk samples was quantified. Given the stability of DNA and the collection of milk samples shortly after pasteurisation, it is anticipated that DNA from non-viable cells will contribute to the measured data. Furthermore, the sequencing read data were not rarefied; however, the samples exhibited a comparable sequencing depth (average: 249,714 reads). For an in-depth discussion about normalisation techniques for amplicon sequencing methods, see McMurdie and Holmes (2013) and Weiss et al. (2017). The observed effects can likely be attributed to the complex microbial composition of the milk samples, where a high diversity of bacterial species with relatively low abundance is anticipated, particularly under conditions of heat stress. In the absence of heat treatment, a short pre-incubation period allowed for the proliferation of certain species, leading to an increase in their relative abundance. Similarly, the addition of adjunct cultures promoted the presence of specific bacterial subpopulations. Due to the inherent limitations of sequencing platforms, including the finite sequencing depth (total number of reads per run), this increase in the

abundance of certain species can result in a subsampling effect, in which the detection of low-abundance species is reduced or entirely excluded.

The effects described above were particularly evident when comparing the raw milk control sample S409 with the thermised milk control sample S413 from the second day of production. Despite having approximately ten times lower viable cell counts, S413 yielded 203,460 reads compared to 154,094 reads in S409. The greater sequencing depth in S413 is likely to have increased the likelihood of detecting rare ASVs that might otherwise be missed, leading to an inflated species richness. In contrast, thermised milk samples with adjunct cultures, where microbial populations have been replenished, showed similar levels of species diversity to raw milk samples with adjunct cultures, despite slight differences in sequencing depth. Additionally, we cannot exclude the possibility that some of the additional ASVs detected in thermised milk samples originated from contamination introduced during/after pasteurisation. In cheese, this effect is not observed because the bacterial populations have had sufficient time to stabilise during ripening. Thus, the observed alpha diversity indices were considered more accurate and reliable, and no significant differences were found between the investigated groups (milk treatment and adjunct cultures).

3.1.4. Beta diversity of vat milk samples

Beta diversity is a term used in (microbial) ecology to describe the variability in microbial community structure across different environments or samples. The index provides a measure of the extent of species differences or shifts in community structure that can be observed at different spatial or temporal scales. A clear clustering of the vat milk samples was observed in the PCoA according to the adjunct culture added, which was based on the Aitchison dissimilarity of the species compositions (Fig. 3). By contrast, the microbiomes of the ripened cheese samples seemed less distinct from each other (Fig. 4). In the PCoA of the vat milk samples, the separation is primarily evident on principal coordinate axis 1, as illustrated by the biplot, which indicates that the primary driver on this axis is the abundance of *L. helveticus*. PERMANOVA based on Aitchison dissimilarity of the species compositions for the vat milk samples demonstrated statistically significant ($p < 0.01$) differences for the different adjunct cultures. However, it is noteworthy that the group dispersions were also found to be significantly different. In general, PERMANOVA is considered a more robust method in the context of the heterogeneity of group dispersions compared to other methods. For a detailed discussion on this topic, see Warton et al. (2012).

A pairwise PERMANOVA for control vat milks and vat milks with adjunct cultures, with p-value adjustment using the Benjamini-Hochberg method (Haynes, 2013), revealed significant differences ($p = 0.038$) between the samples, with the exception of those with added eLI and eLI.s adjunct cultures ($p = 0.057$). Furthermore, the differential abundances of bacterial species were evaluated using the analysis of the composition of microbiomes (ANCOM) method (Mandal et al., 2015). In ANCOM analysis, the *W* statistic represents the number of times the null hypothesis (that a particular feature has the same abundance between groups) is rejected when comparing a given feature with all other features in the dataset. A higher *W* value indicates that a feature's relative abundance is significantly different across the groups being compared. Significant differences in species ratios were observed for the adjunct cultures in the vat milk samples. The species *S. sp. VAR1* ($W = 686$) was found to be highly abundant in vat milk samples with LI adjunct cultures, while *L. helveticus* ($W = 687$) was highly abundant in vat milk samples with eLI and in the samples from the second production day with the eLI.s adjunct cultures (see Fig. 3 and Supplementary Fig. S1 for details).

3.1.5. Alpha diversity of cheese samples

The alpha diversity of the ripened cheese samples did not differ significantly between the control cheeses and those with adjunct cultures (see Supplementary Table S2). Alpha-diversity metrics are typically performed on ASVs. However, at the species level, a difference was observed between the number of species identified in the cheeses made from raw milk (range: 23–47) and those made from thermised milk (range: 161–204; Supplementary Fig. S3).

In addition to the adjunct cultures added to the vat milk for cheese production, a common starter culture consisting of *Lc. lactis, Lc. cremoris*, and *Ln. mesenteroides* was employed. In the control cheese samples, these three species were the most abundant. In the cheese produced from thermised milk, *Lc. cremoris* (6.7 %) and species from genera typically found in cheese rinds, *Corynebacterium* (9.2 %) and *Brevibacterium* (2.4 %), were the most abundant. In the control cheeses made from raw milk, the most abundant species were *Lc. cremoris* (5.9 %), *E. faecalis* (6.4 %), *Lacticaseibacillus* (*L.*) *paracasei* (3.3 %), *Propionibacterium* (*P.*) *freudenreichii* (1.5 %), and species from the *L. plantarum* group (0.6 %). It is noteworthy that *P. freudenreichii* and the *L. plantarum* group spp. were detected in all cheeses made from raw milk but not in any of the cheeses made from thermised milk, even though they were not detected in any of the adjunct cultures or vat milk samples. Similarly, *L. paracasei* was identified in all raw milk cheeses and in those made with the LI adjunct culture but only with a low abundance of 0.1 % in the vat milk sample from the second day of production (Supplementary Fig. S1).

Overall, more than 400 microbial species have been identified in raw

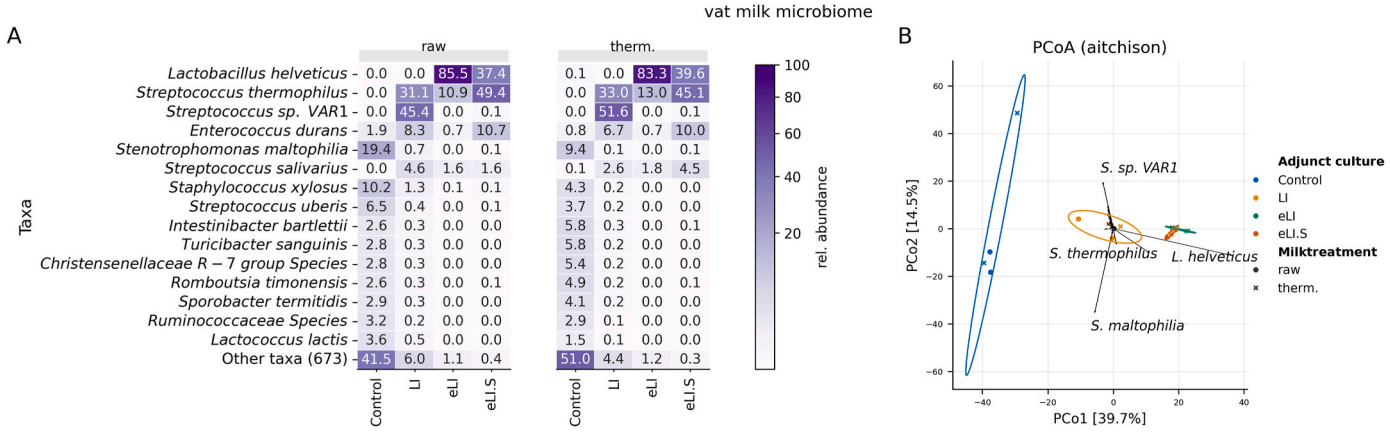

Fig. 3. Heatmap of average relative abundances and PCoA of Aitchison dissimilarities for the microbiomes of vat milk samples with adjunct cultures. Panel A depicts a heatmap of the mean relative abundance of the 15 most prevalent bacterial species in vat milk samples, as determined by 16S rRNA gene amplicon sequencing in terms of species counts. Panel B depicts a principal coordinate analysis of Aitchison dissimilarities for the microbiomes of vat milk samples with adjunct cultures, with the adjunct cultures indicated by colours and the milk treatment (raw/thermised) indicated by shape. Ellipses are used to indicate the range of two standard deviations around the centroids for the control samples (without added adjunct culture) and the samples with adjunct cultures.

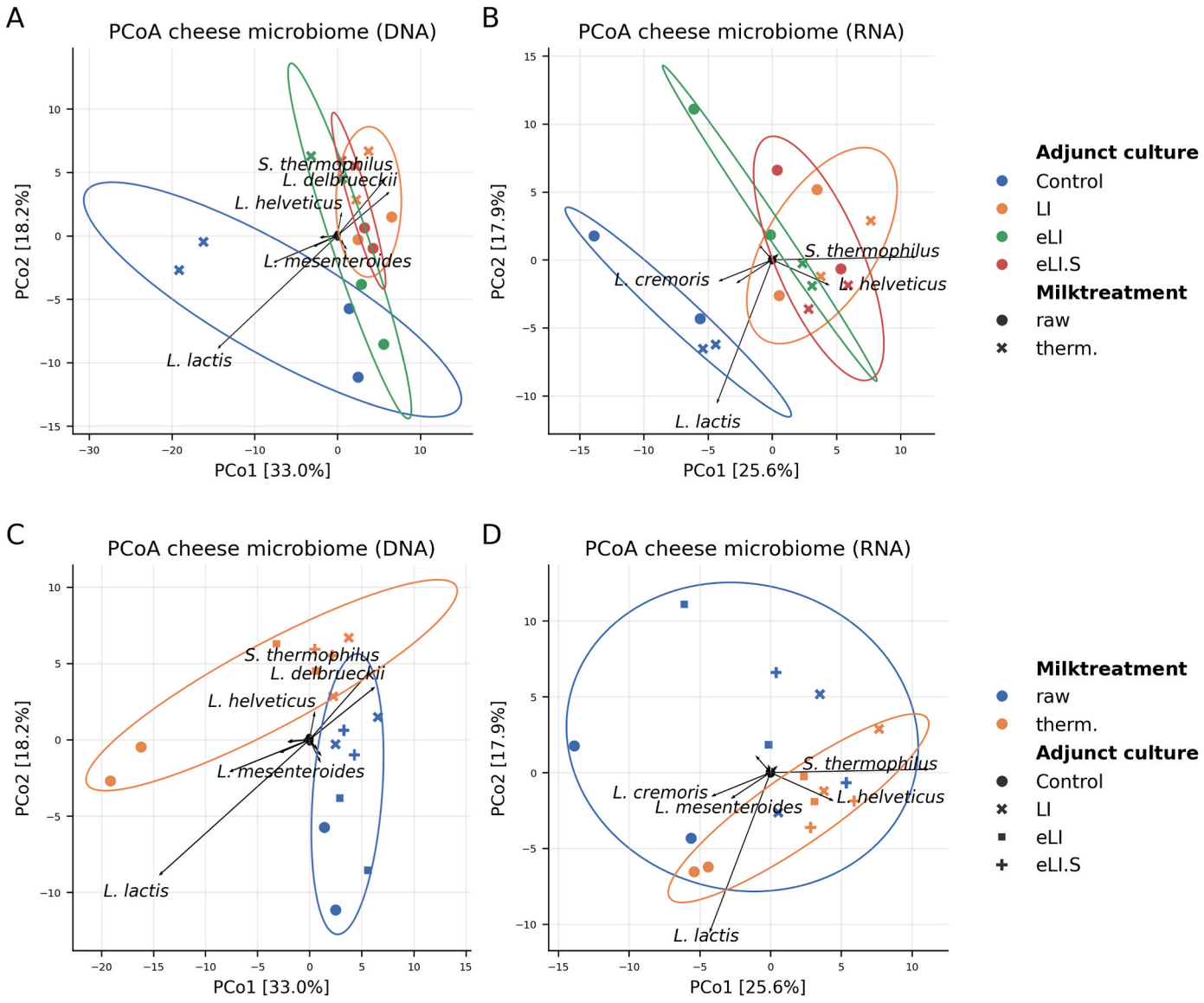

Fig. 4. PCoA of Aitchison dissimilarities for the microbiomes of cheese samples with adjunct cultures. Panels A and B illustrate the results of a principal coordinates analysis (PCoA) based on an Aitchison dissimilarity matrix of species counts (beta-diversity) of the 16S rRNA gene (DNA) and the 16S rRNA (RNA) amplicon sequencing, respectively. The samples are differentiated based on colours for the adjunct cultures and based on shapes for the milk treatment (raw/thermised). Ellipses indicate the range of two standard deviations around the centroids for the control samples (without added adjunct cultures) and the samples with adjunct cultures. Panels C and D show the same results as panels A and B. The samples are differentiated based on colour for the milk treatment and based on shape for the added adjunct cultures. Ellipses indicate the range of two standard deviations around the centroids for cheese samples made from raw milk or thermised milk.

milk (Montel et al., 2014). Biodiversity decreases drastically in cheese cores due to harsh conditions during cheese manufacture, such as the use of starter cultures, heat treatment, and the later addition of salt, limited sources of fermentable carbohydrates, acidic pH, and low temperatures during ripening (De Filippis et al., 2014; Gobbetti et al., 2018). Our findings are well aligned with observations from others describing a small but well-defined core microbiota for a certain cheese type. Zago et al. (2021) found 15 bacterial species with an abundance of more than 1 % in Grana Padano, while De Filippis et al. (2014) identified 6 dominant OTUs and 25 sub-dominant OTUs with an abundance higher than 0.01 % in Mozzarella, Grana Padano, and Parmigiano Reggiano. In Raclette du Valais, a semi-hard cheese made from raw cow's milk, the 21 most prevalent species represented, on average, 99.96 % of the total reads (Dreier et al., 2022).

For the cheese samples in this study, DNA and RNA were extracted and subsequently analysed using amplicon sequencing. It has been established that RNA is less stable than DNA and susceptible to ubiquitous ribonucleases. Consequently, its half-life in the environment is shorter than that of DNA. In the context of cheese, it is commonly assumed that the relative abundance of viable and metabolically active bacterial cells can be approximated by measuring RNA. However, a recent study identified the limitations of RNA-based amplicon sequencing for activity assessment in complex community samples (Wang et al., 2023).

We observed that the alpha diversity of the 16S rRNA gene (DNA) results was significantly higher ($p = 0.03$) than that of the 16S rRNA (RNA) results for the cheese samples (Supplementary Table S2). A PCoA based on Aitchison dissimilarity revealed no clear differentiation between the two groups (Supplementary Fig. S4B). However, a PERMANOVA based on Aitchison dissimilarities showed a significant difference ($p = 0.03$) between the two methods. The Venn diagram (Supplementary Fig. S4C) illustrates the presence of 110 bacterial species identified in the DNA samples, while 105 bacterial species were detected in the RNA samples. The intersection between the two approaches encompasses 68

species. The 68 species accounted for between 98.51 % and 100 % of the reads in the DNA samples and between 98.44 % and 100 % of the reads in the RNA samples.

3.1.6. Beta diversity of cheese samples

The PCoA based on Aitchison dissimilarity for the counts from the DNA samples of ripened cheeses indicated the presence of distinct clusters for the control cheeses and those produced with adjunct cultures. However, the differentiation between the various adjunct cultures was less pronounced (Fig. 4A). The observed differences were statistically significant (PERMANOVA, $p < 0.01$), and the group dispersions were also found to be significantly different. The results of the pairwise PERMANOVA indicated statistically significant differences between all groups, with the exception of the control cheeses and the ripened cheeses made with eLI adjunct cultures ($p = 0.204$) and between cheeses made with eLI and eLI.s adjunct cultures ($p = 0.802$). A more pronounced clustering was observed for the two distinct milk treatments (raw/thermised), with notable differences between the groups as determined by PERMANOVA ($p < 0.01$) and with homogeneity of group dispersions (Fig. 4B).

A differential abundance analysis performed with ANCOM revealed statistically significant differences in species ratios between ripened cheese samples with different adjunct cultures for *S. sp. VAR1* ($W = 102$), *S. thermophilus* ($W = 100$), and *L. helveticus* ($W = 105$). The differentially abundant species for the two milk treatments were identified as members of the *L. plantarum* group ($W = 103$), *L. paracasei* ($W = 97$), and *P. freudenreichii* ($W = 103$).

A PCoA based on Aitchison dissimilarity for the RNA samples from ripened cheeses revealed a similarity in the group dispersions and a greater differentiation between the groups than observed in the DNA samples for the control and adjunct cultures. (Fig. 4B, PERMANOVA; $p < 0.01$). The results of the pairwise PERMANOVA indicated statistically significant differences between all groups, with the exception of those comparing cheeses produced with eLI and eLI.s adjunct cultures, for which the *p*-value was 0.395.

The differential abundance analysis of the RNA samples identified the same species that were found to have significant differences in species ratios between the ripened cheese samples with different adjunct cultures and the DNA samples: *S. sp. VAR1* ($W = 96$), *S. thermophilus* ($W = 101$), and *L. helveticus* ($W = 100$). The differential abundance of species for the two milk treatments differed in the RNA samples, with only two out of three species from the DNA samples also identified as statistically different. These were the species of the *L. plantarum* group ($W = 86$) and *P. freudenreichii* ($W = 90$).

3.2. Culture dependent analysis of bacterial communities

The microbiota was investigated using culture dependent analysis of bacterial communities in vat milk, one-day-old cheeses, and ripened cheeses after two and four months. The aim was to identify differences between raw and thermised milk used for cheese production and to examine the influence of the added adjunct cultures (Table 1, Table 2).

The only notable distinction between raw and thermised vat milk (with adjunct cultures) was the slightly elevated abundance of *Enterobacteriaceae* in the raw milk samples. Additionally, the *Enterobacteriaceae* counts were significantly higher in cheese produced from raw milk after 1 and 60 days of ripening. In the fully ripened cheeses (120 days), the counts of Lactobacilli, facultative heterofermentative LAB, propionic acid bacteria, and *Escherichia coli* were higher in the cheeses made from raw milk.

With regard to the differences between the samples with varying adjunct cultures, the only significant differences were observed in the vat milk samples. The total aerobic plate count was found to be higher in the control samples than in those with an added LI adjunct culture. Significant differences were observed in enterococci counts between the control and LI samples, as well as between the eLI and eLI.s samples. After 60 days of ripening, no pathogens (*Listeria, Salmonella*) were detected in any of the model cheeses (Table 2).

3.3. Cheese production

Cheese cross sections after 120 d of ripening are shown in Supplementary Fig. S5. The use of the natural milk adjunct culture eLI.s in the cheeses made from raw milk resulted in an acceleration of the acidification process during the initial 6 h of cheese manufacture on day one, as indicated by the lower pH values in Supplementary Fig. S6. Between 6 and 24 h, the pH value decreased at a slower rate than that observed in the control sample. The final pH was 5.12, which was slightly above the target range of 4.95–5.01. The natural milk adjunct cultures LI and eLI did not significantly impact the acidification process. However, the final pH values were slightly below the target range, with pH values of 4.94 and 4.92, respectively. The control cheeses, produced from raw and thermised milk without the addition of adjunct cultures, exhibited a final pH value of 4.99.

A comparable observation was made with regard to the natural milk adjunct cultures in cheeses produced from thermised milk. The natural milk adjunct culture eLI.s in the cheeses made from raw milk accelerated the acidification process during the initial 6 h, after which the pH value decreased at a slower rate than the control, reaching a final pH of 5.06. The natural milk adjunct cultures LI did not significantly impact the acidification process; however, the final pH value was slightly below the target range, with a pH value of 4.90. The cheese sample with eLI was within the desired pH range after 24 h, with a pH value of 4.97.

On the second day of cheese production, the acidification process remained consistent across all cheeses made from raw milk, with the pH reaching the target range after 24 h. The acidification process for the cheeses made from thermised milk was also highly similar in all samples. The pH after 6 h was slightly lower than in the control for the eLI and eLI.s adjunct cultures, but the final pH was within the target range for all cheeses after 24 h.

The use of natural milk adjunct cultures did not significantly impact the production parameters, even though curd acidification in the first hours of cheese making was partially increased. The final pH was either above or below the target range on the first day of production but stayed within the target range on the second production day. The differences between the two days may be due to differences in the microbial compositions. The acidifying performance of microbes is known to be strain-dependent (Bancalari et al., 2016). It was surprising to find that most cheeses made with natural milk adjunct cultures were in or above the pH target range, even though a common drawback of adjunct cultures is curd over-acidification due to lactose fermentation in addition to primary starters (Gobbetti et al., 2015).

A natural milk adjunct culture that can be used successfully in cheese production should be safe, as reliably stable as possible, have no negative impact on the production parameters and final quality, and have a positive influence on the aroma profile of the cheese. The three cultures tested in this study can be considered safe, as the microbiological tests conducted after 60 days of ripening did not detect any of the typical pathogens, *Listeria* and *Salmonella*. Additionally, no coagulase-positive staphylococci were identified in the cheese samples.

The quality of raw milk is crucial in determining the effectiveness and safety of the natural milk adjunct cultures used in cheese production. Raw milk serves as the foundational medium for these cultures, influencing their development and activity. High-quality raw milk, characterized by favourable microbial profiles and low contamination levels, provides an optimal environment for the growth and proliferation of beneficial microorganisms. This, in turn, supports the production of robust and consistent adjunct cultures.

Given the current limitations in sample size and the restricted number of trials included in this study, it is not possible to draw definitive conclusions regarding the stability of the adjunct cultures or the selection of the most suitable milk. Further investigation is necessary to

Table 1
Culture dependent analysis of bacterial communities – Average plate counts (log10) data for different treatments. Comparisons between the plate counts of different milk treatments (raw/therm) used for cheese productions in the upper part of the table, significant differences are indicated by an asterisk. Comparisons between the plate counts of the different adjunct cultures added for cheese production in the lower part of the table, significant differences are indicated by letters according to the adjunct cultures that were compared: Ctrl - LI: a, Ctrl - eLI: b, Ctrl - eLI.s: c, LI - eLI: d, LI - eLI.s: e, eLI - eLI.s: f.

		APC	Streptococci	Lactobacilli	FH	PAB	Enterococci	*Enterobacteriaceae*	*E. coli*	Salt tolerant	CPS
Milk treatment (n = 8)											
Vat milk	**Raw**	4.24 ± 0.5	3.03 ± 0.77	2.35 ± 1.09	1.03 ± 0.18	0.95 ± 0.0	2.65 ± 0.92	1.84 ± 0.57*	1.25 ± 0.54	3.22 ± 0.59	0.95 ± 0.0
	Therm	3.78 ± 1.05	2.27 ± 1.33	1.91 ± 1.42	1.08 ± 0.35	0.96 ± 0.02	2.21 ± 1.31	0.95 ± 0.0*	0.95 ± 0.0	2.17 ± 1.32	0.95 ± 0.0
Cheese 1d	**Raw**	6.18 ± 0.0	8.81 ± 0.37	3.26 ± 2.43	2.18 ± 0.43	1.95 ± 0.0	4.84 ± 0.55	4.42 ± 0.2*	3.84 ± 0.63*	5.01 ± 0.73*	1.95 ± 0.0
	Therm	6.18 ± 0.0	8.89 ± 0.21	5.8 ± 3.27	2.16 ± 0.58	1.95 ± 0.0	3.6 ± 1.65	3.5 ± 0.71*	2.3 ± 0.51*	3.36 ± 1.58*	1.95 ± 0.0
Cheese 2M	**Raw**	6.18 ± 0.0	7.87 ± 1.04	7.26 ± 0.28*	6.9 ± 0.53*	4.56 ± 1.1*	5.45 ± 0.44	3.56 ± 0.35*	3.03 ± 0.94*	5.43 ± 0.27*	1.95 ± 0.0
	Therm	6.18 ± 0.0	6.99 ± 0.86	4.33 ± 2.02*	4.1 ± 1.4*	3.29 ± 1.04*	4.6 ± 1.14	2.56 ± 0.7*	0.95 ± 0.0*	5.7 ± 0.15*	1.95 ± 0.0
Cheese 4M	**Raw**	6.17 ± 0.01	7.0 ± 1.61	7.19 ± 0.41*	6.97 ± 0.61*	2.73 ± 1.44*	5.11 ± 0.29	2.48 ± 0.56	2.33 ± 0.53*	5.04 ± 0.29	1.95 ± 0.0
	Therm	6.15 ± 0.08	6.33 ± 1.43	3.53 ± 2.39*	4.16 ± 2.35*	4.33 ± 0.89*	4.5 ± 1.45	2.11 ± 0.44	0.95 ± 0.0*	5.37 ± 1.03	1.95 ± 0.0
Adjunct cultures (n = 4)											
Vat milk	**Ctrl**	3.24 ± 0.97[a]	1.94 ± 1.15	1.33 ± 0.75	1.08 ± 0.26	0.95 ± 0.0	1.45 ± 0.58[ac]	1.27 ± 0.62	0.95 ± 0.0	2.22 ± 0.98	0.95 ± 0.0
	LI	4.77 ± 0.29[a]	2.66 ± 0.58	2.31 ± 1.57	0.97 ± 0.02	0.95 ± 0.0	2.79 ± 0.14[a]	1.32 ± 0.72	1.27 ± 0.62	2.9 ± 0.4	0.95 ± 0.0
	eLI	3.56 ± 0.26	2.23 ± 1.0	2.55 ± 1.39	0.95 ± 0.0	0.95 ± 0.0	1.77 ± 0.55[f]	1.51 ± 0.65	0.95 ± 0.0	1.9 ± 1.09	0.95 ± 0.0
	eLI.s	4.46 ± 0.53	3.78 ± 1.02	2.34 ± 1.27	1.22 ± 0.49	0.97 ± 0.02	3.71 ± 1.17[cf]	1.5 ± 0.64	1.23 ± 0.54	3.78 ± 1.14	0.95 ± 0.0
Cheese 1d	**Ctrl**	6.18 ± 0.0	8.92 ± 0.31	1.95 ± 0.0	1.95 ± 0.0	1.95 ± 0.0	3.4 ± 1.67	3.6 ± 1.22	3.15 ± 1.14	3.67 ± 0.66	1.95 ± 0.0
	LI	6.18 ± 0.0	8.85 ± 0.36	3.5 ± 3.1	2.59 ± 0.79	1.95 ± 0.0	4.96 ± 0.47	4.01 ± 0.51	3.17 ± 1.03	5.21 ± 0.47	1.95 ± 0.0
	eLI	6.18 ± 0.0	8.94 ± 0.4	6.71 ± 3.22	1.95 ± 0.0	1.95 ± 0.0	3.43 ± 1.1	4.11 ± 0.37	2.75 ± 0.92	4.03 ± 0.9	1.95 ± 0.0
	eLI.s	6.18 ± 0.0	8.69 ± 0.07	5.95 ± 2.87	2.19 ± 0.47	1.95 ± 0.0	5.1 ± 1.18	4.11 ± 0.52	3.21 ± 1.13	3.82 ± 2.7	1.95 ± 0.0
Cheese 2M	**Ctrl**	6.18 ± 0.0	7.62 ± 0.92	5.2 ± 2.06	5.2 ± 2.09	4.44 ± 0.63	4.26 ± 0.82	3.16 ± 0.83	1.92 ± 1.34	5.62 ± 0.13	1.95 ± 0.0
	LI	6.18 ± 0.0	7.11 ± 1.0	7.35 ± 1.12	6.23 ± 0.68	3.67 ± 1.78	5.81 ± 0.06	2.91 ± 0.74	1.93 ± 1.36	5.63 ± 0.17	1.95 ± 0.0
	eLI	6.18 ± 0.0	7.29 ± 1.01	5.71 ± 1.82	5.57 ± 1.94	4.14 ± 1.27	4.64 ± 0.83	3.11 ± 0.86	2.18 ± 1.47	5.51 ± 0.33	1.95 ± 0.0
	eLI.s	6.18 ± 0.0	7.7 ± 1.45	4.92 ± 2.78	5.01 ± 2.43	3.46 ± 1.22	5.4 ± 1.05	3.06 ± 0.87	1.95 ± 1.4	5.5 ± 0.4	1.95 ± 0.0
Cheese 4M	**Ctrl**	6.18 ± 0.0	6.94 ± 1.17	4.57 ± 3.02	4.52 ± 2.96	2.74 ± 1.35	4.07 ± 1.45	2.2 ± 0.5	1.7 ± 0.96	4.54 ± 0.58	1.95 ± 0.0
	LI	6.18 ± 0.0	7.16 ± 1.43	7.31 ± 0.81	6.9 ± 0.31	3.16 ± 1.39	5.62 ± 0.29	2.56 ± 0.71	1.72 ± 0.98	5.56 ± 0.34	1.95 ± 0.0
	eLI	6.12 ± 0.11	6.25 ± 2.06	4.96 ± 2.92	4.97 ± 2.99	3.95 ± 1.39	4.25 ± 0.9	2.2 ± 0.5	1.7 ± 0.96	5.08 ± 0.24	1.95 ± 0.0
	eLI.s	6.17 ± 0.01	6.31 ± 1.71	4.61 ± 2.55	5.87 ± 1.42	4.28 ± 1.55	5.26 ± 0.59	2.2 ± 0.5	1.45 ± 0.58	5.65 ± 1.12	1.95 ± 0.0

APC: Aerobic plate count, FH: Facultative heterofermentative LAB, PAB: Propionic acid bacteria, CPS: Coagulase positive staphylococci, N.D.: Not determined.

Table 2
Culture dependent analysis of bacterial communities – Average plate counts (log10) data for different treatments (Part 2). Comparisons between the plate counts of different milk treatments (raw/therm.) used during cheese productions in the upper part of the table. Significant differences are indicated by an asterisk. Comparisons between the plate counts of the different adjunct cultures added during cheese productions in the lower part of the table, significant differences are indicated by letters according to the adjunct cultures that were compared: Ctrl - LI: a, Ctrl - eLI: b, Ctrl - eLI.s: c, LI - eLI: d, LI - eLI.s: e, eLI - eLI.s: f, N.D.: Not determined.

		Moulds	Yeasts	Butyric spores	*Salmonella* spp.	*L. monocytogenes*	Other listeria
Milk treatment (n = 8)							
Vat milk	**Raw**	0.95 ± 0.0	0.95 ± 0.0	1.69 ± 0.02	N.D.	N.D.	N.D.
	Therm	0.95 ± 0.0	0.95 ± 0.0	1.73 ± 0.13	N.D.	N.D.	N.D.
Cheese 1d	**Raw**	1.95 ± 0.0	1.95 ± 0.0	2.68 ± 0.02	N.D.	N.D.	N.D.
	Therm	1.95 ± 0.0	1.95 ± 0.0	2.68 ± 0.0	N.D.	N.D.	N.D.
Cheese 2M	**Raw**	4.16 ± 0.57	2.88 ± 0.62	2.68 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	Therm	3.61 ± 0.8	2.57 ± 0.69	2.68 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Cheese 4M	**Raw**	1.95 ± 0.0	1.95 ± 0.0	2.68 ± 0.0	N.D.	N.D.	N.D.
	Therm	1.95 ± 0.0	1.95 ± 0.0	2.68 ± 0.02	N.D.	N.D.	N.D.
Adjunct cultures (n = 4)							
Vat milk	**Ctrl**	0.95 ± 0.0	0.95 ± 0.0	1.78 ± 0.17	N.D.	N.D.	N.D.
	LI	0.95 ± 0.0	0.95 ± 0.0	1.68 ± 0.0	N.D.	N.D.	N.D.
	eLI	0.95 ± 0.0	0.95 ± 0.0	1.7 ± 0.02	N.D.	N.D.	N.D.
	eLI.s	0.95 ± 0.0	0.95 ± 0.0	1.68 ± 0.0	N.D.	N.D.	N.D.
Cheese 1d	**Ctrl**	1.95 ± 0.0	1.95 ± 0.0	2.68 ± 0.0	N.D.	N.D.	N.D.
	LI	1.95 ± 0.0	1.95 ± 0.0	2.69 ± 0.02	N.D.	N.D.	N.D.
	eLI	1.95 ± 0.0	1.95 ± 0.0	2.68 ± 0.0	N.D.	N.D.	N.D.
	eLI.s	1.95 ± 0.0	1.95 ± 0.0	2.68 ± 0.0	N.D.	N.D.	N.D.
Cheese 2M	**Ctrl**	3.66 ± 1.14	3.13 ± 0.3	2.68 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	LI	3.6 ± 0.26	2.55 ± 0.69	2.68 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	eLI	4.15 ± 0.9	2.59 ± 0.77	2.68 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	eLI.s	4.13 ± 0.34	2.63 ± 0.84	2.68 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Cheese 4M	**Ctrl**	1.95 ± 0.0	1.95 ± 0.0	2.69 ± 0.02	N.D.	N.D.	N.D.
	LI	1.95 ± 0.0	1.95 ± 0.0	2.68 ± 0.0	N.D.	N.D.	N.D.
	eLI	1.95 ± 0.0	1.95 ± 0.0	2.68 ± 0.0	N.D.	N.D.	N.D.
	eLI.s	1.95 ± 0.0	1.95 ± 0.0	2.68 ± 0.0	N.D.	N.D.	N.D.

N.D.: Not determined.

gain a more profound understanding of these parameters. However, there were notable variations in the composition of the adjunct cultures on different production days. This indicates that the production parameters for the cultures could be further optimised to more effectively control variability. Given the limited number of samples in this pilot study, further research involving a broader range of milk samples from multiple dairy farms is necessary to fully understand the reproducibility of the production process and the microbiome of the cultures. Expanding the sample size will provide a more comprehensive view of how different milk sources and environmental factors impact the microbial diversity and flavour profiles of cheese. Additionally, investigating a wider array of adjunct cultures and milk treatments could reveal more nuanced interactions that affect cheese quality and safety. Such studies are essential for developing improved protocols to produce natural milk adjunct cultures made from raw milk, which could enhance the consistency and quality of artisanal cheeses.

3.4. Physical, biochemical, and chemical parameters

A total of 28 chemical and biochemical parameters were evaluated in ripened cheese samples. The 17 parameters that were found to be significantly different between one of the investigated groups (milk treatment or adjunct cultures) are displayed in Table 3. The concentrations of L-lactic acid, iso-butyric acid, and total nitrogen were observed to be lower in cheeses produced from raw milk compared to those produced from thermised milk. Conversely, the concentrations of acetic acid, propionic acid, putrescine, and phenylethylamine were found to be higher in cheese produced from raw milk.

To investigate the textural properties of the cheeses, the force at fracture of the cheeses was measured using a uniaxial compression test. As expected, the results indicated that cheeses made from thermised milk were firmer than those made from raw milk. This finding can be attributed, at least in part, to the lower water content (water in fat-free cheese) of the thermised milk cheeses (Fröhlich-Wyder et al., 2023; Guggisberg et al., 2017). The water content might also have an influence on the ripening conditions.

The greatest differences were observed in the composition of cheeses produced with adjunct cultures, particularly in the case of eLI adjunct cultures. In these samples, the concentrations of formic acid, iso-valeric acid, citric acid, cadaverine, and isopentylamine were found to be elevated in comparison to the control samples. Tyramine and cadaverine are frequently detected in commercially available raw milk cheeses with low cooking temperatures and extended ripening times (data not shown). However, the additional culture eLI exhibited elevated levels of cadaverine of over 150 mg kg^{-1}. A substantial increase in biogenic amine concentration by an adjunct culture is not desirable, as susceptible consumers may be deterred to avoid experiencing adverse reactions.

Furthermore, elevated levels of L-leucine aminopeptidase activity and higher concentrations of non-protein and water-soluble nitrogen were observed, indicating enhanced proteolysis in cheeses produced with the eLI adjunct culture. The observed increased proteolysis can probably be attributed to the high relative abundance of *L. helveticus* in the cheeses produced with the eLI adjunct cultures. Despite the high relative abundance of *L. helveticus* in the cheeses made with eLI.s, proteolysis seemed lower than in the cheeses made with the eLI adjunct culture but higher than in the controls. However, since amplicon sequencing approaches provide only semi-quantitative (relative abundance) results, we were unable to determine the absolute abundance of *L. helveticus* in the samples. Nevertheless, it is likely that additional microbial species are involved in the metabolic processes responsible for the increased proteolysis observed in these samples.

The principal component analysis of all 28 measured biochemical and chemical parameters shows a clear separation between the clusters of cheese samples made from raw and thermised milk and, to a lesser extent, between the cheese samples made with different adjunct cultures (Fig. 5A and B). Separation between adjunct cultures is mainly on principal component axis 1 and between milk treatments on principal component axis 2. The ordination of the samples also differed based on the production day (and therefore probably due to the cultures and the

Table 3
Chemical and biochemical parameters of ripened cheeses for different treatments. Comparisons between biochemical parameters of ripened cheese (4 months) for the different adjunct cultures added during cheese productions. In the upper part (Milk treatment) of the table, significant differences are indicated by an asterisk. In the lower part of the table (Adjunct cultures), significant differences are indicated by letters according to the adjunct cultures that were compared: Ctrl - LI: a, Ctrl - eLI: b, Ctrl - eLI.s: c, LI - eLI: d, LI - eLI.s: e, eLI - eLI.s: f, N.D.: Not determined.

	L-Lactic acid	Formic acid	Acetic acid	Propionic acid	Isobutyric acid	Isovaleric acid	Citric acid	Force at fracture
	[mmol kg^{-1}]							[N]
Milk treatment (n = 8)								
Raw	29.95 ± 10.94*	2.51 ± 1.53	32.36 ± 4.39*	32.54 ± 9.33*	0.07 ± 0.04*	0.23 ± 0.12	0.04 ± 0.07	9.06 ± 1.38*
Therm	58.05 ± 10.35*	2.78 ± 1.01	13.56 ± 3.99*	0.95 ± 0.33*	0.16 ± 0.09*	0.35 ± 0.17	0.71 ± 1.01	11.05 ± 0.89*
Adjunct cultures (n = 4)								
Control	49.12 ± 24.5	1.48 ± 0.3b	19.03 ± 11.25	17.1 ± 18.78	0.06 ± 0.05	0.18 ± 0.09b	0.0 ± 0.0b	10.44 ± 1.04
LI	42.25 ± 19.44	2.62 ± 1.0	23.36 ± 11.89	20.65 ± 22.76	0.1 ± 0.06	0.22 ± 0.08	0.0 ± 0.0d	8.67 ± 2.0
eLI	45.05 ± 13.59	3.84 ± 1.26b	26.38 ± 11.46	13.32 ± 16.24	0.18 ± 0.1	0.46 ± 0.15b	1.12 ± 1.14bd	10.94 ± 1.46
eLI.s	39.58 ± 18.78	2.64 ± 1.25	23.06 ± 10.84	15.9 ± 19.02	0.13 ± 0.1	0.3 ± 0.16	0.38 ± 0.75	10.16 ± 0.72

	Cadaverine	**Isopentylamine**	**Putrescine**	**Phenylethylamine**	**LAP**	**Total nitrogen**	**WSN**	**NPN**	**Wff**
	[mg kg^{-1}]				[IU kg^{-1}]	[g kg^{-1}]			
Milk treatment (n = 8)									
Raw	93.92 ± 59.8	3.48 ± 5.21	17.22 ± 9.16*	71.2 ± 26.62*	12.16 ± 15.77	36.04 ± 1.07*	22.18 ± 5.73	7.49 ± 1.16	614.12 ± 12.44*
Therm	75.5 ± 67.15	2.16 ± 6.12	1.06 ± 3.01*	33.21 ± 34.19*	14.42 ± 17.85	37.35 ± 0.83*	24.51 ± 5.88	7.62 ± 1.25	598.62 ± 9.71*
Adjunct cultures (n = 4)									
Control	43.82 ± 36.29^{b}	0.0 ± 0.0^{b}	6.92 ± 9.11	26.28 ± 32.83	2.85 ± 0.75^{b}	37.85 ± 0.72	17.73 ± 6.11^{bc}	6.98 ± 0.48^{b}	593.5 ± 13.72
LI	47.32 ± 13.65^{d}	0.0 ± 0.0^{d}	5.5 ± 6.35	65.28 ± 21.56	3.72 ± 1.98	35.9 ± 1.41	20.35 ± 2.27^{e}	6.7 ± 0.51^{d}	615.5 ± 14.2
eLI	177.0 ± 23.85^{bdf}	10.05 ± 7.25^{bd}	15.3 ± 14.11	53.38 ± 48.6	37.78 ± 13.61^{b}	36.4 ± 0.91	26.65 ± 3.08^{b}	8.88 ± 0.62^{bd}	609.25 ± 12.01
eLI.s	70.7 ± 39.18^{f}	1.23 ± 2.46	8.84 ± 12.93	63.9 ± 33.04	8.8 ± 6.89	36.62 ± 0.71	28.65 ± 2.57^{ce}	7.67 ± 1.46	607.25 ± 4.79

LAP: L-leucine aminopeptidase, NPN: Non-protein nitrogen, WSN: Water-soluble nitrogen, Wff: Water in fat free cheese.

milk used for cheese making), especially for the cheese samples made with the adjunct culture eLI.s (Fig. 5B).

The present study revealed that the adjunct cultures were mainly enriched with *S. thermophilus* and specific lactobacilli, namely *L. helveticus* and *L. delbrueckii*. The results indicate that proteolysis is higher in cheeses with the adjunct cultures and that this probably correlates with the presence of *L. helveticus*. Increased proteolysis may enhance flavour development in ripened cheeses. However, *L. paracasei*, *L. plantarum* group *spp*., *and P. freudenreichii* species were identified to be of raw milk origin and were not introduced by the adjunct cultures. Previous studies have shown that adjunct cultures of *L. paracasei* and *L. plantarum* can enhance proteolysis and increase free amino acid levels in cheddar cheese (Lynch et al., 1999; Stefanovic et al., 2018). NSLAB strains have the potential to increase during the ripening process, contributing to the production of 87a range of volatile compounds, including carboxylic acids, esters, and branched-chain compounds resulting from amino acid degradation (Blaya et al., 2018; Bluma et al., 2017; Gobbetti et al., 2015; Yavuz et al., 2021). Therefore, an optimised procedure to enhance the growth of NSLAB, including *L. paracasei* and *L. plantarum*, in natural milk adjunct cultures could positively impact the flavour profile.

P. freudenreichii plays a crucial role in the production of Swiss-type cheeses; it contributes to flavour and eye formation. When added to Raclette cheese, *P. freudenreichii* enhances flavour intensity and produces characteristic compounds, such as acetate, propionate, and branched-chain volatiles, without causing undesired eye formation (Thierry et al., 2005). Propionic acid concentrations in commercially available Vacherin Fribourgeois cheese (data not shown) indicate that the milk used for cheese making is a reliable source of *P. freudenreichii* and promoting its growth in an adjunct culture is not required and may harbour unwanted side effects, such as splits and cracks or increased eye sizes.

3.5. Volatilome

The principal component analysis of the eight volatile aroma compounds that were identified as the primary factors contributing to the differentiation between the milk treatment groups in the VIP analysis is shown in Fig. 5, panels C and D. As was observed for the biochemical and chemical parameters, the separation of the groups between the milk treatments was distinct (the VIP analysis was performed on the milk treatment groups), while no clear separation based on the adjunct cultures used for cheese production was observed in the cheese samples.

The majority of the compounds were identified at higher concentrations in the raw milk cheeses (Fig. 6). This finding is consistent with the consensus that cheeses produced from raw milk are perceived as having a richer flavour profile and more complex flavour compositions (Beuvier & Buchin, 2004; Montel et al., 2014). Ethanol is frequently detected during headspace analysis of cheeses, including Raclette-type cheeses, Cheddar, Gouda, and La Serena (Andriot et al., 2024; Arora et al., 1995; Carbonell et al., 2002; Shiota et al., 2015). However, it does not significantly impact the overall aroma of the cheese due to its higher perception threshold compared to more potent aroma compounds, such as esters, aldehydes, and sulphur compounds. The volatile compound 4-methylpentanoic acid, which is associated with a fruity flavour, was not identified in the control cheeses or in those made with eLI.s-added cultures from thermised milk. The compound 2-butanol was identified

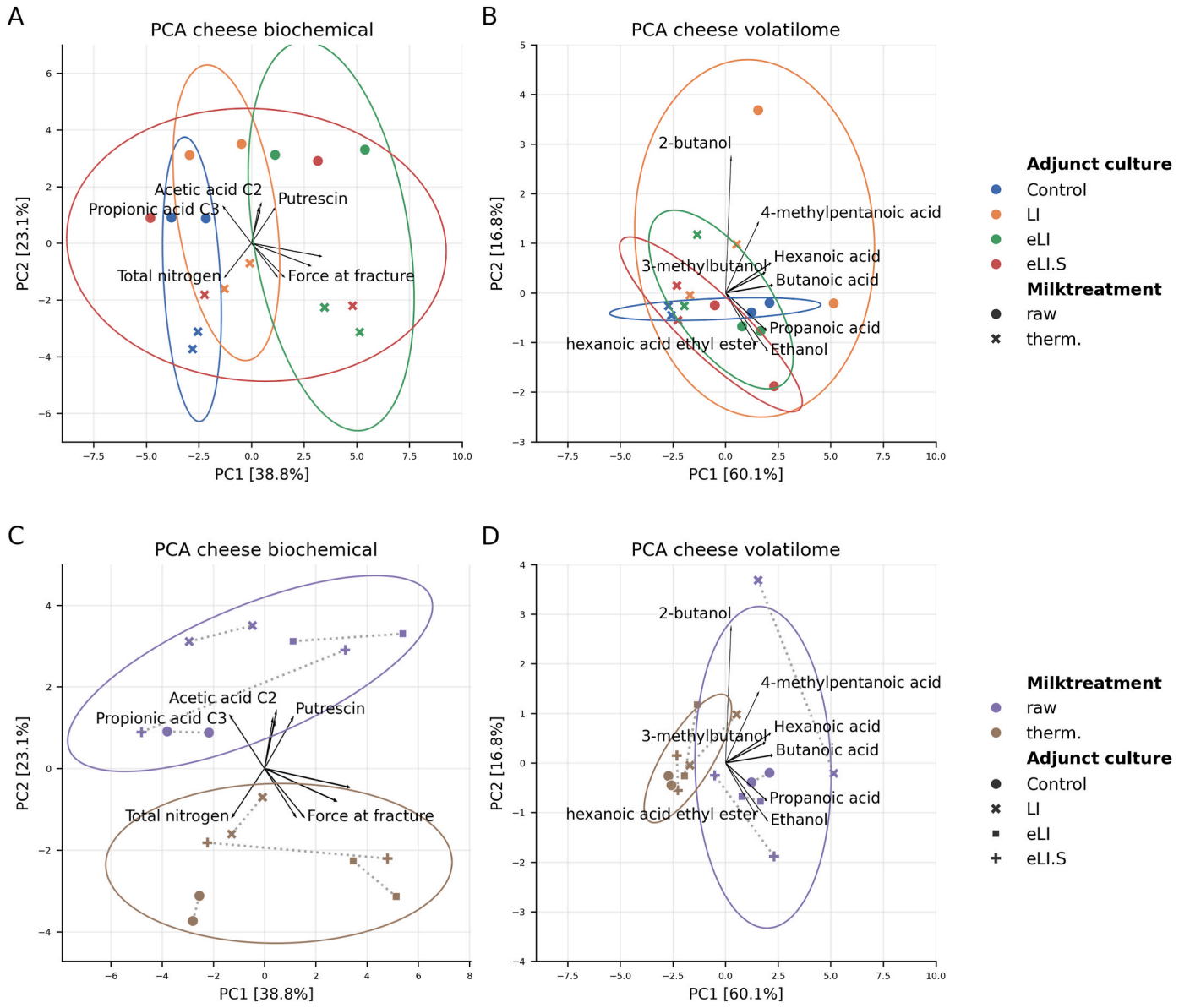

Fig. 5. PCA for biochemical parameters (features = 28) and volatilome data (features = 8) in cheese samples. Panels A and B illustrate the results of a principal component analysis (PCA) for 28 biochemical parameters in panel A and cheese volatilome data (8 features) in panel B for the cheese samples. The eight features for cheese volatilome were selected based on the Variable Importance in Projection (VIP) procedure (see Material and methods section). The samples are differentiated based on colours for the adjunct cultures and based on shapes for the milk treatment (raw/thermised). Ellipses indicate the range of two standard deviations around the centroids for the control samples and the samples with added adjunct cultures. Panels C and D show the same PCA results as panels A and B. The samples are differentiated based on colours for milk treatment and based on shapes for the added adjunct cultures. Ellipses indicate the range of two standard deviations around the centroids for the cheese samples made from raw milk or thermised milk.

in three distinct groups of cheeses: one produced with LI adjunct cultures made from raw milk and two produced with eLI and eLI.s adjunct cultures made from thermised milk.

The GC-MS method and the chemical approach used for the analysis of the volatile carboxylic acids showed good agreement for the proportions of propionic acid in the cheeses. However, the two methods contradicted each other for butanoic acid (butyric acid) and hexanoic acid. The GC-MS method identified a greater amount of butanoic and hexanoic acids in cheeses made from raw milk, whereas the quantitative chemical analysis demonstrated approximately equal concentrations for these compounds in cheeses made from raw milk and cheeses made from thermised milk.

The two methods used to measure volatile carboxylic acids differ significantly in their approach, which is a consequence of their distinct methodologies. The first method involves acidifying the homogenised cheese samples and extracting the acids, including those potentially trapped in cells, via steam distillation. The acids are then derivatised into ethyl esters, which facilitates their detection by GC-FID. However, this method has the potential to overestimate the ester content, as esterification can occur naturally in cheese, leading to an accumulation of both naturally occurring esters and those produced by the method procedure. By contrast, the GC-MS method is designed to capture the aroma of the cheese by directly extracting volatile compounds from homogenised cheese using headspace analysis. This approach provides a more accurate reflection of the cheese's volatile profile without altering the natural ester balance. Whereas the first method yields absolute results (concentrations) of the carboxylic acids, the volatilome analysis is semi-quantitative, with a focus on the differences in the ratios of the volatile compounds within the overall aroma profile.

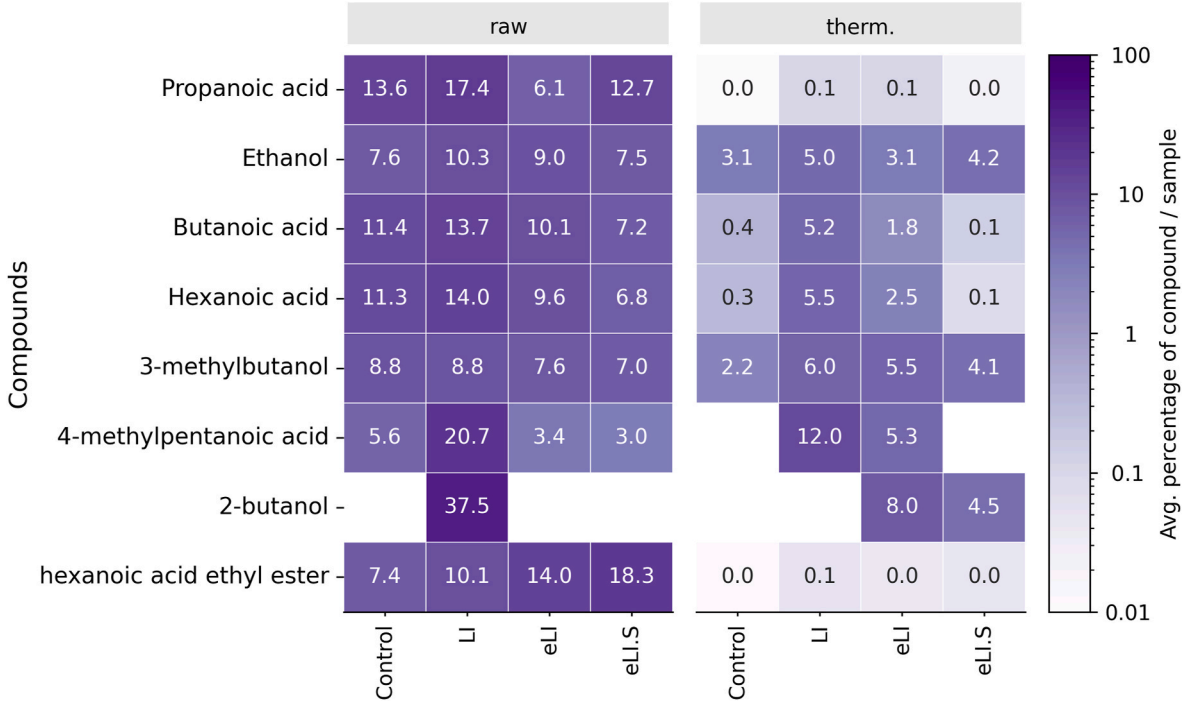

Fig. 6. Heatmap of volatilome data, average abundance percentage (normalised per compound). Each row represents the average relative abundance of a unique volatile compound across grouped samples, with the total abundance of each compound across all samples normalised to 100%. Values are displayed as percentages, allowing for the comparison of compound abundance across different sample groups within each row. Note that comparisons should only be made within rows and not between rows, as normalisation was performed independently for each compound.

4. Conclusion

The study reveals that adjunct cultures and milk treatment significantly influence the microbial composition and, to a lesser extent, the flavour profiles of semi-hard Vacherin Fribourgeois model cheeses. Our findings indicate that these cultures can enhance and help control microbial diversity, thereby supporting the expression of terroir in raw milk cheeses, which consistently exhibit greater diversity and more complex flavor profiles compared to cheeses made from thermised milk. Notably, while lattoinnesto-like cultures displayed variability across production days, enriched lattoinnesto-like cultures—particularly with added salt—produced distinct microbial profiles characterized by a predominance of *L. helveticus*. Furthermore, eLI adjunct cultures promoted enhanced proteolysis, leading to higher concentrations of flavor-related compounds. Overall, the findings underscore the critical role of milk treatment and adjunct cultures in shaping the flavour and quality of cheese, offering insights for optimising artisanal cheese production processes. However, given the limited number of samples and replicates in this pilot study, further research involving a broader range of milk samples from multiple dairy farms is necessary to fully understand the reproducibility of the production process and the microbiome of the cultures and refine culture production parameters.

CRediT authorship contribution statement

Matthias Dreier: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Luca Bettera:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Conceptualization. **Hélène Berthoud:** Writing – review & editing, Writing – original draft, Resources, Methodology, Investigation. **Pascal Fuchsmann:** Writing – review & editing, Writing – original draft, Resources, Methodology, Investigation. **Lucie K. Tintrop:** Writing – original draft. **Hans-Peter Bachmann:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. **Dominik Guggisberg:** Writing – review & editing, Writing – original draft. **Remo S. Schmidt:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

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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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.idairyj.2025.106249.

Data availability

The data presented in the study are deposited in the "Code and Data for Manuscript: How raw milk-based adjunct cultures influence the microbial diversity in cheese" repository, doi:

10.5281/zenodo.13882370. The NGS data presented in the study are deposited in the Sequence Read Archive (SRA), BioProject ID: PRJNA1162746.

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