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A taste of North Macedonia: Seasonal variation in the microbiota, physico-chemical traits, and morpho-textural profile of a traditional brined raw goat's milk cheese

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ABSTRACT

This study provides a comprehensive characterization of a traditional Macedonian brined raw goat's milk cheese, focusing on how seasonal production (spring vs. autumn) shapes its physicochemical traits, morpho-textural properties, and microbial ecology. Cheese samples produced in autumn exhibited stronger acidification, higher titratable acidity, lower water activity, and higher NaCl content than spring cheeses, reflecting variability associated with artisanal, non-standardized processing. Texture profile analysis showed that cohesiveness and springiness were significantly affected by season, whereas hardness and adhesiveness remained comparable across batches. A combined culture-dependent and 16S rRNA gene–based metataxonomic approach revealed seasonally distinct microbiota. Viable microbial populations composed of mesophilic aerobes (up to 6.51 log cfu g^{-1} at 60 days of ripening), presumptive mesophilic lactobacilli and lactococci (up to 6.51 and 7.18 log cfu g^{-1} at 60 days of ripening, respectively), presumptive coagulase-negative and coagulase-positive staphylococci (up to 6.97 and 1.76 log cfu g^{-1} at 60 days of ripening, respectively), and Enterobacteriaceae (up to 1.25 log cfu g^{-1} at 60 days of ripening) were detected. Spring cheeses were characterized by higher relative abundances of Carnobacteriaceae, *Enterococcus*, *Serratia*, and *Tetragenococcus halophilus*, whereas autumn cheeses were dominated by *Companilactobacillus* and *Lactococcus*, alongside various Enterobacteriaceae. Beta-diversity analysis confirmed significant clustering of cheese microbiota by season. In total, 134 lactic acid bacteria isolates were obtained from the dairy environment, milk, brine, and cheese. These included *Lactococcus lactis*, *Levilactobacillus brevis*, multiple *Enterococcus* species, *Pediococcus pentosaceus*, *Lacticaseibacillus paracasei*, *Marinilactibacillus psychrotolerans*, and *Companilactobacillus alimentarius*. Many isolates showed strong proteolytic activity, several produced exopolysaccharides, and a subset exhibited lipolytic capacity, underscoring their technological potential. Screening for the histidine decarboxylase gene *hdcA* revealed that only the *C. alimentarius* isolate was positive, excluding this strain from consideration as an adjunct culture, whereas all other isolates were *hdcA*-negative and therefore suitable candidates from a histamine-safety perspective. Overall, this integrated analysis highlights the rich microbial diversity and seasonal variability of this artisanal cheese and supports the selection of safe autochthonous lactic acid bacteria for future product valorization.

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1. Introduction

Goat milk is a dietary staple for more than three-quarters of the global population, valued for its high-quality protein, essential minerals such as potassium and calcium, and vitamin A (ALKaisy et al., 2023). In addition, the fat and protein in goat milk are more easily digested and less allergenic than those in cow's milk, making it a preferred choice for certain consumers (Singh et al., 2021). Interestingly, casein micelles in goat milk are generally smaller, more soluble, sediment more slowly, have lower heat stability, and contain higher concentrations of calcium, phosphorus and iron compared to those in cow milk (dos Santos et al., 2023; Tilocca et al., 2020).

Milk from cows, ewes, or goats is highly susceptible to microbial contamination and spoilage, which makes prompt refrigeration necessary after milking. Nevertheless, even when stored at low temperatures, fresh milk typically remains stable for only a few days. Converting milk into cheese represents one of the most effective approaches to prolonging its shelf life.

Cheesemaking involves the addition of a coagulating agent derived from microbial cultures or from rennet of animal or plant origin. Traditionally, rennet is obtained from the abomasum of unweaned calves, then dried and processed for dairy applications. In addition to animal rennet, microbial coagulants are extensively used, particularly aspartic proteases produced by *Rhizomucor miehei*. Owing to their three-dimensional structure, which closely resembles that of chymosin (Wiśniewski et al., 2025), these enzymes are considered effective alternatives to conventional rennet. After coagulation, the curd is cut and the whey drained, then molded and salted. The molded curd subsequently undergoes fermentation, driven either by the natural microbiota present in raw milk or by selected starter cultures when pasteurized milk is used. This may be followed by a ripening phase, the duration and conditions of which vary according to the cheese type and local tradition (Arias-Roth et al., 2022).

The microbial community of raw milk is typically composed of saprophytic microorganisms originating from the udder and the dairy environment. Pathogenic species may also be present, depending on animal health and hygiene practices during milking. The natural milk microbiota is therefore recognized as an important factor influencing both the safety and the sensory properties of cheese (Tilocca et al., 2020). During fermentation, pro-technological bacteria (primarily lactic acid bacteria) although representing only a minor fraction of the initial microbiota, acidify the curd through the production of organic acids such as lactic and acetic acid. This process not only creates conditions unfavorable for spoilage and pathogenic microorganisms but also allows lactic acid bacteria to prevail. Furthermore, their proteolytic and lipolytic activities generate volatile compounds that contribute to the distinctive sensory characteristics of cheese. However, lactic acid bacteria are also capable of producing biogenic amines in dairy products, including histamine (Moradiyan Tehrani & Goli, 2025). This metabolite, formed through the decarboxylation of histidine, can cause scombroid poisoning, a foodborne intoxication characterized by symptoms such as headache, palpitations, and vomiting (Belleggia et al., 2021). This reaction is catalyzed by histidine decarboxylase, an enzyme encoded within a gene cluster that includes the *hdcA* gene (Belleggia et al., 2021).

According to Tilocca et al. (2020), the bacterial groups most commonly associated with goat cheese include *Lactococcus*, *Levilactobacillus, Lactiplantibacillus*, *Lacticaseibacillus, Companilactobacillus*, and *Enterococcus*, followed by Enterobacteriaceae and Micrococcaceae. Furthermore, halophilic species have also been reported (Quigley et al., 2013).

Recent studies have demonstrated that cheese microbiota and quality traits are strongly shaped by seasonal dynamics affecting both raw milk and resident environmental populations. In particular, Ferrocino et al. (2026) showed that season-dependent shifts in stable facility-associated microbiota can influence microbial succession during ripening, ultimately impacting texture, aroma development, and sensory identity.

Today, a wide range of goat milk–based products is commercially available, such as yogurt, fermented milk and milk powder, and cheese. Interestingly, goat milk and its derived cheeses are notable for the presence of bioactive compounds, such as beta-carotene, lutein, and terpenoids, which play a role in both flavor and potential health benefits (Kontodimos et al., 2023). Across much of the Balkan Peninsula, cheeses made from raw goat's milk are traditionally prepared in household settings without the use of starter cultures (Terzić-Vidojević et al., 2020). These traditional practices not only preserve local culinary heritage but also embody artisanal techniques that shape the microbiological and sensory properties of the final products. In rural households, dairy production commonly includes fresh cheese and white brined cheese, both typically crafted from raw milk (Terzić-Vidojević et al., 2020).

It is noteworthy that a key political goal of Western Balkan countries is accession to the European Union. Achieving this requires substantial efforts to enhance the performance and competitiveness of the agricultural sector, as well as to align it with EU standards, demanding considerable resources and policy attention (Martinovska Stojcheska et al., 2024). Agriculture remains vital to the region's social and economic structure, serving as a major source of employment and income, particularly given the high proportion of the population residing in rural areas and employed in farming activities (Martinovska Stojcheska et al., 2024). Within this framework, North Macedonia is among the few Western Balkan countries that provide financial support, though still modest, for the advancement of rural communities (Martinovska Stojcheska et al., 2024).

Despite growing evidence that cheese quality and microbiota are influenced by seasonal factors and by resident environmental populations, integrated studies simultaneously addressing physico-chemical traits, texture, and microbial dynamics in traditional raw goat's milk cheeses remain limited. This study aimed to comprehensively characterize a traditional brined raw goat's milk cheese produced by an artisanal dairy in North Macedonia by investigating its physico-chemical traits, morpho-textural properties, and microbial dynamics during ripening, with particular emphasis on seasonal production (spring and autumn). A key objective was the extensive isolation and characterization of lactic acid bacteria from milk, brine, cheese, and the dairy environment, combining classical microbiological analyses, 16S rRNA gene–based metataxonomics, and phenotypic assessment of proteolytic, lipolytic, and exopolysaccharide-producing activities. Safety attributes of the isolates were also considered. For this reason, all isolates were screened for the presence of the *hdcA* gene (encoding for the enzyme histidine decarboxylase) to assess their potential involvement in histamine formation.

2. Materials and methods

2.1. Cheese manufacturing and sampling

Cheese was produced from raw goat's milk collected immediately after milking, without heat treatment before or after coagulation. Production was carried out in two seasons, spring (April) and autumn (November) 2023, using milk from mixed-breed local goats reared in Selce village, Tetovo, North Macedonia. Coagulation was performed at 37 °C for 60 min using liquid microbial rennet "Super Maja" (5000 International Milk Clotting Units - IMCU) (Super Group, Skopje, North Macedonia), containing the enzyme mucorpepsin derived from *R. miehei*. The curd was pressed for 2 h, cut into ~250 g blocks, and dry salted for 24 h. Subsequently, the cheese was immersed in 10% (w v^{-1}) NaCl brine and ripened at 12–15 °C for 60 days. Apart from the milk, the same batches of rennet and salt were used in both cheesemaking trials. The flow chart of cheese production is reported in Fig. 1.

Two seasonal productions were carried out, one in spring (S) and one in autumn (A). For each season, two independent cheese production batches were produced, identified as S1 and S2 for spring and A1 and A2

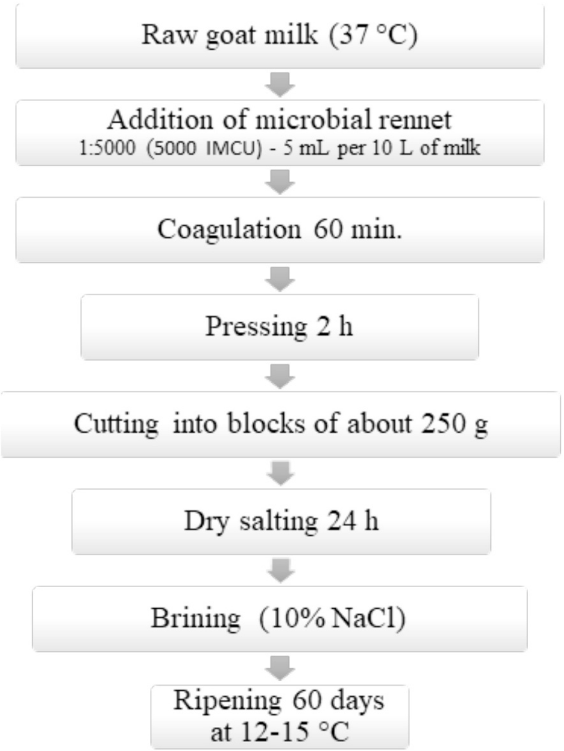

Fig. 1. Production flow chart of traditional brined raw goat's milk cheese.

for autumn. Within each batch, raw milk (M) and cheeses (C) at 0, 30, and 60 days of ripening (C_t0, C_t30, C_t60) were sampled and analyzed. The resulting cheese was rindless, white, and semi-hard in texture (Fig. 2). The cheeses were preserved in brine after production and collected prior to packaging. Milk, brine, and cheese samples were stored at +4 °C and analyzed within 2 days of sampling.

Meteorological data recorded in the Selce area during the cheese production period are presented in Supplementary Table 1.

2.2. Collection of environmental samples

To enable the isolation of pro-technological microorganisms of environmental origin, equipment and dairy surfaces were swabbed through the Swab Rince Kit (Oxoid, Basingstoke, UK), which consists of a sterile cotton stick and a test-tube with 10 mL of physiological solution (Osimani et al., 2011). The following surfaces were swabbed: walls of the dairy plant, curd knife, spoon, milk tank, and cheese vat. For each surface, a 25 cm^2 area was sampled, and swabs were stored at +4 °C and analyzed within 2 days of sampling.

Fig. 2. Ready-to-eat traditional brined raw goat's milk cheese.

2.3. Physico-chemical measurements

The pH of milk and cheese samples was determined using a HI2031 solid electrode (Hanna Instruments, Padova, Italy) by direct insertion of the electrode into the sample.

Water activity (a_w) was evaluated in line with ISO 18787:2017 using a AwTherm instrument (Rotronic, Bassersdorf, Germany).

The NaCl content (%) was measured using a salinity meter (LAQUAtwin salt-22, HORIBA Ltd., Kyoto, Japan).

Titratable acidity was measured using 10 g of cheese homogenized in 90 mL of deionized water, and the results were reported as % lactic acid equivalents, following the procedure described by Rampanti et al. (2023). For each parameter, samples were analyzed in triplicate, and the results were expressed as the mean ± standard deviation.

Total nitrogen was determined by the Kjeldahl method. Briefly, samples were digested with concentrated sulfuric acid (VWR, Leuven, Belgium) in the presence of a catalyst mixture consisting of potassium sulfate (VWR) and copper sulfate (VWR) to convert organically bound nitrogen into ammonium sulfate. After digestion, the solution was rendered alkaline by the addition of sodium hydroxide (VWR), and the liberated ammonia was distilled. The ammonia was collected in a boric acid (VWR) solution and titrated with standardized hydrochloric acid (0.1 N) (VWR). Total nitrogen content was calculated based on the volume of titrant consumed. Protein content was estimated by multiplying total nitrogen by the appropriate nitrogen-to-protein conversion factor (6.25) (Dimitrellou et al., 2010).

2.4. Morpho-textural analyses

Color measurements were conducted using a Konica Minolta CR-310 chroma meter (Ramsey, NJ, USA) coupled with a Data Processor (DP-301) and interfaced with a personal computer via an RS232 serial connection. Each sample was analyzed in triplicate, and the mean value of three readings was recorded for each measurement. The color attributes were expressed as L^*, a^*, and b^* parameters.

Regarding texture analysis, for each cheese sample, cylindrical specimens (15 mm in height and 20 mm in diameter) were prepared and subjected to uniaxial compression using a CT3–4500 texture analyzer (Brookfield Engineering Laboratories Inc., Middleboro, MA, USA). The instrument was fitted with a 36 mm diameter cylindrical probe (model TA-AACC36) and operated at a crosshead speed of 1.5 mm·s^{-1}, applying a non-destructive deformation of 40% as described by Cardinali et al. (2024). During testing, the specimens were placed between the load cell and the base platform of the analyzer, utilizing a 4500 g load cell. Hardness (N) was defined as the maximum force recorded during the deformation process, while cohesiveness, springiness, and adhesiveness were derived from the corresponding force–time curve peaks. The analysis was performed in triplicate at 25 °C (Akbar et al., 2025) to better simulate mouthfeel conditions. Each sample was analyzed in triplicate, and the mean value of three readings was recorded for each measurement.

2.5. Microbiological analyses

For microbial counts, 10 mL of goat milk or 10 g of each cheese sample was aseptically mixed with 90 mL of sterile peptone water (1 g L^{-1}). Homogenization was carried out for 2 min at 260 rpm using a Stomacher 400 Circulator (International PBI, Milan, Italy). Serial tenfold dilutions were subsequently prepared, and microbial enumeration was conducted for different microorganisms following the methodologies outlined in Table 1. In addition, the detection of *Listeria monocytogenes* and *Salmonella* spp. was carried out according to UNI EN ISO 11290-1:2017 and UNI EN ISO 6579-1:2020 standard methods,

Table 1
Microbial counts growth media and incubation conditions.

Microorganisms	Growth medium	Supplement	Incubation temperature	Incubation time	Incubation conditions	Reference
Total aerobic mesophilic count	Plate Count Agar (VWR Prolabo Chemicals, Leuven, Belgium)	n.a.	30 °C	72 h	Aerobiosis	UNI EN ISO 4833-1:2022
Coagulase-positive staphylococci	Mannitol Salt Agar (VWR Prolabo Chemicals)	Rabbit Plasma Fibrinogen (BP-RPF) (VWR Prolabo Chemicals)	37 °C	48 h	Aerobiosis	UNI EN ISO 6888-2:2023
Coagulase-negative staphylococci	Mannitol Salt Agar (VWR Prolabo Chemicals)	n.a.	37 °C	48–72 h	Aerobiosis	Cardinali et al. (2025)
Escherichia coli	REBECCA™ Agar (bioMérieux, Marcy-l'Étoile, France)	EB supplement (bioMérieux)	37 °C	24 h	Aerobiosis	AFNOR AES 10/06–01/08
Enterobacteriaceae	REBECCA™ Agar (bioMérieux)	EB supplement (bioMérieux)	37 °C	24 h	Aerobiosis	AES 10/07–01/08
Presumptive mesophilic lactobacilli	De Man, Rogosa and Sharpe (MRS) Agar (VWR Prolabo Chemicals)	Cycloheximide (250 mg L^{-1})	37 °C	48–72 h	Aerobiosis	Cardinali et al. (2025)
Presumptive mesophilic lactococci	M17 Agar (VWR Prolabo Chemicals)	Cycloheximide (250 mg L^{-1})	22 °C	48–72 h	Aerobiosis	Cardinali et al. (2025)

n.a. not added.

respectively.

Viable cell counts were performed exclusively on non-enriched milk and cheese samples in order to reflect microbial populations under actual cheesemaking and ripening conditions.

In contrast, samples collected within the dairy plant and brine were initially enriched to facilitate the recovery of stressed or injured lactic acid bacteria cells (Garofalo et al., 2015). For this purpose, the entire content of each Swab Rinse Kit tube (10 mL) or 10 mL of brine were transferred into 90 mL of sterile de Man, Rogosa and Sharpe (MRS) broth (VWR) and incubated at 37 °C for 5 days. After enrichment, aliquots of the MRS broth were streaked onto MRS agar (VWR) plates and incubated at 37 °C for 72 h.

2.6. *Staphylococcal enterotoxins detection*

Staphylococcal enterotoxins in cheese samples were analyzed following UNI EN ISO 19020:2017 using the VIDAS® system with the SET2 kit (bioMérieux, Marcy-l'Étoile, France) (Cesaro et al., 2022). Briefly, 10 g of sample were homogenized with 40 mL of pre-warmed distilled water (38 ± 2 °C) in filter bags, held at room temperature for 40 min with intermittent mixing, acidified (pH 3.5–4.0), and centrifuged (4000 rpm, 15 min, 4 °C). The supernatant was neutralized (pH 7.4–7.6), centrifuged again, filtered, and concentrated overnight at 4 °C by dialysis (MWCO 7000 Da) against 30% (*w/v*) PEG. The extract was recovered in distilled water and immediately analyzed. The SET2 kit detects enterotoxins A, B, C, D and E collectively, and results were expressed as Test Values (TV), automatically calculated by the VIDAS® system; samples with TV ≥ 0.13 were considered positive (Cesaro et al., 2022).

2.7. *Metataxonomic analysis*

Cell pellets were obtained by centrifuging 1.5 mL of milk samples and cheese homogenates (10^{-1} dilution) as described by Rampanti, Cantarini, et al. (2024). Total genomic DNA was then extracted using the Quick-DNA™ Fecal/Soil Microbe Miniprep Kit (Zymo Research, Irvine, CA, USA), according to the manufacturer's instructions. DNA concentration was measured with a Qubit 4.0 Fluorometer using the Qubit™ dsDNA HS Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA).

Bacterial community profiling was carried out by amplifying the V3–V4 hypervariable regions of the 16S rRNA gene, using primers previously validated by Klindworth et al. (2013). Amplicon libraries were prepared according to the Illumina 16S Metagenomic Sequencing Library Preparation workflow (Illumina Inc., San Diego, CA, USA) and sequenced on an Illumina MiSeq platform (Illumina Inc.) using paired-end reads (2 × 250 bp).

Raw sequencing reads (FASTQ files) were processed with the QIIME2 pipeline (Bolyen et al., 2019). Primer and adapter sequences were trimmed, and reads were quality filtered and denoised using the DADA2 algorithm (Callahan et al., 2016) to generate amplicon sequence variants (ASVs). The taxonomic assignment of the bacterial ASVs was performed using the SILVA database (release 138, 99% identity reference sequences). Sequencing depth adequacy was assessed through alpha rarefaction analysis and Good's coverage estimation using QIIME2. Alpha rarefaction curves were generated to evaluate the relationship between sequencing depth and diversity metrics, while Good's coverage was calculated to estimate sampling completeness. The corresponding results are shown in Supplementary Fig. 1.

All sequence data were deposited in the Sequence Read Archive of the National Center for Biotechnology Information (NCBI) under the Bioproject accession number PRJNA1321184.

ASVs tables were analyzed by MicrobiomeAnalyst (https://www.microbiomeanalyst.ca/) for downstream ecological and statistical analyses. Alpha-diversity was assessed by calculating Chao1 richness, Shannon diversity, and Simpson diversity indices. Boxplots were generated to visualize differences between seasonal groups (spring vs. autumn), and significance was evaluated using the Wilcoxon rank-sum test.

Beta-diversity was assessed using Bray–Curtis's dissimilarity matrices. Principal Coordinate Analysis (PCoA) plot was used to visualize variation in community composition, and the significance of clustering by season was tested using PERMANOVA.

Differential abundance analysis across four experimental groups (spring batch 1 "S1", spring batch 2 "S2", autumn batch 1 "A1", and autumn batch 2 "A2") was carried out in the R environment (version 4.5.1; R Core Team, Vienna, Austria) using the Kruskal–Wallis's test (stats package), followed by false discovery rate (FDR) correction with the Benjamini–Hochberg procedure. Only taxa detected in at least two samples with a relative abundance ≥0.5% were retained. Data visualization was performed with the ggplot2 package.

2.8. *Isolation and characterization of lactic acid bacteria from dairy environment, brine, and cheese*

2.8.1. *Isolation and identification*

Colonies grown on MRS agar (VWR) were randomly picked and re-streaked on the same medium to ensure purity. Pure cultures were stored at −80 °C until further use.

Before DNA extraction, frozen cultures were thawed and sub-cultured twice on MRS agar (VWR) at 37 °C for 48–72 h. DNA was then extracted following the method described by Osimani et al. (2015). Briefly, after centrifugation of bacterial suspensions, cell pellets were subjected to enzymatic lysis with lysozyme, followed by proteinase K treatment and heat inactivation. Detailed buffer compositions and incubation conditions are provided in Osimani et al. (2015). The concentration and quality of the DNA were assessed using a NanoDrop ND 1000 spectrophotometer (Thermo Fisher Scientific) and adjusted to 100 ng µL^{-1}. Standardized DNA samples were amplified by PCR in a 25 µL reaction volume using MyFi™ DNA Polymerase (2× Master Mix, Bioline, Meridian Bioscience) and universal primers P27f and P1495r targeting the 16S rRNA gene. Amplifications were carried out in a MyCycler Thermal Cycler (Bio-Rad Laboratories, Hercules, USA) under cycling conditions adapted from Osimani et al. (2015). PCR products were verified by agarose gel electrophoresis (Osimani et al., 2015) and submitted to Genewiz (Leipzig, Germany) for purification and Sanger sequencing. The obtained sequences were compared against 16S rRNA gene sequences of type strains in the GenBank database (http://www.ncbi.nlm.nih.gov/) using BLAST. Accession numbers were assigned to the isolates following submission of the sequences to GenBank.

2.8.2. *Proteolytic and lipolytic activity*

Proteolytic and lipolytic activities were determined following the methodology of Linares-Morales et al. (2020), with slight modifications. Before testing, isolates were retrieved from cryo-preserved stocks and sub-cultured twice on the original isolation medium.

Proteolysis was assessed on skim milk agar prepared as described by Rampanti, Cardinali, et al. (2024), whereas lipolysis was evaluated on tributyrin agar (Rampanti, Cardinali, et al., 2024). For each strain, 5 µL of an active culture were spotted in duplicate onto the respective media and incubated at 30 °C for 48 h. The development of a clear halo surrounding the inoculated area was considered indicative of enzyme activity. Halo sizes were classified as follows: + (1 mm), ++ (1–2 mm), and +++ (>2 mm) (Rampanti, Cardinali, et al., 2024).

2.8.3. *Exopolysaccharides (EPS) production*

EPS production was evaluated according to Hilbig et al. (2019), with minor adjustments. After recovery from cryo-preserved stocks, isolates were sub-cultured twice under the same growth conditions used for their initial isolation. Subsequently, 5-µL aliquots of each culture were inoculated in duplicate onto two EPS-inducing media: (i) MRS agar supplemented with sucrose (80 g L^{-1}) to induce homopolysaccharides (HoPS) synthesis; (ii) MRS agar supplemented with yeast extract (10 g L^{-1}), meat extract (10 g L^{-1}), lactose (20 g L^{-1}), and galactose (20 g L^{-1}) to promote heteropolysaccharides (HePS) synthesis (Rampanti, Cardinali, et al., 2024).

After incubation for 48 h at 30 °C, isolates were classified as EPS-producers when colonies exhibited either filamentous "ropy" behavior (detectable by stretching with a sterile toothpick) or a mucoid, glossy appearance.

2.8.4. *hdcA gene detection*

DNA extracted from each isolate was screened for the presence of the *hdcA* gene by PCR. Amplification targeted a 174 bp fragment using primers Hdc1 and Hdc2 (Belleggia et al., 2021). PCR reactions were conducted on a MyCycler Thermal Cycler (BioRad Laboratories) as reported by Rampanti, Nikolovska Nedelkoska, et al. (2024). PCR products were visualized by agarose gel electrophoresis, using HyperLadder™ 100 bp (Meridian Bioscience) as a molecular size marker. Each run included a no-template control and a positive control strain (*Lactobacillus parabuchneri* DSM 5987).

2.9. *Statistical analysis*

Statistical analyses of the microbiological, physico-chemical, color, and texture parameters were conducted to assess differences among the samples using JMP software version 11.0.0 (SAS Institute Inc., Cary, NC, USA). A one-way analysis of variance (ANOVA) was applied, and mean comparisons were performed with Tukey–Kramer's Honest Significant Difference (HSD) test at a 5% significance level.

3. Results and discussion

Understanding how artisanal brined raw goat's milk cheese develops its complex identity requires exploring the delicate interactions between raw materials, traditional practices, and the microbial communities that emerge during production and ripening. When these factors are examined across different seasons and through complementary analytical approaches, they reveal a rich and dynamic ecosystem that is far more intricate than expected. This study unveils how a traditional brined raw goat's milk cheese from North Macedonia develops its distinctive microbiological and technological identity, revealing dynamics that have not been previously documented. By integrating both classical microbiological and high-resolution metataxonomics methos, the study offers a rare opportunity to observe the inner workings of an artisanal cheese ecosystem, inviting deeper exploration into the mechanisms that drive its uniqueness. Altogether, the findings shed light on a culturally rooted dairy product whose microbial biodiversity has remained largely unexplored, opening new perspectives for its scientific characterization and future valorization.

3.1. *Physico-chemical analyses*

The results of physico-chemical analyses are reported in Table 2.

When considering cheese samples produced in spring, a gradual acidification was observed during ripening, with pH values decreasing from 6.28 at t_0 to 5.35 after 60 days of ripening. In contrast, cheeses produced in autumn exhibited a more pronounced acidification trend, with pH values declining from 5.76 at t_0 to 4.62 after 60 days. The differences between the two production periods were statistically significant, with autumn-produced cheeses consistently showing lower pH values compared to those produced in spring ($p < 0.05$). Notably, the pH values detected in samples produced in spring were generally higher than those reported by Kocak et al. (2020) for white brined goat milk cheese produced without starter cultures, which showed pH values of 4.95, 4.79, and 4.72 after 1, 30, and 60 days of ripening, respectively. Interestingly, the values reported by Kocak et al. (2020) were comparable to those recorded for the autumn-produced samples in the present study. Furthermore, the pH values observed here were consistent with those reported by Kondyli et al. (2016) for the same type of cheese, which exhibited an average pH of 4.61 after 60 days of ripening. During cheese production, the decrease in pH reflects curd acidification mediated by lactic acid bacteria naturally present in the curd and the dairy environment. In this study, the differences observed between samples produced in different seasons likely reflect the absence of standardized procedures and the use of empirical, artisanal production methods. However, seasonal differences between spring and autumn cheeses are likely influenced not only by the artisanal and non-standardized production process, but also by environmental conditions during manufacture and ripening. Although direct temperature profiles of milk, curd, and ripening rooms were not recorded, meteorological data for the Selce area provide a useful proxy. In more detail, spring production occurred under milder conditions, whereas autumn cheesemaking and ripening took place at lower average temperatures and higher precipitation. These conditions may have indirectly affected curd cooling, brine temperature, and microbial dynamics.

Considering titratable acidity (TA), low values ranging from 0.08% to 0.12% lactic acid equivalents were recorded in the milk samples, with no statistically significant differences observed between the two seasons examined ($p > 0.05$). These values were slightly lower than those suggested by Wang et al. (2025) for a good cheesemaking, attesting between 0.15% and 0.17% when expressed as lactic acid. TA values recorded in cheese samples showed statistically significant seasonal variation, with cheeses produced in autumn exhibiting higher mean values regardless of ripening duration ($p < 0.05$). Overall, titratable acidity increased progressively with ripening time, which may be attributed to intensified microbial activity and lactic acid production. These processes promote

Table 2
Physico-chemical parameters of milk and cheese samples at different ripening times (t0, t30 days, t60 days).

Parameter	Samples	S1	S2	Overall mean	A1	A2	Overall mean
pH	M	6.74 ± 0.01	6.68 ± 0.01	6.71 ± 0.03^{a}	6.58 ± 0.01	6.58 ± 0.01	6.58 ± 0.01^{b}
	C_t_{0}	6.47 ± 0.06	6.09 ± 0.07	6.28 ± 0.22^{a}	5.69 ± 0.24	5.83 ± 0.05	5.76 ± 0.16^{b}
	C_t_{30}	5.67 ± 0.01	5.20 ± 0.06	5.43 ± 0.28^{a}	4.73 ± 0.08	4.68 ± 0.11	4.70 ± 0.08^{b}
	C_t_{60}	5.35 ± 0.03	5.35 ± 0.02	5.35 ± 0.02^{a}	4.66 ± 0.05	4.58 ± 0.00	4.62 ± 0.05^{b}
a_w	M	0.99 ± 0.00	0.99 ± 0.00	0.99 ± 0.00^{a}	0.99 ± 0.00	0.99 ± 0.00	0.99 ± 0.00^{a}
	C_t_{0}	0.94 ± 0.00	0.96 ± 0.00	0.95 ± 0.01^{a}	0.92 ± 0.01	0.89 ± 0.00	0.90 ± 0.02^{b}
	C_t_{30}	0.94 ± 0.00	0.94 ± 0.00	0.94 ± 0.00^{a}	0.90 ± 0.01	0.91 ± 0.00	0.90 ± 0.01^{b}
	C_t_{60}	0.94 ± 0.00	0.94 ± 0.00	0.94 ± 0.00^{a}	0.87 ± 0.01	0.87 ± 0.01	0.87 ± 0.01^{b}
NaCl (%)	M	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	C_t_{0}	4.60 ± 0.01	4.60 ± 0.01	4.60 ± 0.01^{b}	6.25 ± 0.21	5.90 ± 0.14	6.08 ± 0.25^{a}
	C_t_{30}	2.82 ± 0.01	2.81 ± 0.01	2.81 ± 0.01^{b}	6.70 ± 0.42	7.25 ± 0.35	6.98 ± 0.45^{a}
	C_t_{60}	3.73 ± 0.06	2.97 ± 0.01	3.35 ± 0.44^{b}	6.90 ± 0.57	5.80 ± 0.14	6.35 ± 0.72^{a}
Titratable Acidity (% lactic acid equivalents)	M	0.08 ± 0.01	0.09 ± 0.00	0.08 ± 0.01^{a}	0.13 ± 0.00	0.11 ± 0.01	0.12 ± 0.01^{a}
	C_t_{0}	0.10 ± 0.02	0.17 ± 0.01	0.14 ± 0.04^{b}	0.37 ± 0.05	0.37 ± 0.09	0.37 ± 0.05^{a}
	C_t_{30}	0.46 ± 0.06	0.90 ± 0.13	0.68 ± 0.27^{b}	1.51 ± 0.03	1.32 ± 0.04	1.42 ± 0.11^{a}
	C_t_{60}	0.92 ± 0.01	0.92 ± 0.10	0.92 ± 0.06^{b}	1.50 ± 0.04	1.73 ± 0.10	1.61 ± 0.15^{a}
Crude protein (%)	M	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	C_t_{0}	19.52 ± 0.13	18.79 ± 0.21	19.15 ± 0.52^{b}	20.64 ± 0.04	20.89 ± 0.32	20.77 ± 0.18^{a}
	C_t_{30}	19.48 ± 0.06	20.51 ± 0.33	19.99 ± 0.73^{a}	18.62 ± 0.12	$18,53 \pm 0.17$	18.58 ± 0.07^{a}
	C_t_{60}	18.41 ± 0.09	18.56 ± 0.04	18.48 ± 0.11^{b}	22.21 ± 0.11	20.50 ± 0.10	21.35 ± 1.22^{a}

Values are presented as means ± standard deviation. Different uppercase letters indicate significant differences ($p < 0.05$) between overall means of spring and autumn samples. Abbreviations: S, spring; A, autumn; 1 and 2, production batches; M, milk; C: cheese. Times indicate cheese samples at different ripening stages. n.a.: not analyzed.

proteolysis and play a crucial role in flavor development and cheese preservation (Kholif & Kholif, 2025).

Regarding a_w, a slight decrease was observed in cheese samples produced during spring, reaching an average value of 0.94 after 60 days of ripening. In contrast, cheeses produced in autumn showed a more pronounced reduction, with an average a_w of 0.87 at the same stage. Statistically significant differences were found between the two production periods, with autumn samples exhibiting the lowest a_w values ($p < 0.05$). In brined cheeses, a_w is largely affected by the diffusion of NaCl into the curd during brining. As noted by Santapaola et al. (2013), salting is a key step in cheese production, influencing sensory attributes and controlling microbial and enzymatic activities through its effect on a_w. Consistent with this, the NaCl content measured in the present study corresponded closely with a_w trends, being highest in autumn-produced samples (6.35% at 60 days) and lowest in those manufactured in spring (3.35% at 60 days) ($p < 0.05$).

At 60 days of ripening, crude protein content ranged from 18.48% in cheeses produced in spring to 21.35% in those produced in autumn, with the latter being significantly higher ($p < 0.05$). The values observed in spring-produced samples were consistent with those reported by Kondyli et al. (2016) for white-brined goat milk cheese (16.01–17.45%). In contrast, the protein levels measured in autumn-produced samples aligned with the range reported by Wu et al. (2025) for goat milk Halloumi-type cheese (20.83–22.02%). Seasonal differences in crude protein content may also be linked to variations in goat feeding regimes (Park et al., 2007). During spring, goats mainly graze on fresh pasture, whereas autumn feeding often includes more conserved forages, which may result in higher milk protein concentration due to reduced dilution effects and changes in nutrient intake.

3.2. Morpho-textural analyses

Results of color evaluation are reported in Table 3.

Regarding lightness (L^*), which can range from 0 (black) to 100 (white), similar values were generally observed among cheese samples, regardless of the production season. However, after 60 days of ripening, the average L^* value of cheeses produced in autumn was significantly lower ($p < 0.05$). The L^* values recorded at 60 days were consistent with those reported by Pavia et al. (1999) for brine-salted Manchego-type cheese, which exhibited an L^* value of approximately 85 at the end of ripening. The L^* values obtained in the present study were also within the range reported by Buffa et al. (2001) for salt-brined raw goat milk cheese, which showed an L^* value of ~91 after 60 days of ripening.

Regarding the a^* parameter (redness), which represents the green (negative) to red (positive) color axis, statistically significant differences were observed between samples produced in spring and those produced in autumn ($p < 0.05$). However, all measured values were located on the negative side of the scale, indicating a tendency toward green. The recorded values were in accordance with those reported by Pavia et al. (1999) for brine-salted Manchego-type cheese and Buffa et al. (2001) for salt-brined raw goat milk cheese, which showed greenish hues.

Regarding the b^* parameter (yellowness), which represents the blue (negative) to yellow (positive) color axis, statistically significant differences were observed between cheeses produced in spring and those

Table 3
Color parameters of cheese samples at different ripening times (t0, t30 days, t60 days).

Parameter	Sampling time	SC1	SC2	Overall mean	AC1	AC2	Overall mean
L^*	t_{0}	86.37 ± 2.39	81.32 ± 2.75	83.85 ± 3.60^{a}	82.86 ± 4.97	85.71 ± 1.69	84.29 ± 3.67^{a}
	t_{30}	84.33 ± 0.98	86.70 ± 1.62	85.51 ± 1.77^{a}	86.16 ± 2.22	82.07 ± 1.77	84.12 ± 2.87^{a}
	t_{60}	83.20 ± 1.18	85.39 ± 2.70	84.30 ± 2.21^{a}	81.05 ± 2.05	79.31 ± 3.08	80.18 ± 2.53^{b}
a^*	t_{0}	-0.66 ± 0.09	-0.28 ± 0.09	-0.47 ± 0.22^{a}	-1.20 ± 0.11	-1.45 ± 0.30	-1.33 ± 0.24^{b}
	t_{30}	-0.28 ± 0.05	-0.33 ± 0.17	-0.31 ± 0.11^{a}	-1.74 ± 0.13	-1.72 ± 0.25	-1.73 ± 0.18^{b}
	t_{60}	-0.83 ± 0.10	-0.31 ± 0.17	-0.57 ± 0.31^{a}	-0.52 ± 0.09	-0.39 ± 0.12	-0.45 ± 0.11^{b}
b^*	t_{0}	11.00 ± 0.07	11.13 ± 1.74	11.07 ± 1.10^{b}	12.75 ± 0.41	13.64 ± 0.93	13.19 ± 0.81^{a}
	t_{30}	10.72 ± 0.65	11.34 ± 0.16	11.03 ± 0.54^{b}	12.68 ± 0.38	12.00 ± 0.53	12.34 ± 0.56^{a}
	t_{60}	10.39 ± 0.23	10.54 ± 0.23	10.47 ± 0.22^{a}	10.63 ± 1.15	10.81 ± 0.60	10.72 ± 0.82^{a}

Values are presented as means ± standard deviation. Different uppercase letters indicate significant differences ($p < 0.05$) between overall means of spring and autumn samples. Abbreviations: S, spring; A, autumn; C: cheese; 1 and 2, production batches. Times indicate cheese samples at different ripening stages.

produced in autumn at t_0 and t_{30}. However, no significant differences were found between the two groups at t_{60} ($p < 0.05$). Overall, the recorded values indicated a tendency toward yellowish hues (approximately 10), consistent with the findings of Pavia et al. (1999) for brine-salted Manchego-type cheese and Buffa et al. (2001) for salt-brined raw goat milk cheese, which reported positive b^* values of around 15 and 10, respectively.

Cheese texture is a sensory attribute characterized by a set of descriptive terms that reflect the perception experienced during mastication (Foegeding & Drake, 2007). In cheese texture analysis, fundamental mechanical tests apply controlled stresses or strains to samples of defined geometry to assess their physical and rheological properties (Foegeding & Drake, 2007). In the present study, hardness, cohesiveness, springiness, and adhesiveness were determined through Texture Profile Analysis (TPA).

The results of the texture evaluation are reported in Table 4.

Hardness refers to the maximum force detected during the first compression cycle, representing the sample's resistance to the initial bite (Foegeding & Drake, 2007). In the present study, no statistically significant differences were observed among samples ($p < 0.05$), regardless of the production season. The values recorded at 60 days of ripening were in the range of those reported by Türkmen and Güler (2022) for brined Turkish white cheeses, approximately 10 N. Similarly, hardness values were in accordance with those reported by Miloradovic et al. (2018) for goat white brined cheese in 6% brine, also around 10 N. In cheese, hardness is generally influenced by factors such as water content, ripening conditions, and salting method (e.g., dry salting or brining) as well as salt concentration. As reviewed by Foegeding and Drake (2007), the hardness of the cheese gel affects both chewing behavior and the associated physiological responses, which together influence the timing and pattern of flavor release.

Cohesiveness refers to a product's ability to maintain its structural integrity when subjected to a second deformation relative to the first. From a sensory perspective, it represents the extent to which the masticated cheese sample remains intact after five chews (Foegeding & Drake, 2007). In the present study, statistically significant differences were observed between cheeses produced in spring and those produced in autumn, with the latter exhibiting higher cohesiveness values ($p < 0.05$). The values recorded for autumn-produced samples were consistent with those reported by Miloradovic et al. (2018) for goat white brined cheese, approximately 0.6. In contrast, the cohesiveness values of cheeses produced in spring were significantly higher than those reported by Miloradovic et al. (2018), but closely aligned with those described by Türkmen and Güler (2022) for brined Turkish white cheeses, which reached about 0.80 after 60 days of ripening.

Among texture parameters, springiness indicates how effectively a product regains its original structure after being compressed once and allowed to rest for a specified time before the next compression (Foegeding & Drake, 2007). In the present study, samples produced in spring exhibited significantly lower values than those manufactured in autumn ($p < 0.05$). Overall, the values obtained were higher than those reported by Türkmen and Güler (2022) for brined Turkish white cheeses and by Miloradovic et al. (2018) for goat white brined cheese, which were approximately 0.9 after 60 and 50 days of ripening, respectively.

As noted by Foegeding and Drake (2007), adhesiveness reflects the tendency of the masticated sample to adhere to oral surfaces after five chewing cycles. In the samples analyzed in the present study, no statistically significant differences in adhesiveness were observed after 60 days of ripening, regardless of the production season. In all the analyzed samples positive values close to zero were observed, these results were in contrast with those obtained by Türkmen and Güler (2022) for brined Turkish white cheeses, which showed negative values close to zero. Negative cohesiveness values were also reported by Alkhalaileh (2025) for fresh white brined cheese produced with goat milk. As reported by Zheng et al. (2016), an optimal level of adhesiveness is beneficial for cheese consumption, as it contributes to flavor and taste perception. Nevertheless, excessive adhesiveness may cause practical issues, including sticking to the packaging and the development of undesirable textural defects.

The seasonal variation observed in texture parameters may be associated with differences in salt uptake, a_w, and acidification kinetics. In particular, the higher NaCl content and lower a_w observed in autumn cheeses, together with enhanced acidification, may have contributed to modifications of the casein network and its viscoelastic behavior, potentially affecting cohesiveness and springiness (Foegeding & Drake, 2007).

3.3. Study of cheese microbiota

Artisanal brined raw goat's milk cheeses represent an invaluable reservoir of microbial diversity that warrants in-depth investigation. In this study, a combined approach of viable cell counting and metataxonomic analysis was employed to obtain a comprehensive overview of the bacterial communities present throughout cheese manufacture, providing insights into both dominant and minor taxa, including cultivable and uncultivable microorganisms. Furthermore, viable lactic acid bacteria were isolated from milk, cheese, brine, and the dairy environment to characterize their lipolytic and proteolytic activities, exopolysaccharide production, and the presence of the *hdcA* gene responsible for histamine biosynthesis.

3.3.1. Viable counts

The results of viable counts are reported in Table 5.

Viable counts were obtained by direct plating of non-enriched samples. High counts of total mesophilic aerobic bacteria were detected in the milk samples, reaching a maximum of 6.5 log cfu g^{-1} during the spring season. The milk microbiota is known to be complex and affected by various factors, including animal health, diet, farming practices,

Table 4
Texture parameters of cheese samples at different ripening times (t0, t30 days, t60 days).

Parameter	Sampling time	SC1	SC2	Overall mean	AC1	AC2	Overall mean
Hardness [N]	t_0	7.33 ± 0.05	7.43 ± 0.59	7.38 ± 0.34^b	11.01 ± 2.22	11.10 ± 3.23	11.06 ± 2.26^a
	t_{30}	13.59 ± 0.29	14.34 ± 3.07	13.97 ± 1.83^a	12.77 ± 3.95	15.60 ± 6.05	14.18 ± 4.48^a
	t_{60}	14.65 ± 1.50	11.96 ± 0.14	13.31 ± 1.78^a	23.83 ± 3.04	13.55 ± 1.64	18.69 ± 6.26^a
Cohesiveness	t_0	0.87 ± 0.08	0.80 ± 0.07	0.83 ± 0.07^a	0.85 ± 0.02	0.83 ± 0.06	0.84 ± 0.04^a
	t_{30}	0.84 ± 0.01	0.79 ± 0.04	0.81 ± 0.04^a	0.73 ± 0.04	0.74 ± 0.06	0.73 ± 0.04^b
	t_{60}	0.84 ± 0.01	0.81 ± 0.01	0.83 ± 0.02^a	0.55 ± 0.02	0.69 ± 0.00	0.62 ± 0.08^b
Springiness	t_0	2.25 ± 0.21	2.10 ± 0.57	2.18 ± 0.36^a	3.05 ± 0.64	2.70 ± 0.28	2.88 ± 0.45^a
	t_{30}	2.20 ± 0.00	2.00 ± 0.14	2.10 ± 0.14^b	3.20 ± 0.00	3.65 ± 0.07	3.43 ± 0.26^a
	t_{60}	2.10 ± 0.14	2.15 ± 0.07	2.13 ± 0.10^b	3.30 ± 0.14	3.05 ± 0.07	3.18 ± 0.17^a
Adhesiveness	t_0	0.52 ± 0.18	1.34 ± 0.87	0.93 ± 0.70^a	0.48 ± 0.05	0.58 ± 0.12	0.53 ± 0.09^a
	t_{30}	0.58 ± 0.06	0.60 ± 0.05	0.59 ± 0.04^b	0.54 ± 0.04	0.52 ± 0.01	0.53 ± 0.02^a
	t_{60}	0.64 ± 0.03	0.62 ± 0.04	0.63 ± 0.03^a	0.70 ± 0.18	0.57 ± 0.04	0.64 ± 0.13^a

Values are presented as means ± standard deviation. Different uppercase letters indicate significant differences ($p < 0.05$) between overall means of spring and autumn samples. Abbreviations: S, spring; A, autumn; C: cheese; 1 and 2, production batches. Times indicate cheese samples at different ripening stages.

Table 5
Viable counts of milk and cheese samples at different ripening times (t0, t30 days, t60 days).

Microbial group	Sample	S1	S2	Overall mean	A1	A2	Overall mean
Total mesophilic aerobes	M	6.13 ± 0.07	6.68 ± 0.03	6.40 ± 0.32^a	4.99 ± 0.02	5.84 ± 0.03	5.41 ± 0.49^b
	C_t$_0$	7.10 ± 0.02	8.76 ± 0.03	7.93 ± 0.96^a	6.94 ± 0.02	6.85 ± 0.02	6.90 ± 0.05^a
	C_t$_{30}$	7.60 ± 0.02	7.52 ± 0.02	7.56 ± 0.05^a	5.72 ± 0.04	3.40 ± 0.11	4.56 ± 1.34^b
	C_t$_{60}$	6.50 ± 0.01	6.53 ± 0.02	6.51 ± 0.02^a	7.03 ± 0.04	5.56 ± 0.12	6.30 ± 0.85^a
Presumptive mesophilic lactobacilli	M	2.91 ± 0.15	3.40 ± 0.13	3.15 ± 0.30^b	4.84 ± 0.08	4.86 ± 0.07	4.85 ± 0.06^a
	C_t$_0$	4.63 ± 0.14	7.50 ± 0.01	6.06 ± 1.66^a	6.77 ± 0.01	6.58 ± 0.07	6.68 ± 0.12^a
	C_t$_{30}$	6.67 ± 0.01	7.52 ± 0.06	7.10 ± 0.50^a	5.12 ± 0.08	3.65 ± 0.07	4.39 ± 0.85^b
	C_t$_{60}$	7.44 ± 0.04	5.59 ± 0.10	6.51 ± 1.07^a	6.65 ± 0.01	4.91 ± 0.06	5.78 ± 1.00^a
Presumptive mesophilic lactococci	M	4.55 ± 0.03	4.65 ± 0.04	4.60 ± 0.07^b	4.99 ± 0.01	5.18 ± 0.01	5.08 ± 0.11^a
	C_t$_0$	7.19 ± 0.18	7.72 ± 0.01	7.45 ± 0.33^a	6.92 ± 0.02	6.69 ± 0.08	6.80 ± 0.14^b
	C_t$_{30}$	6.92 ± 0.00	7.65 ± 0.06	7.28 ± 0.42^a	5.21 ± 0.05	3.58 ± 0.09	4.39 ± 0.94^b
	C_t$_{60}$	7.57 ± 0.12	6.80 ± 0.01	7.18 ± 0.45^a	6.80 ± 0.01	5.41 ± 0.10	6.11 ± 0.81^b
Presumptive coagulase-negative staphylococci	M	4.39 ± 0.04	4.57 ± 0.03	4.48 ± 0.11^a	2.75 ± 0.04	3.15 ± 0.04	2.95 ± 0.23^b
	C_t$_0$	5.34 ± 0.05	7.93 ± 0.22	6.64 ± 1.50^a	<1	<1	<1^b
	C_t$_{30}$	6.26 ± 0.37	6.94 ± 0.03	6.60 ± 0.45^a	3.52 ± 0.06	<1	1.76 ± 2.03^b
	C_t$_{60}$	7.05 ± 0.05	6.89 ± 0.01	6.97 ± 0.10^a	5.87 ± 0.12	<1	2.93 ± 3.39^b
Presumptive coagulase-positive staphylococci	M	3.18 ± 0.07	3.54 ± 0.09	3.36 ± 0.22^a	1.52 ± 0.06	2.30 ± 0.17	1.91 ± 0.46^b
	C_t$_0$	3.02 ± 0.04	6.50 ± 0.06	4.76 ± 2.01^a	5.80 ± 0.49	5.81 ± 0.02	5.80 ± 0.29^a
	C_t$_{30}$	<1	5.70 ± 0.01	2.85 ± 3.29^a	3.67 ± 0.37	<1	1.70 ± 1.98^a
	C_t$_{60}$	<1	<1	<1^b	3.46 ± 0.09	<1	1.76 ± 2.04^a
Enterobacteriaceae	M	<1	2.16 ± 0.06	1.08 ± 1.25^b	3.48 ± 0.01	3.49 ± 0.04	3.49 ± 0.02^a
	C_t$_0$	6.09 ± 0.07	6.25 ± 0.03	6.17 ± 0.10^a	5.68 ± 0.04	4.99 ± 0.02	5.34 ± 0.40^b
	C_t$_{30}$	2.07 ± 0.04	4.85 ± 0.01	3.46 ± 1.61^a	3.23 ± 0.07	<1	1.61 ± 1.86^a
	C_t$_{60}$	<1	<1	<1^b	2.60 ± 0.14	<1	1.25 ± 1.45^a
Escherichia coli	M	<1	2.02 ± 0.09	1.01 ± 1.17^a	<1	2.41 ± 0.10	1.20 ± 1.39^a
	C_t$_0$	2.10 ± 0.03	7.24 ± 0.02	4.67 ± 2.97^a	4.43 ± 0.07	3.89 ± 0.06	4.16 ± 0.32^a
	C_t$_{30}$	<1	5.10 ± 0.02	2.55 ± 2.94^a	2.36 ± 0.08	<1	1.21 ± 1.40^a
	C_t$_{60}$	<1	3.37 ± 0.02	1.68 ± 1.94^a	2.16 ± 0.06	<1	1.06 ± 1.22^a

Values are expressed as Log cfu g^{-1} and presented as means ± standard deviation. Different uppercase letters indicate significant differences ($p < 0.05$) between overall means of spring and autumn samples. Abbreviations: S, spring; A, autumn; 1 and 2, production batches; M, milk; C: cheese. Times indicate cheese samples at different ripening stages.

season, and storage conditions (Parente et al., 2020). The total mesophilic aerobic counts obtained in the present study are comparable to those reported by Picon et al. (2016) for raw goat milk collected across different production areas and seasons, ranging from approximately 6.0 to 6.3 log CFU g^{-1} in spring and autumn samples, respectively. Notably, the counts observed in the current study exceeded the microbiological limits established by Regulation (EC) No. 853/2004 of the European Parliament and of the Council laying down specific hygiene rules for food of animal origin, which specifies that raw milk from species other than cows intended for the manufacture of products without heat treatment should not exceed 500.000 cfu mL^{-1}. Regarding the cheese samples, total mesophilic aerobic bacteria counts reached up to 6.5 log cfu g^{-1} after 60 days of ripening, with no significant differences observed between samples collected in the two seasons ($p > 0.05$). The counts observed in the samples analyzed in the present study, produced in 10% brine, were lower than those reported by Miloradovic et al. (2018) for goat white brined cheese manufactured in 3% or 6% brine, suggesting a stronger bacteriostatic effect associated with the higher salt concentration used in this study. Although total mesophilic aerobic counts provide only a general indication of the viable microbiota present in food products, they are widely regarded as a useful parameter for assessing overall microbiological quality. In cheese, these counts reflect the combined presence of viable pro-technological bacteria, as well as potential spoilage and pathogenic microorganisms (Pothakos et al., 2012), thereby highlighting the need for further investigation to clarify the qualitative composition of the microbial community and better understand their ecological and technological roles in this cheese.

Regarding lactic acid bacteria, the counts of presumptive mesophilic lactobacilli showed no statistically significant differences among samples after 60 days of ripening across the two seasons ($p > 0.05$), with mean values reaching 6.5 and 5.7 log cfu g^{-1} in spring and autumn, respectively. In contrast, presumptive mesophilic lactococci exhibited significantly higher mean counts in samples collected during spring compared to those collected in autumn ($p < 0.05$), reaching values up to 7.1 log cfu g^{-1}. Regarding the seasonal difference in lactococci counts observed between spring- and autumn-produced cheeses at 60 days of ripening, the higher counts detected in spring samples may reasonably be associated with the milder environmental conditions recorded at the production site during spring, characterized by higher average atmospheric temperatures and humidity compared to autumn. Although ripening was conducted under the same controlled temperature conditions in both seasons, differences in the initial microbiota at t$_0$ and in the broader environmental context may have contributed to supporting lactococcal growth and persistence in spring-produced cheeses.

The counts of lactic acid bacteria detected in the present study were generally lower than those reported by Miloradovic et al. (2018) for goat white brined cheese, attesting at ~9 log cfu g^{-1} after 50 days of ripening. Interestingly, the counts of mesophilic lactobacilli observed in the present study were comparable to those reported by Halici Demir and Kaptan (2025) in Edirne white cheese made from goat milk after 90 days of ripening, which reached 6.6 log cfu g^{-1}. In contrast, the counts of mesophilic lactococci were lower than those reported in the same study, where values of approximately 9 log cfu g^{-1} were detected. Lactic acid bacteria constitute the primary microbial group responsible for cheese fermentation. Their natural presence in milk, and consequently in cheese, originates mainly from the animal's udder surface and the surrounding dairy environment. After raw milk is transformed into curd, lactic acid bacteria metabolize carbohydrates predominantly into lactic acid, which accounts for more than 50% of the total fermentation products (Chen et al., 2025). Homolactic species produce lactic acid as the main organic acid, whereas heterofermentative species generate lactic acid along with varying amounts of acetic acid, ethanol, and carbon dioxide (CO_2). The acidification of cheese curd plays a crucial role in determining the safety and quality of cheeses made from raw milk by inhibiting the growth of pathogenic and spoilage microorganisms. In addition, proteolytic activity of lactic acid bacteria contributes to the generation of volatile compounds from the cheese protein fraction, thereby enhancing the aromatic profile of the final product. In the present study, lactic acid bacteria isolates were obtained from raw milk, cheese samples, and the dairy environment, and subsequently characterized. The results of these analyses are discussed in the following sections.

Presumptive coagulase-negative staphylococci exhibited statistically significant seasonal variation, with the highest counts observed in spring (reaching approximately 7 log cfu g^{-1} after 60 days of ripening). Coagulase-negative staphylococci are typically nonpathogenic members of the normal commensal flora. They exhibit strong tolerance to extreme environments, being capable of growing across a wide pH range (4.8–9.4) and enduring high concentrations of NaCl (7.5–10%) (Kürekci, 2016). Notably, these bacteria have been identified as dominant taxa in cheese brining baths, suggesting an important role in cheese ripening and aroma development (Hammer et al., 2019). Several species of coagulase-negative staphylococci have been identified in goat-associated environments, including the skin, nares, mucous membranes, milk, and cheese (Becker et al., 2014), which may account for the high counts observed in the present study. Again, the seasonal variation in coagulase-negative staphylococci detected between spring- and autumn-produced cheeses at 60 days of ripening could be linked to the more favorable environmental conditions typical of spring, including higher average temperatures and increased humidity.

Enterobacteriaceae are widely recognized as indicators of inadequate hygiene practices during milk handling and cheesemaking, while *Escherichia coli* is commonly regarded as a marker of fecal contamination. In the present study, Enterobacteriaceae counts were on average higher in samples produced during autumn ($p < 0.05$), with a mean value of 1.25 log cfu g^{-1} after 60 days of ripening. *E. coli* counts did not show statistically significant differences between the two seasons, with mean values ranging between 1.06 and 1.68 log cfu g^{-1}after 60 days of ripening. A general decrease in Enterobacteriaceae and *E. coli* counts was observed throughout cheese ripening. Notably, the milk used for cheesemaking exhibited appreciable levels of both Enterobacteriaceae and *E. coli*, likely resulting from improper milking practices that led to contamination of the curd and, consequently, the cheese. It is plausible that brining and acidification by lactic acid bacteria created a hostile environment that contributed to the reduction of Enterobacteriaceae and *E. coli* loads (Vernozy-Rozand et al., 2005).

Finally, presumptive coagulase-positive staphylococci detected in milk showed significantly higher counts in samples produced during spring compared with those produced in autumn ($p < 0.05$), with mean values reaching 3.36 log cfu g^{-1}. Interestingly, previous studies have highlighted a pronounced seasonal pattern for *Staphylococcus aureus*, with significantly lower concentrations and diversity detected in indoor air during winter compared to warmer seasons (Madsen et al., 2018). Since this microorganism is commonly associated with human skin, such variation may be related to behavioral and physiological factors, including heavier clothing in colder months and increased perspiration in summer, which could influence its shedding and subsequent transfer into the dairy environment and milk by milking personnel (Madsen et al., 2018).

Conversely, the average counts of coagulase-positive staphylococci in cheese samples after 60 days of ripening were lowest in those produced during spring ($p < 0.05$). The presence of coagulase-positive staphylococci in milk and cheese poses a potential risk to human health, as this group of Gram-positive bacteria includes toxigenic species such as *Staphylococcus intermedius*, *Staphylococcus schleiferi* subsp. *coagulans*, and *Staphylococcus aureus* (Samanta & Bandyopadhyay, 2020). *Staphylococcus aureus* is a well-known contaminant of milk and a primary causative agent of mastitis in dairy animals, rendering milk from infected individuals unfit for human consumption. Mastitis continues to be a major concern in the dairy industry due to the widespread prevalence of *S. aureus* and its ease of transmission among animals, which can ultimately lead to contamination of milk and dairy products. Additionally, cross-contamination may occur through insufficiently sanitized equipment or improper handling during milking and processing (Léguillier et al., 2024). In the present study, no clinical cases of mastitis were diagnosed in the goats that supplied the milk; however, the possibility that some animals suffered from an undiagnosed subclinical form cannot be excluded. Of note, *S. aureus* is a commensal microorganism that commonly inhabits the skin, nasal passages, and gastrointestinal tract of humans. Therefore, its potential presence in the analyzed cheese samples may have resulted from cross-contamination associated with inadequate hygiene practices during production. Staphylococcal food poisoning is a gastrointestinal illness caused by the ingestion of food containing enterotoxins, even when occurring in minimal amounts (<1 μg), produced by *S. aureus* (Bencardino et al., 2021; Shalaby et al., 2024). These enterotoxins can pass through the gastrointestinal tract largely unaffected, as they are resistant to degradation by proteolytic enzymes. Once absorbed into the bloodstream, they can induce symptoms such as nausea and vomiting (Bencardino et al., 2021). Notably, *S. aureus* can grow and produce enterotoxins at temperatures exceeding those normally used for refrigerated foods. Although adequate heat treatment can inactivate *S. aureus* cells, the preformed enterotoxins are highly heat resistant and cannot be eliminated by common cooking methods such as pasteurization, boiling, steaming, baking, or frying (Cesaro et al., 2022). Based on the counts of coagulase-positive staphylococci detected in the analyzed milk and cheese samples, staphylococcal enterotoxins A, B, C, D, and E were collectively assessed. No enterotoxins were detected in any of the samples, indicating the absence of toxin-producing coagulase-positive staphylococci. These findings comply with the requirements established by Commission Regulation (EC) No. 2073/2005 on microbiological criteria for foodstuffs.

Notably, neither *Salmonella* spp. nor *L. monocytogenes* was detected in any of the samples analyzed, confirming the safety of the cheese examined in this study.

3.3.2. Metataxonomic analysis

Overall, 89,257 reads were used for the metataxonomic analysis of the bacterial biota, with an average of 2,625 sequences per sample. The results of the microbiota composition, expressed as relative frequency of bacterial ASVs, are shown in Fig. 3, with taxa reported at the highest confidently assigned taxonomic level.

Milk used for spring cheesemaking (S_M1 and S_M2) was characterized by the dominance of *Pseudomonas* spp. (76.65 and 71.05% of the relative frequency, respectively), followed by *Deinococcus* spp. (13.62% and 10.98% of the relative frequency, respectively), and *Micrococcaceae* (4.67% and 4.49% of the relative frequency, respectively). Sample S_M2 also showed the presence of *Chryseobacterium* spp. (1.50%), *Lactococcus* spp. (2.50%), *Moraxellaceae* (1.66%), and *Sphingobacterium faecium* (1.33%).

Autumn milk also revealed the dominance of Pseudomonadaceae and *Pseudomonas* spp. (>40% of the relative frequency). Moreover, both A_M1 and A_M2 showed the presence of Enterobacteriaceae (3.32% and 6.58% of the relative frequency, respectively), *Escherichia* spp. (1.26% and 2.41% of the relative frequency, respectively), *Chryseobacterium* spp. (1.78% and 3.56% of the relative frequency, respectively), and *Sphingobacterium* spp. (0.80% and 1.60%, respectively). A_M1 was also characterized by the presence of *Bacillus* spp. (15.55% of the relative frequency), *Lacticaseibacillus paracasei* (3.81%), and *Streptococcus thermophilus* (30.30%).

Pseudomonas spp. represented the major bacterial taxa observed in milk samples. Regarding this genus, it includes psychrotrophic bacteria known to contribute to spoilage of raw milk, including that from goats (Scatamburlo et al., 2015). *Pseudomonas* spp. possess high genetic and metabolic adaptability, allowing them to persist in diverse environments and on dairy equipment such as pipelines, bulk tanks, and milking systems (Scatamburlo et al., 2015). Scatamburlo et al. (2015) highlighted the importance of controlling *Pseudomonas* contamination during the production and storage of goat milk.

As for *Deinococcus* spp., Decadt et al. (2025) recently detected its presence in brine samples from dairy environments, suggesting a similar source in the present study. Initially classified as *Micrococcus*, these

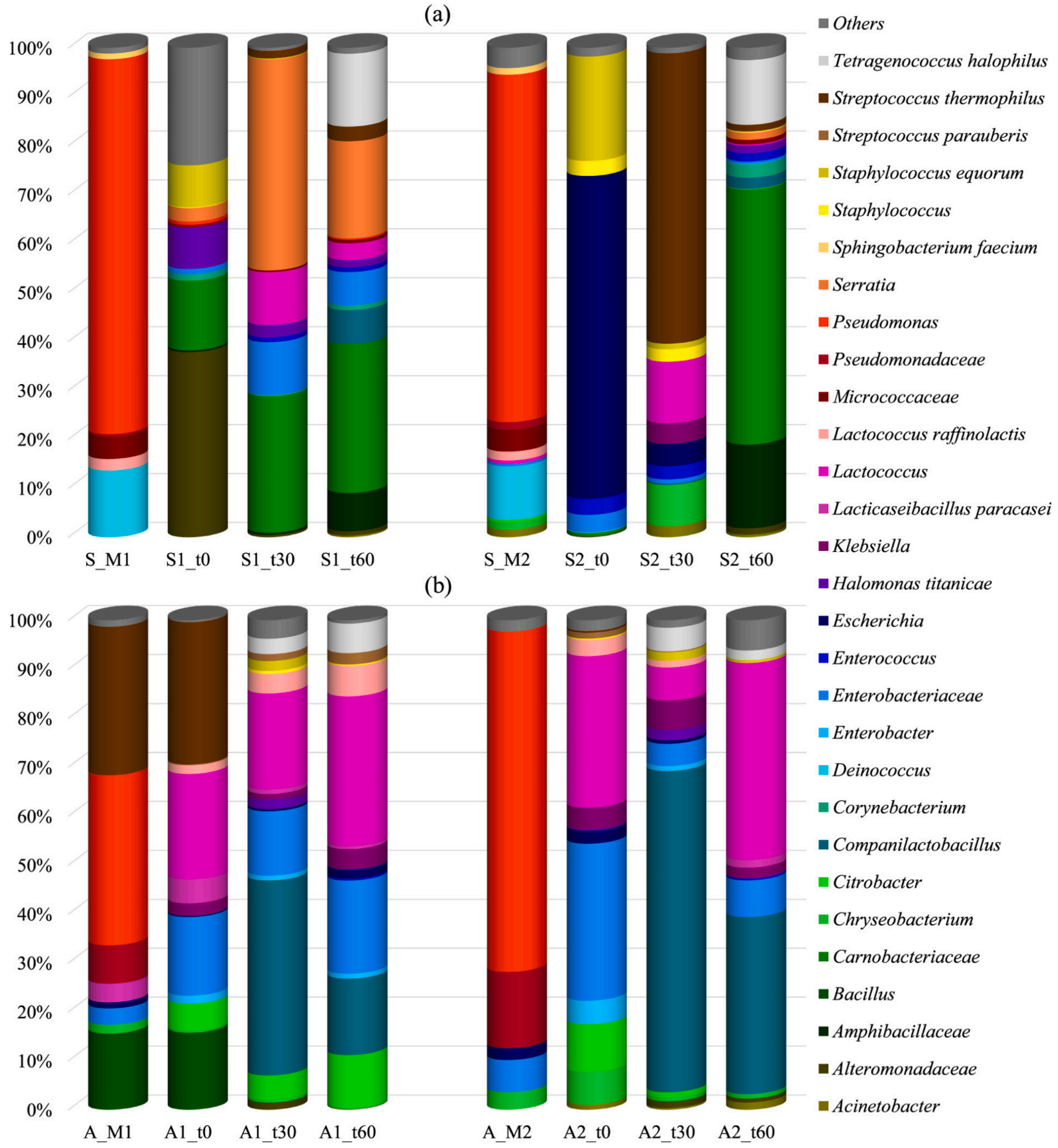

Fig. 3. Relative frequency of bacterial Amplicon Sequence Variants (ASVs). (a) Spring production (S). (b) Autumn production (A).
Two independent batches were analyzed: batch 1 (S1 and A1, respectively) and batch 2 (S2 and A2, respectively). Within each batch, raw milk samples (M1 and M2) and cheeses at 0, 30, and 60 days of ripening (t_0, t_{30}, t_{60}) were evaluated. Bars represent the relative abundance (%) of bacterial taxa identified by 16S rRNA gene sequencing. Only taxa with a relative abundance >1% in at least two samples are shown; the remaining taxa are grouped as "Others".

bacteria are coccoid or rod-shaped, non-sporulating organisms noted for their exceptional stress tolerance and their uniquely effective mechanisms for repairing DNA damage (Gerber et al., 2015).

Among the lactic acid bacteria detected in raw milk, the presence of *Lactococcus* reflects its strong adaptation to the dairy environment. In cheeses produced from unpasteurized milk, it generally becomes the dominant taxon, suppressing spoilage and pathogenic bacteria (Cavanagh et al., 2015). *L. paracasei* has also been reported in raw milk by Bettera et al. (2023) as a non-starter lactic acid bacterium with potential use as an adjunct culture. Similarly, the occurrence of *S. thermophilus* in raw milk has previously been documented by Delgado et al. (2013).

The occurrence of Enterobacteriaceae and *Escherichia* ASVs in the analyzed milk samples reflects the results of viable counts already discussed.

Notably, the detection of *Bacillus* spp. in autumn milk samples warrants attention in terms of cheese safety and quality. Although the presence of *Bacillus cereus* cannot be inferred from the present data, species belonging to the genus *Bacillus* are known to include spoilage- and safety-relevant microorganisms in dairy environments due to their ability to persist on processing surfaces and form biofilms (Kumari & Sarkar, 2016).

Regarding cheese samples, alpha-diversity indices did not differ significantly between spring (S) and autumn (A) samples ($p > 0.05$)

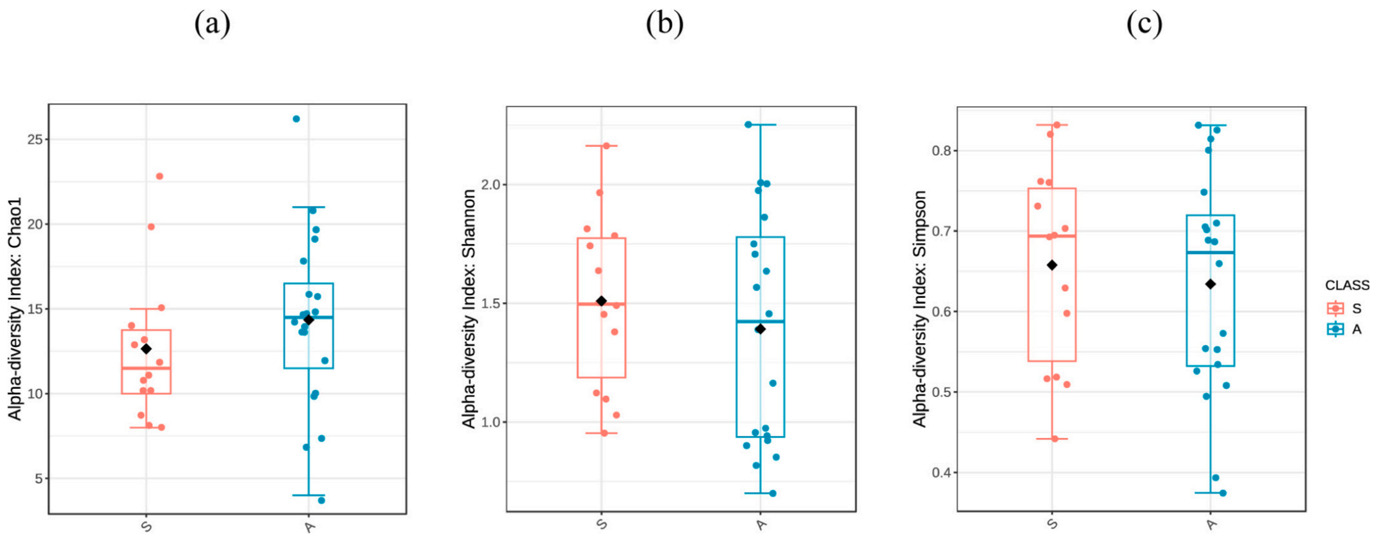

Fig. 4. Comparison of alpha-diversity indices between spring (S, red) and autumn (A, blue) cheesemaking samples. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
(a) Chao1 richness index, (b) Shannon diversity index, and (c) Simpson diversity index. Each boxplot shows the distribution of diversity values with individual sample points overlaid; black diamonds represent the mean values.

(Fig. 4). Autumn samples showed higher richness (Chao1) (Fig. 4, panel a), whereas Shannon (Fig. 4, panel b) and Simpson (Fig. 4, panel c) diversity indices were slightly lower compared to spring, but none of these differences reached statistical significance ($p > 0.05$). In contrast, beta-diversity analysis based on Bray–Curtis's dissimilarities revealed a clear separation between spring and autumn cheese samples (Fig. 5). The first three axes of the PCoA explained 26.9%, 18.1%, and 13.6% of the variance, respectively, and PERMANOVA confirmed that microbial community composition differed significantly between seasons ($p < 0.05$).

The data presented in this study are in agreement with Ferrocino et al. (2026), who reported that cheese microbiomes are shaped by seasonal dynamics influencing both raw materials and the processing environment. Ferrocino et al. (2026) also highlighted how specific facility sites act as reservoirs that drive microbial transfer during production, ultimately affecting texture, and overall cheese quality. These aligned observations emphasize the need to account for environmental and seasonal variability to ensure consistent product characteristics.

In the present study, cheese samples from both spring and autumn cheesemaking shared the presence of *Chryseobacterium* spp. (overall mean 1.64% of the relative frequency), Enterobacteriaceae (overall mean 8.42% of the relative frequency), *Halomonas* spp. (overall mean 1.24% of the relative frequency), and *Staphylococcus equorum* (overall mean 2.38% of the relative frequency), further supporting the presence of a relatively stable microbiome, in agreement with previous observations by Ferrocino et al. (2026).

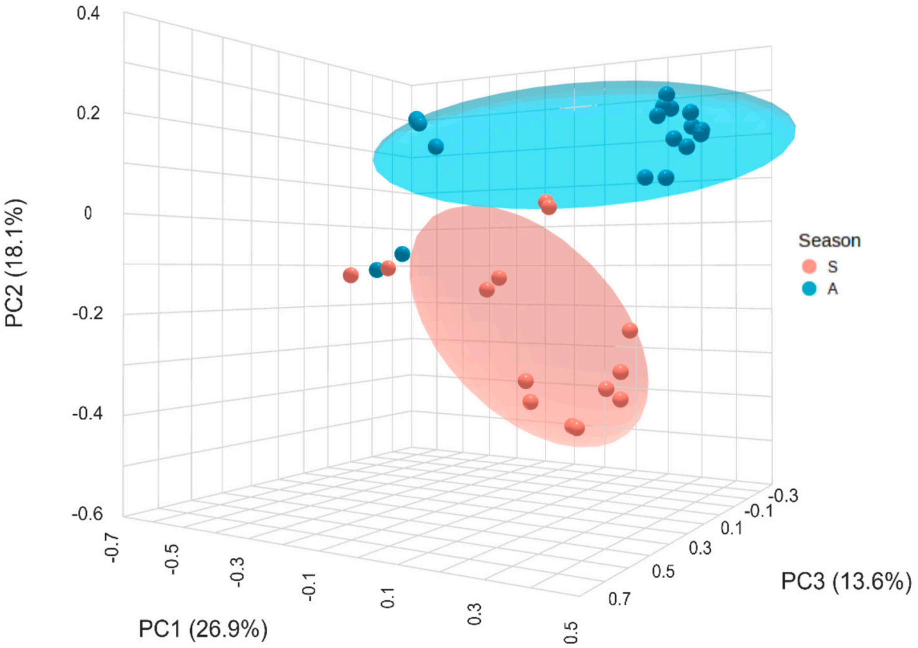

Fig. 5. Principal Coordinate Analysis (PCoA) of bacterial communities in cheesemaking samples from spring (S, red) and autumn (A, blue). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
The plot shows separation of samples along PC1 (26.9%), PC2 (18.1%), and PC3 (13.6%), with ellipses indicating the clustering of samples by season.

Interestingly, *Chryseobacterium* was already found in the microbiota originating from wooden tools and floors of dairy plants (Ruta et al., 2025), suggesting the same origin in the analyzed cheese samples.

Regarding *Halomonas*, its presence reflects the ability of this haloalkaliphilic bacterium to thrive in the high-salt environments typical of halophilic foods such as brined cheeses (Vermote et al., 2018). Vermote et al. (2018) also reported *Halomonas* in brine samples, suggesting that it may be introduced via the sea salt used during cheese production, as seawater is a natural reservoir for these marine bacteria.

Regarding *S. equorum* ASVs, this species has previously been isolated from brine baths in Germany (Hammer et al., 2019), which may explain its presence in the samples herein analyzed. This finding supports the viable-count data for coagulase-negative cocci and reinforces the value of combining culture-dependent methods with DNA-based approaches.

As expected, lactic acid bacteria constituted a key component of the core microbiota in the analyzed cheese samples. *S. thermophilus* showed an overall mean relative abundance of 8.33%, while *T. halophilus* averaged 2.93%. *Lactococcus* spp. was also detected in samples from both seasons, though with notably higher abundance in autumn-produced cheeses (21.36%) compared with those produced in spring (3.97%).

Regarding *S. thermophilus* ASVs, their presence in the analyzed cheese samples is very likely linked to the milk microbiota previously discussed. The occurrence of *S. thermophilus* in cheese has been associated with increased levels of free fatty acids, protein degradation, and higher concentrations of free amino acids, all of which can strongly influence cheese aroma (Zhao, Zhang, et al., 2025). Notably, since dry salting is known to reduce the viability of *S. thermophilus* compared with brine salting (Hickey et al., 2017), the use of brine salting in the cheeses examined here may have supported the persistence of this taxon in the final products.

T. halophilus is a salt-tolerant lactic acid bacterium frequently reported as a core member of the microbiota in fermented fish products and soy sauce (Belleggia & Osimani, 2023; Zhang et al., 2024). Its occurrence in cheese, however, appears to be uncommon. Nonetheless, *Tetragenococcus* species have been identified in several dairy-related environments, including cheese brines and raw milk cheeses such as Mexican Cotija and French Brie de Meaux (Rodríguez et al., 2022). More recently, they were also reported in traditional Spanish blue-veined cheeses produced from raw milk and Turkish herby cheeses (Rodríguez et al., 2022; Rüstemoğlu et al., 2023).

Regarding *Lactococcus* spp. ASVs, this genus of lactic acid bacteria includes *Lactococcus lactis*, comprising the subspecies *lactis* and *cremoris*, which is widely used as a starter culture in the dairy industry, including for brined cheese production (Altin et al., 2024; Cavanagh et al., 2015). *Lactococcus* species originate from diverse environments, including raw milk, raw-milk cheeses, and non-dairy habitats (Cavanagh et al., 2015). By converting lactose into lactic acid, this key genus gains a competitive advantage in milk, supported by genes that enhance lactose utilization. Notably, *Lactococcus* exhibited strain-dependent effects on casein proteolysis in Turkish white-brined cheese, influencing free amino acid levels (Hayaloglu et al., 2004) and likely altering the cheese's volatile profile (Zhao, Zheng, et al., 2025).

The occurrence of Enterobacteriaceae ASVs in all cheese samples underscores the necessity of improving hygienic conditions throughout the cheesemaking process.

The bacterial taxa with significantly different relative abundances across spring and autumn cheese samples are shown in Fig. 6. In detail, taxa significantly associated with spring cheeses included

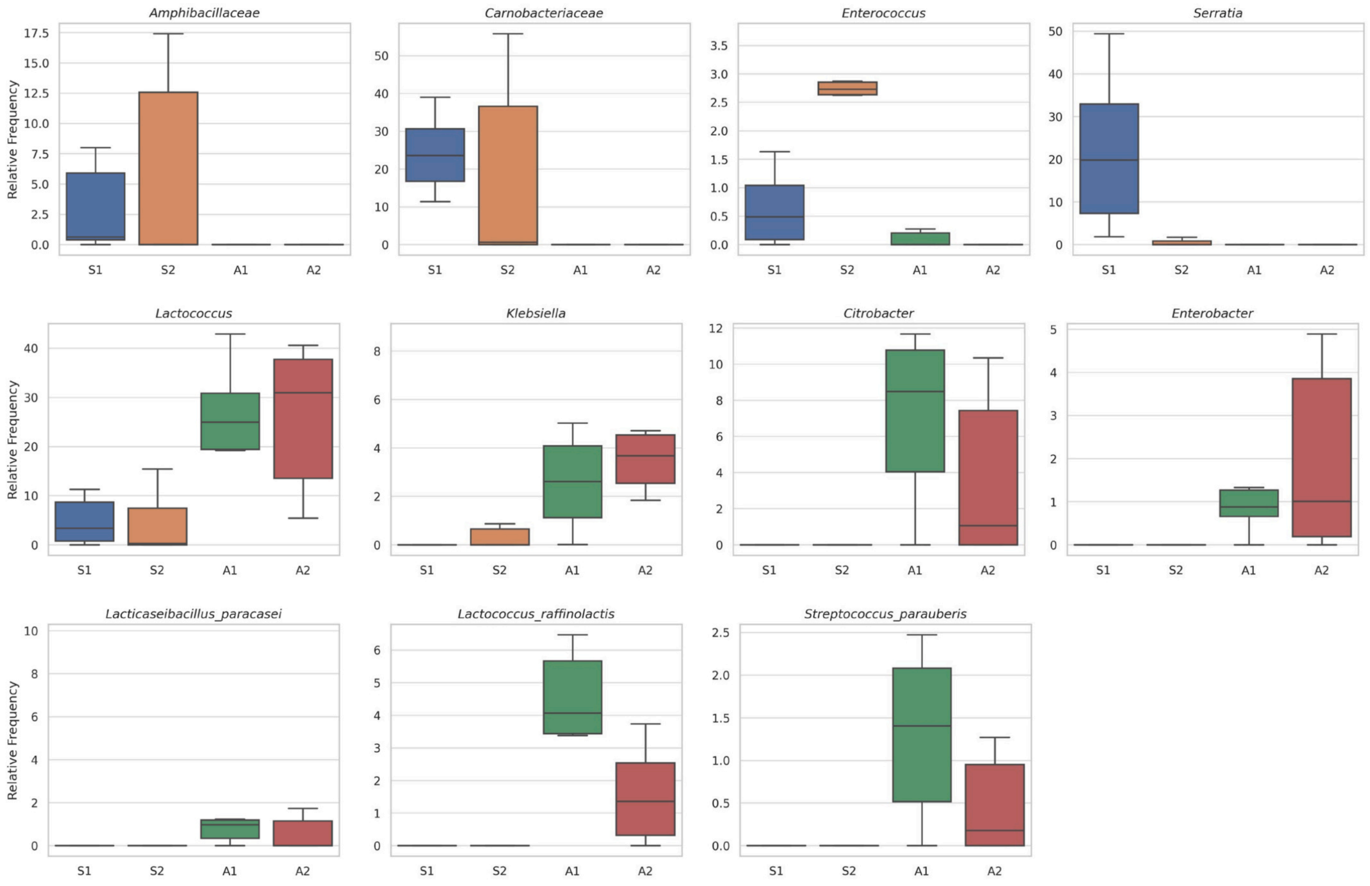

Fig. 6. Boxplot of bacterial taxa with significantly different relative abundances across experimental groups.
Each panel represents a bacterial taxon that showed significant differences (FDR-adjusted $p < 0.05$) in relative abundance among the four groups (S1, S2, A1, A2), based on the Kruskal–Wallis test.

Amphibacillaceae (overall mean 3.67% of the relative frequency), Carnobacteriaceae (17.91%), and *Enterococcus* spp. (1.37%). Moreover, cheese samples from the first batch of the spring production (S1) showed particularly high abundance of *Serratia* spp. This taxon was found with a relative abundance of 2.74% at t_0, increasing up to 43.12% at t_{30}, and with a final value of 19.84% (t_{60}). The sample S1_t_0 showed a high abundance of Alteromonadaceae (37.81%), decreasing to values <1% in the following sampling times.

To the author's knowledge, species of the Amphibacillaceae family have rarely been reported in cheese, and the present study reveals a previously unrecognized biological niche for these bacteria. Notably, *Amphibacillus* has been detected in brines used for Spanish-style green table olives (Correa-Galeote et al., 2022). This genus comprises halophilic bacteria that cannot grow at NaCl concentrations below 6%. Therefore, the presence of Amphibacillaceae ASVs in the analyzed cheese samples may be related to the high-salt conditions characteristic of brine ripening.

Carnobacteriaceae have been isolated from cheese brines in Danish dairies (Haastrup et al., 2018). Within this family, *Carnobacterium* species have been reported in several cheese types, including French surface-mold–ripened soft cheeses, French soft-ripened and red-smear cheeses made from cow's, ewe's, or goat's milk, as well as Mozzarella (Afzal et al., 2010). Because *Carnobacterium* species acidify milk slowly, they are not suitable as starter cultures and are instead classified as non-starter lactic acid bacteria, contributing to cheese aroma development during ripening (Afzal et al., 2010).

Regarding *Enterococcus* spp., their occurrence and role will be discussed in the following paragraph, as enterococcal isolates from the cheese and environmental samples in this study were obtained and characterized. Although the role of enterococci in cheese remains controversial due to their association with nosocomial infections and antimicrobial resistance, this group of lactic acid bacteria has long been recognized for its pro-technological potential, including contributions to aroma development and the production of bacteriocins active against human foodborne pathogens (Favaro et al., 2014).

The high occurrence of *Serratia* in the spring-produced samples likely reflects the high Enterobacteriaceae counts, as this family may have included species belonging to this spoilage taxon.

Cheese from the autumn cheese-making was characterized by a higher occurrence of *Citrobacter* spp. (overall mean 4.60% of the relative frequency), *Enterobacter* spp. (1.33%), *Klebsiella* spp. (2.88%), *Lacticaseibacillus paracasei* (1.17%), *Lactococcus raffinolactis* (2.38%), and *Streptococcus parauberis* (0.76%) (Fig. 6). The sample A1_t_0 showed a high abundance of *Bacillus* spp. (15.73%), subsequently not detected in the following sampling times. Conversely, *Companilactobacillus,* not detected at t_0, resulted as a dominant taxon at both t_{30} and t_{60}, with an overall mean value of 22.47% of the relative frequency.

L. paracasei belongs to the group of non-starter lactic acid bacteria with a heterofermentative metabolism, contributing to aroma development and increased organic acid production in cheese (Zhao, Zhang, et al., 2025).

As for L. *raffinolactis*, Kimoto-Nira et al. (2012) described a commensal relationship between this species and *L. lactis* that may contribute to enhanced acid production in cheese.

S. parauberis has recently been identified as part of the minor microbiota in Maltese sheep cheese and in Torta del Casar (Ihsan et al., 2025), although its specific contribution to cheese remains poorly understood. Nevertheless, a positive association has been reported between *S. parauberis* and the formation of aroma compounds, contributing to a more complex cheese flavor (Ihsan et al., 2025).

Companilactobacillus (basonym *Lactobacillus alimentarius*) is a lactic acid bacterium with a facultatively heterofermentative metabolism, for which ecological data remain limited (Tabanelli et al., 2024). *Companilactobacillus* has previously been linked to dairy ecosystems and showed a probiotic potential in goats, where its administration was associated with improved intestinal health and an increased proportion of unsaturated fatty acids in the milk (Tabanelli et al., 2024). Interestingly, species within *Companilactobacillus* genus have been reported to produce bacteriocins with broad antimicrobial activity, acting against both Gram-positive and Gram-negative pathogenic bacteria (Honrada Perez et al., 2022).

The detection of *Citrobacter*, *Enterobacter*, and *Klebsiella* in the analyzed cheese samples further supports the Enterobacteriaceae counts previously discussed.

The presence of *Bacillus* spp. in cheese is also consistent with the metataxonomic results obtained for autumn-produced milk.

Several taxa were also sporadically detected at low relative frequencies, including *Acinetobacter* spp., *Chromohalobacter* spp., *Corynebacterium* spp., *Kocuria* spp., and *Rothia* spp.

Overall, the results showed that after 60 days of ripening, Carnobacteriaceae, Enterobacteriaceae, *Serratia*, and *T. halophilus* formed the core microbiota of cheese samples produced in spring, whereas *Companilactobacillus* and *Lactococcus* were the dominant taxa in autumn-produced samples at the same ripening stage.

3.3.3. *Identification and characterization of lactic acid bacteria isolates*

The isolation and characterization of lactic acid bacteria represent crucial steps in understanding the microbial ecology of artisanal raw-milk cheeses and the technological functions carried by their cultivable fraction. While culture-independent approaches provide a comprehensive overview of the overall microbiota, only the recovery of viable isolates enables the assessment of culture-specific traits with direct implications for cheese quality and safety. In traditional cheese-making environments, where raw milk, dairy surfaces, and equipment act as natural reservoirs of microorganisms, lactic acid bacteria may exhibit diverse metabolic activities, including proteolysis, lipolysis, exopolysaccharide synthesis, and even the potential to produce biogenic amines. Characterizing these isolates is therefore essential not only to elucidate their contribution to ripening and sensory development but also to evaluate their technological potential and identify strains that may pose safety concerns.

In this study, lactic acid bacteria isolates obtained from the dairy environment were taxonomically identified and examined for key technological and safety-related properties, providing insight into their functional roles within the cheese ecosystem.

Table 6 reports the results of the identification and characterization of lactic acid bacteria isolated from the dairy environment. A total of 21 isolates were recovered from wall and tool swabs, comprising 9 cultures most closely related to *Levilactobacillus brevis*, 3 to *Enterococcus faecium*, 4 to *Enterococcus faecalis*, 1 to *Enterococcus pingfangensis/Enterococcus xiangfangensis*, 3 to *Pediococcus pentosaceus*, and 1 to *Lactiplantibacillus paraplantarum.* All isolates were evaluated for lipase and protease activities, EPS production, and the presence of the *hdca* gene.

Among the obtained isolates, *L. brevis* is a microaerophilic, strictly heterofermentative lactic acid bacterium that occurs in a wide range of environments (Teixeira, 2014). From a dairy technology perspective, *L. brevis* is recognized as belonging to the non-starter lactic acid bacteria group (Georgalaki et al., 2025). To the best of the authors' knowledge, there is limited scientific information available on *L. brevis* cultures originating from environmental sources. Notably, the ability of *L. brevis* to form biofilms through the production of EPS may help explain its persistence in the dairy processing settings used for cheese manufacturing (Rezaei et al., 2023), even when standard hygiene practices were applied by cheese producer. Supporting this, 4 out of the 9 isolates produced sucrose-dependent EPS, whereas 1 strain produced sucrose-independent EPS, confirming the biofilm-forming potential of *L. brevis*. Only 1 out of the 9 *L. brevis* studied pure cultures showed lipase activity. Although lipase production is uncommon in lactic acid bacteria, lipase activity present in dairy starter or adjunct cultures is important because these enzymes can substantially influence the flavor development of fermented dairy products (Meyers et al., 1996). Of note, *L. brevis* strains already proved to have lipase with pro-technological

Table 6
Identification and characterization of lactic acid bacteria isolates from dairy environment.

Source	Isolate	Closest relative	% Identity[1]	Accession number[2]	Lipase activity*	Protease activity*	*hdca* gene	EPS***	
								Sucrose-dependent	Sucrose-independent
Walls	E1	*Levilactobacillus brevis*	100.00%	NR_116238	−	+	−	−	−
	E2	*Levilactobacillus brevis*	99.90%	NR_116238	−	++	−	−	−
	E3	*Lactiplantibacillus paraplantarum* group	99.42%	NR_025447	−	+	−	+	−
	E4	*Levilactobacillus brevis*	100.00%	NR_116238	−	+	−	+	−
	E5	*Levilactobacillus brevis*	99.81%	NR_116238	−	+	−	+	+
	E6	*Enterococcus faecium*	99.27%	NR_113904	+	++	−	−	−
	E7	*Enterococcus faecium*	99.91%	NR_113904	+	+	−	−	−
	E8	*Enterococcus faecium*	99.24%	NR_113904	+	+	−	−	−
Tools	T1	*Levilactobacillus brevis*	99.88%	NR_116238	−	+	−	+	−
	T2	*Levilactobacillus brevis*	99.31%	NR_116238	−	+	−	−	−
	T3	*Levilactobacillus brevis*	100.00%	NR_116238	+	+	−	−	−
	T4	*Levilactobacillus brevis*	100.00%	NR_116238	−	+	−	+	−
	T5	*Pediococcus pentosaceus*	99.79%	NR_042058	−	+	−	+	−
	T6	*Levilactobacillus brevis*	99.50%	NR_116238	−	+	−	−	−
	T7	*Pediococcus pentosaceus*	99.42%	NR_042058	−	++	−	−	−
	T8	*Pediococcus pentosaceus*	99.72%	NR_042058	−	+	−	−	−
	T9	*Enterococcus faecalis*	94.87%	NR_113902	+	+	−	−	−
	T10	*Enterococcus faecalis*	99.90%	NR_113902	+	+	−	−	−
	T11	*Enterococcus pingfangensis* *Enterococcus xiangfangensis*	99.83%	NR_174226 NR_133741	+++	+	−	−	−
	T12	*Enterococcus faecalis*	98.94%	NR_113902	++	+	−	−	−
	T13	*Enterococcus faecalis*	98.31%	NR_113902	++	+	−	−	−

[1] Percentage of identical nucleotides in the sequence obtained from the bacterial isolates and the sequence of the closest relative found in the GenBank database.

[2] Accession number of the sequence of the closest relative found by BLAST search.

[*] -, negative; +, positive (1 mm halo); ++, positive (1–2 mm halo); +++, positive (> 2 mm halo).

[**] M, mucoid colonies.

applications (Khan et al., 2017). All *L. brevis* isolates showed notably high protease activity. The proteolytic system of lactic acid bacteria contributes to acid development and flavor formation by breaking down proteins and releasing amino acids. According to Ebadi Nezhad et al. (2020), cultures with strong proteolytic capacity are typically selected for hard cheese ripening, whereas those with milder activity are better suited for fresh or semi-hard cheeses.

As for the enterococci, the presence of species from this bacterial group in the dairy environment has already been reported by Alp Gundog et al. (2025), who isolated *E. faecium* and *E. faecalis* from raw bulk-tank milk and equipment surfaces. Enterococci have previously been isolated from traditional Montenegrin brined cheeses (Ruppitsch et al., 2020) and from home-made white brined cheese (Favaro et al., 2014). Among the characterized isolates in the present study, all *E. faecium* cultures exhibited lipase activity. Lipase-producing *E. faecium* strains have also been described by Ramakrishnan et al. (2012) and Merih-Kivanc (2022), further confirming the technological potential of these microorganisms for use in the food industry. A notable protease activity was also observed in all *E. faecium* isolates. Interestingly, proteolytic *E. faecium* strains obtained by Moayedi et al. (2025) from traditional cheeses significantly increased the free amino acid content of whey-based experimental beverages and produced pleasant volatile compounds. No EPS production was observed in any of the *E. faecium* isolates examined in the present study.

E. faecalis isolates showed both lipase and protease activity. With regard to lipase production, to the authors' knowledge this enzymatic activity has rarely been reported for *E. faecalis*, making comparisons with previously published data difficult. Nevertheless, Carrasco de Mendoza et al. (1992) observed lipolytic activity for this species. As for protease activity, *E. faecalis* var. *liquefaciens* is commonly found in cheeses that show extensive protein breakdown, as this microorganism is highly efficient at degrading casein and other milk proteins, even at refrigeration temperatures (García et al., 2002). No EPS production was observed in any of the *E. faecalis* isolates studied in the present research.

Enterococcus isolate T11 could not be unequivocally identified at the molecular level; sequence analysis indicates that it is closely related to *E. pingfangensis*/*E. xiangfangensis* species. Both of these closely related species were originally isolated from Chinese pickles (Li et al., 2014; Li & Gu, 2019). To the authors' knowledge, *E. xiangfangensis* has already been reported in dairy products, specifically in spontaneously fermented cottage cheese from Mongolia and dairy products from Russia (Yu et al., 2021). In addition, an isolate putatively identified as *E. pingfangensis* was obtained by Nelli et al. (2023) from Gidotyri goat cheese, suggesting that goat cheese may represent a potential habitat for this species, although the identification was not definitive. Isolate T11 exhibited markedly high lipase and protease activities, whereas no EPS production was detected.

Regarding *P. pentosaceus,* strains recovered from the udder of cattle milk offered fresh insights into the selection of cultures with food-protective properties due to production of bacteriocins (Fischer et al., 2025). Notably, the demand for *P. pentosaceus* strains in the dairy sector has increased due to their strong potential as both probiotic and starter-culture microorganisms (Mgomi et al., 2022). Therefore, the characterization of the isolates obtained in the present study may provide suitable candidates for improving cheese quality. Among the *P. pentosaceus* isolates analyzed, none exhibited lipase activity, whereas all showed protease activity. As noted by Vafopoulou-Mastrojiannaki et al. (1994), *P. pentosaceus* isolated from traditional Greek cheeses showed high proteinase, aminopeptidase, and dipeptidyl aminopeptidase activities. In the present study, 1 out of the 3 isolates of *P. pentosaceus* showed the production of sucrose-dependent EPS, whereas none of them produced sucrose-independent EPS. In relation to this trait, EPS-producing *P. pentosaceus* strains with antioxidant activity have already been isolated from wheat, rice, and various vegetables (Sharma et al., 2024).

L. paraplantarum is a facultatively heterofermentative species that grows within a temperature range of 15–37 °C and withstands sodium chloride levels of up to 8%. This organism is categorized as a non-starter lactic acid bacterium and has previously been detected in cheeses such as Caciocavallo (Ciocia et al., 2013) and Brazilian semi-hard artisanal varieties (Winkelströter et al., 2015). In the isolate examined in the present study, lipase activity was absent, whereas protease activity was evident. Notably, *L. paraplantarum* strains have been reported to possess proteolytic capacities, including leucine arylamidase, valine arylamidase, and cysteine arylamidase activities (Prasev et al., 2025). The isolate analyzed here also produced sucrose-dependent exopolysaccharides (EPS), consistent with earlier reports describing ropy EPS formation by L. *paraplantarum* isolated from a Serbian soft, white, homemade cheese (Nikolic et al., 2012).

The recovery of lactic acid bacteria directly from raw milk, brine, and cheese samples provides essential insight into the fermentative microbiota actively driving curd acidification and ripening. Unlike environmental isolates, these isolates reflect the core microbial consortium that persists and adapts throughout cheese manufacture. Their taxonomic identification and functional characterization help clarify their technological relevance and contribution to the sensory and biochemical evolution of the final product.

The results of identification and characterization of lactic acid bacteria isolates from milk, cheese, and brine are reported in Table 7. Again, all isolates were assessed for lipase and protease activities, EPS production, and the presence of the *hdcA* gene.

In total, 113 isolates were obtained from brine and cheese samples. These included 8 cultures most closely related to *L. lactis,* 3 to *Leuconostoc mesenteroides*, 1 to *Carnobacterium gallinarum*, 1 to *Enterococcus mundtii*, 2 to *Lactococcus cremoris*, 35 to *E. faecium*, 3 to *E. faecium/Enterococcus durans*, 5 to *Marinilactibacillus psychrotolerans*, 5 to *E. faecalis*, 9 to *E. durans*, 3 to *Enterococcus lactis*, 8 to *Enterococcus pseudoavium/Enterococcus devriesei*, 9 to *E. pseudoavium/Enterococcus viikkiensis*, 7 to *Enterococcus italicus*, 1 to *Enterococcus innesii/Enterococcus gallinarum*, 4 to *Lactiplantibacillus plantarum* group, 4 to L. *brevis*, 1 to *Companilactobacillus alimentarius*, 2 to *E. pingfangensis/E. xiangfangensis*, 2 to *Lacticaseibacillus paracasei* subsp. *tolerans*.

The detection of several species in cheese and brine that were also recovered from the dairy environment suggests the occurrence of cross-contamination between processing surfaces, raw materials, and final products. This overlap supports the hypothesis that microbial transfer takes place throughout the production chain. Such interactions may influence both the technological performance and the microbial stability of artisanal goat cheese.

To avoid redundancy with the previously examined environmental isolates, the following section focuses exclusively on the microbial species recovered from milk, brine, and cheese that were not already discussed in the context of the dairy environment. Although several taxa (including, *L. brevis*, *E. faecium*, *E. faecalis*, *E. durans*, *E. pingfangensis/E. xiangfangensis*, *P. pentosaceus*, and L. *paraplantarum*) were isolated from both sources, their technological traits and ecological roles have been comprehensively addressed above. The subsequent discussion therefore concentrates on the remaining species uniquely represented, or taxonomically distinct, within the brine- and cheese-derived collection, thereby providing an interpretation of their contribution to the fermentative dynamics and ripening processes characteristic of artisanal goat cheese.

Enterococci isolates represented one of the major microbial groups recovered, confirming the results obtained from the environmental samples.

One isolate closely related to *E. mundtii* was identified; this species has previously been isolated from yellow cheese (Farías et al., 1996) and has been successfully applied as an antilisterial culture in fresh Minas cheese (Vera Pingitore et al., 2012). Interestingly, bacteriocinogenic *E. mundtii* has also been isolated from Slovak raw goat milk by Lauková et al. (2020). The isolate examined in the present study did not exhibit lipase activity; however, it tested positive for protease, suggesting a potential contribution to the production of free amino acids and the enhancement of the cheese's sensory attributes. The studied isolate of *E. mundtii* also showed the production of mucoid colonies in growth medium containing sucrose, indicating exopolysaccharide (EPS) synthesis as previously demonstrated by Vallejo et al. (2018).

In the present study, *E. lactis* was also recovered from the cheese samples. This species has previously been isolated from raw milk cheese by Fu et al. (2022). Moreover, a probiotic *E. lactis* strain studied by Tadesse et al. (2024) produced bacteriocins with a potential food-protective effect. Two of the three *E. lactis* isolates examined in this study exhibited lipase activity. Similarly, Morandi et al. (2013) reported low lipolytic activity in *E. lactis* strains isolated from Bitto PDO raw milk cheese. The same authors also observed low proteolytic activity in these strains, consistent with findings for other enterococcal species (Sarantinopoulos et al., 2001) and with the protease activity results obtained in the present study. No EPS production was observed for any of the *E. lactis* isolates.

As for *E. italicus*, this species has previously been isolated from Turkish white cheese samples by İspirli et al. (2017) and, more recently, detected in the microbiota of traditional “Bieno” cheese produced in North Macedonia (Josifovska et al., 2024). All *E. italicus* isolates examined in the present study exhibited lipase activity, consistent with the lipase activity reported by Cardinali et al. (2025) for *E. italicus* isolated from smoked cheese made from raw goat's milk. The isolates also showed protease activity, suggesting a potential positive contribution to flavor development. No EPS production was observed for any of the *E. italicus* isolates.

Unambiguous identification of the *E. pseudoavium/E. devriesei* and *E. pseudoavium/E. viikkiensis* isolates was not achieved, likely due to the very close phylogenetic relationships among these species (Li et al., 2014; Švec et al., 2005). Interestingly, *E. devriesei* has previously been isolated from Cueva de la Magahá and Torta Arochena cheeses produced from goats' milk (Martín-Platero et al., 2009). *E. pseudoavium* has also been isolated from cheese whey used as a starter culture for Mina artisanal cheese (Castro et al., 2016), whereas *E. viikkiensis* has been identified in ewe's milk (Dapkevicius et al., 2021). In the present study, most isolates exhibited lipase activity, and all were positive for protease activity, suggesting a potential contribution to cheese flavor development. No EPS production was detected in any of the *E. pseudoavium/E. devriesei* or *E. pseudoavium/E. viikkiensis* isolates.

E. innesii/E. gallinarum are very closely related phylogenetically (Gooch et al., 2021); therefore, unambiguous identification between these two species was also not achieved. Notably, *E. innesii* was only recently defined following its isolation from the wax moth *Galleria mellonella* (Gooch et al., 2021). *E. gallinarum* has previously been isolated from Caciocavallo Palermitano cheese (Guarrasi et al., 2017). The isolate examined in the present study exhibited both lipase and protease activities, whereas no EPS production was observed.

In the present study, a few *L. lactis* isolates exhibited lipolytic activity. Notably, Yu et al. (2025) reported that a lipase-producing probiotic *L. lactis* strain enhanced sour-cream production from raw cow's milk by altering the fatty acid profile and pH, resulting in pronounced sensory changes. *L. lactis* can degrade lipids through extracellular and intracellular lipases, releasing fatty acids and glycerol. It can also modify fatty acids via reactions such as saturation, hydration, dehydration, and isomerization (Akpoghelie et al., 2025). Accordingly, the lipase-producing isolates identified in this study were likely to have contributed to the sensory properties of the cheese samples. All *L. lactis* isolates exhibited protease activity, a functional trait that allows this pro-technological species to hydrolyze milk proteins during fermentation (Zhao, Zhang, Simpson and Gänzle, 2025), thereby contributing to cheese flavor development through the release of free amino acids. Regarding EPS production, only 1 out of the 8 *L. lactis* isolates produced mucoid colonies, indicative of exopolysaccharide synthesis. Notably, EPS from L. *lactis* has been reported to possess antioxidant, antitumor, antibacterial, and immunomodulatory activities, along with valuable technological properties such as emulsification and water retention (Sharma et al., 2024).

Among the lactococci, isolates closely related to *L. cremoris* were also recovered. This microorganism, recently reclassified from *L. lactis* subsp. *cremoris* to species level (Li et al., 2021), is known to impart buttery aromas to cheese through its production of acetoin and diacetyl (Tapia

Table 7
Identification and characterization of lactic acid bacteria isolates from milk, cheese, and brine.

Source	Isolate	Closest relative	% Identity[1]	Accession number[2]	Lipase activity*	Protease activity*	*hdcA* gene	EPS***	
								Sucrose-dependent	Sucrose-independent
SM	S1	*Lactococcus lactis*	100.00%	NR_113960	−	+	−	−	−
	S2	*Leuconostoc mesenteroides*	100.00%	NR_074957	−	+	−	−	−
	S3	*Lactococcus lactis*	98.96%	NR_113960	+	+	−	M	−
	S4	*Carnobacterium gallinarum*	98.81%	NR_042093	+	+	−	M	−
	S5	*Enterococcus mundtii*	99.29%	OM090208	−	+	−	M	−
SC1_t0	S6	*Lactococcus cremoris*	99.74%	NR_113925	−	+	−	−	−
	S7	*Lactococcus cremoris*	99.57%	NR_113925	−	+	−	−	−
	S8	*Enterococcus faecium*	99.64%	NR_113904	+	+	−	−	−
	S9	*Marinilactibacillus psychrotolerans*	99.80%	NR_024794	−	+	−	−	−
	S10	*Marinilactibacillus psychrotolerans*	99.51%	NR_024794	+	+	−	−	−
	S11	*Enterococcus faecium*	99.38%	NR_113904	+	+	−	−	−
	S12	*Enterococcus faecium*	99.26%	NR_113904	+	+	−	−	−
SC2_t0	S13	*Enterococcus faecium*	99.72%	NR_113904	+	++	−	−	−
	S14	*Enterococcus faecalis*	99.90%	NR_113902	+	++	−	−	−
	S15	*Enterococcus faecium*	99.41%	NR_113904	−	++	−	−	−
	S16	*Enterococcus faecium*	99.77%	NR_113904	−	+	−	−	−
	S17	*Enterococcus faecium*	99.64%	NR_114742	−	+	−	−	−
	S18	*Enterococcus faecium*	99.16%	NR_113904	+	+	−	−	−
	S19	*Enterococcus faecium*	99.91%	NR_113904	+	+	−	−	−
SC1_t30	S20	*Enterococcus faecalis*	99.22%	NR_113902	−	+	−	−	−
	S21	*Enterococcus faecalis*	98.87%	NR_113902	−	+	−	−	−
	S22	*Enterococcus faecium*	98.94%	NR_113904	−	+	−	−	−
	S23	*Enterococcus faecium*	98.84%	NR_113904	−	+	−	−	−
	S24	*Enterococcus faecium*	99.63%	NR_113904	−	+	−	−	−
	S25	*Enterococcus faecium*	98.93%	NR_113904	−	+	−	−	−
	S26	*Enterococcus durans*	99.33%	NR_113900	−	+	−	−	−
SC2_t30	S27	*Enterococcus faecium*	98.91%	NR_113904	−	+	−	−	−
	S28	*Enterococcus faecium*	99.53%	NR_113904	−	+	−	−	−
	S29	*Enterococcus faecium*	99.26%	NR_113904	−	+	−	−	−
	S30	*Enterococcus durans*	98.83%	NR_036922	−	+	−	−	−
	S31	*Enterococcus faecium*	99.84%	NR_113904	+	+	−	+	−
	S32	*Enterococcus durans*	99.03%	NR_036922	−	+	−	−	−
	S33	*Enterococcus durans*	99.03%	NR_036922	−	+	−	−	−
SC1_t60	S34	*Enterococcus lactis*	99.56%	NR_117,562	−	+	−	−	−
	S35	*Enterococcus durans*	99.72%	NR_036922	−	+	−	−	−
	S36	*Enterococcus faecium*	98.80%	NR_113904	−	+	−	−	−
	S37	*Enterococcus durans*	99.72%	NR_036922	−	+	−	−	−
	S38	*Enterococcus durans*	98.84%	NR_036922	−	+	−	−	−
	S39	*Enterococcus faecium*	99.37%	NR_113904	−	+	−	−	−
	S40	*Enterococcus faecium*	99.09%	NR_113904	−	+	−	−	−
SC2_t60	S41	*Levilactobacillus brevis*	98.97%	NR_116238	−	+	−	−	−
	S42	*Levilactobacillus brevis*	99.45%	NR_116238	−	+	−	−	−
	S43	*Marinilactibacillus psychrotolerans*	99.26%	NR_024794	−	−	−	−	−
	S44	*Enterococcus faecium*	99.29%	NR_113904	−	+	−	−	−
	S45	*Enterococcus faecium*	99.03%	NR_113904	−	+	−	−	−
	S46	*Enterococcus lactis*	99.05%	NR_117,562	+	+	−	−	−
	S47	*Enterococcus faecium*	99.25%	NR_113904	−	+	−	−	−
Brine (S)	S48	*Enterococcus faecium*	99.58%	NR_113904	−	+	−	−	−
	S49	*Leuconostoc mesenteroides*	99.23%	NR_074957	−	+	−	M	−
	S50	*Enterococcus faecium*	99.77%	NR_113904	+	+	−	−	−
	S51	*Leuconostoc mesenteroides*	99.87%	NR_074957	−	−	−	M	−
	S52	*Marinilactibacillus psychrotolerans*	99.26%	NR_024794	−	+	−	−	−
	S53	*Enterococcus faecium*	99.58%	NR_113904	+	+	−	−	−
	S54	*Enterococcus faecium*	99.33%	NR_113904	−	+	−	−	−
	S55	*Enterococcus faecium*	99.35%	NR_113904	+	+	−	−	−
	S56	*Lactiplantibacillus plantarum* group	99.52%	NR_104573	−	+	−	−	−
	S57	*Lactiplantibacillus plantarum* group	99.28%	NR_104573	−	+	−	−	−
	S58	*Levilactobacillus brevis*	99.77%	NR_116238	−	+	−	−	−
	S59	*Marinilactibacillus psychrotolerans*	99.17%	NR_113872	−	+	−	−	−
	S60	*Enterococcus faecium*	99.20%	NR_113904	+	++	−	−	−
	S61	*Enterococcus faecalis*	99.54%	NR_113902	+	+	−	−	−
	S62	*Enterococcus faecalis*	98.91%	NR_113902	−	−	−	−	−
	S63	*Enterococcus faecium*	98.97%	NR_114742	+	+	−	−	−
AM	A1	*Enterococcus pseudoavium* *Enterococcus viikkiensis*	98.81% 98.81%	NR_113907 NR_117976	−	+	−	−	−
	A2	*Lactococcus lactis*	99.77%	NR_040955	+	+	−	−	−
	A3	*Enterococcus pseudoavium* *Enterococcus viikkiensis*	100.00%	NR_113907 NR_117976	−	++	−	−	−
	A4	*Enterococcus pingfangensis* *Enterococcus xiangfangensis*	99.83%	NR_174226 NR_133741	+	++	−	−	−

(*continued on next page*)

Table 7 (*continued*)

Source	Isolate	Closest relative	% Identity[1]	Accession number[2]	Lipase activity[*]	Protease activity[*]	*hdcA* gene	EPS[***]	
								Sucrose-dependent	Sucrose-independent
	A5	*Enterococcus pseudoavium* *Enterococcus viikkiensis*	97.36%	NR_113907 NR_117976	+	++	−	−	−
	A6	*Enterococcus pseudoavium* *Enterococcus viikkiensis*	99.50%	NR_113907 NR_117976	+	++	−	−	−
	A7	*Enterococcus pseudoavium* *Enterococcus viikkiensis*	97.73%	NR_113907 NR_117976	+	++	−	−	−
AC1_t0	A8	*Lactococcus lactis* *Lactococcus lactis*	99.31%	NR_040955 NR_113960	+	+	−	−	−
	A9	*Enterococcus pseudoavium* *Enterococcus viikkiensis*	99.13%	NR_113907 NR_117976	++	++	−	−	−
	A10	*Enterococcus pseudoavium* *Enterococcus pseudoavium* *Enterococcus devriesei*	99.18%	NR_113907 NR_115762 NR_042389	++	++	−	−	−
AC2_t0	A11	*Enterococcus pseudoavium* *Enterococcus devriesei*	99.34%	NR_113907 NR_042389	+	+	−	−	−
	A12	*Enterococcus pseudoavium* *Enterococcus devriesei*	97.39%	NR_113907 NR_042389	+++	++	−	−	−
AC1_t30	A13	*Enterococcus pseudoavium* *Enterococcus viikkiensis*	99.00%	NR_113907 NR_117976	−	++	−	−	−
	A14	*Enterococcus italicus*	98.21%	NR_104571	+	+	−	−	−
	A15	*Enterococcus italicus*	96.58%	NR_025625	+	+	−	−	−
	A16	*Enterococcus italicus*	97.10%	NR_025625	+	+	−	−	−
	A17	*Enterococcus pseudoavium* *Enterococcus viikkiensis*	98.98%	NR_113907 NR_117976	++	++	−	−	−
	A18	*Enterococcus pseudoavium* *Enterococcus pseudoavium* *Enterococcus devriesei*	97.47%	NR_113907 NR_115762 NR_042389	++	++	−	−	−
	A19	*Enterococcus italicus*	97.44%	NR_025625	++	++	−	−	−
	A20	*Enterococcus innesii* *Enterococcus gallinarum*	97.50%	NR_181755 NR_104559	+	++	−	−	−
AC2_t30	A21	*Enterococcus faecium*	97.09%	NR_113904	+	+	−	−	−
	A22	*Lactiplantibacillus plantarum* group	100.00%	NR_112690	−	+	−	−	−
	A23	*Enterococcus durans*	97.55%	NR_113257	+	+	−	−	−
	A24	*Enterococcus faecium* *Enterococcus durans*	98.09%	NR_113904 NR_113257	+	+	−	−	−
	A25	*Lactiplantibacillus plantarum* group	98.69%	NR_042394	++	+	−	−	−
	A26	*Enterococcus durans*	99.14%	NR_113257	++	+	−	−	−
	A27	*Levilactobacillus brevis*	98.64%	NR_116238	+++	+	−	−	−
	A28	*Companilactobacillus alimentarius*	96.89%	NR_044701	+	+	+	−	−
AC1_t60	A29	*Enterococcus italicus*	97.98%	NR_104571	+	+	−	−	−
	A30	*Enterococcus pseudoavium* *Enterococcus devriesei*	97.67%	NR_113907 NR_042389	−	++	−	−	−
	A31	*Enterococcus italicus*	99.66%	NR_104571	+	+	−	−	−
	A32	*Enterococcus pseudoavium* *Enterococcus devriesei*	99.18%	NR_113907 NR_042389	−	+	−	−	−
	A33	*Enterococcus pseudoavium* *Enterococcus viikkiensis*	97.27%	NR_113907 NR_117976	++	++	−	−	−
	A34	*Enterococcus italicus*	97.40%	NR_104571	++	++	−	−	−
	A35	*Enterococcus pseudoavium* *Enterococcus devriesei*	97.85%	NR_113907 NR_042389	++	++	−	−	−
AC2_t60	A36	*Enterococcus faecium* *Enterococcus durans*	97.51%	NR_113904 NR_113257	+	+	−	−	−
	A37	*Enterococcus faecium*	99.01%	NR_113904	+	+	−	−	−
	A38	*Enterococcus faecium* *Enterococcus durans*	98.00%	NR_113904 NR_113257	+	+	−	−	−
	A39	*Enterococcus faecium*	99.34%	NR_114742	+	+	−	−	−
	A40	*Enterococcus pingfangensis* *Enterococcus xiangfangensis*	100.00%	NR_174226 NR_133741	++	+	−	−	−
	A41	*Enterococcus faecium*	97.01%	NR_042054	++	+	−	−	−
	A42	*Enterococcus faecium*	97.10%	NR_114742	++	+	−	−	−
	A43	*Enterococcus lactis*	99.33%	NR_117,562	++	+	−	−	−
Brine (A)	A44	*Lactococcus lactis*	99.48%	NR_040955	+	++	−	−	−
	A45	*Lactococcus lactis*	98.52%	NR_040955	+	++	−	−	−
	A46	*Enterococcus pseudoavium* *Enterococcus devriesei*	98.93%	NR_115762 NR_042389	++	++	−	−	−
	A47	*Lactococcus lactis*	99.39%	NR_040955	++	+	−	−	−
	A48	*Lactococcus lactis*	98.68%	NR_040955	++	+	−	−	−
	A49	*Lacticaseibacillus paracasei* subsp. *tolerans*	99.00%	NR_113823	++	+	−	−	−
	A50	*Lacticaseibacillus paracasei* subsp. *tolerans*	99.31%	NR_113823	++	+	−	−	−

[1] Percentage of identical nucleotides in the sequence obtained from the bacterial isolates and the sequence of the closest relative found in the GenBank database.

[2] Accession number of the sequence of the closest relative found by BLAST search.

[*] -, negative; +, positive (1 mm halo); ++, positive (1–2 mm halo); +++, positive (> 2 mm halo).

[**] M, mucoid colonies.

et al., 2025), thereby enhancing the sensory profile of the final product. For the two *L. cremoris* isolates, only protease activity was detected, while lipase activity and EPS production were not observed. Of note, proteolytic strains of *L. cremoris* used as adjunct cultures in experimental cheese production resulted in increased levels of free amino acids such as glutamic acid, asparagine, serine, glutamine, and citrulline as well as a firmer cheese structure (Børsting et al., 2015).

Only three isolates closely related to *L. mesenteroides* were recovered. Consistent with this finding, Colombo et al. (2010) also reported isolating only a limited number of strains of this heterofermentative species from artisanal Italian goat cheese, confirming its sporadic yet recurring presence in traditional dairy products. None of the isolates examined in the present study showed lipolytic activity, whereas two out of the three isolates exhibited protease activity. Notably, proteolytic *L. mesenteroides* strains have also been documented by Bonomo and Salzano (2013) in Pecorino di Filiano cheese. The marked proteolytic potential of leuconostocs contributes to the production of metabolites such as lactate, acetaldehyde, and acetate, traits that support their use as non-starter LAB adjunct cultures (Sharma et al., 2024). Moreover, two out of the three *L. mesenteroides* isolates produced mucoid colonies, indicating exopolysaccharide (EPS) synthesis, a characteristic commonly associated with this species, which is known to generate alternan, fructans, dextran, and glucan- or levan-type EPS (Sharma et al., 2024).

Although information on *C. gallinarum* remains limited, this lactic acid bacterium has been previously reported in meat and fish (Leisner et al., 2007). Its detection in the analyzed samples could therefore be linked to cross-contamination. Nonetheless, the metataxonomic analysis revealed the presence of Carnobacteriaceae ASVs, suggesting that this family may play a more substantial role in the cheese ecosystem and warranting further investigation to clarify its contribution within the cheese matrix. In the present study, the isolate exhibited both lipase and protease activities, indicating a potential influence on the cheese's sensory attributes. In addition, its colonies displayed a mucoid appearance when grown on MRS agar supplemented with sucrose, suggesting the production of HoPS.

Among Carnobacteriaceae, also five cultures closely related to *M. psychrotolerans* were recovered from brine and cheese samples. This species of non-starter lactic acid bacteria has previously been isolated from surface-ripened soft cheese by Suzuki et al. (2021) and from Raclette-type cheese by Roth et al. (2011). Notably*, M. psychrotolerans* is a halophilic and alkaliphilic lactic acid bacterium well adapted to saline environments (Ishikawa et al., 2003), which explains its presence in the brine and brined cheese examined in this study. Interestingly, *M. psychrotolerans* also exhibited inhibitory activity against *L. monocytogenes* (Montel et al., 2014), indicating that further characterization of the isolates for potential bacteriocin production is warranted. In the present study, one out of the five isolates exhibited lipase activity, whereas four out of the five *M. psychrotolerans* isolates tested positive for protease activity. To the authors' knowledge, the limited scientific literature prevents further comparison of these characteristics; however, the present study contributes to the current understanding of the enzymatic activities of *M. psychrotolerans*. No EPS production was observed for any of the isolates.

The *L. plantarum* group comprises species with strong genetic and phenotypic similarity, including *L. plantarum* subsp. *plantarum*, *L. plantarum* subsp. *argentoratensis*, *L. paraplantarum*, and *L. pentosus* (Kim et al., 2021). In the present study, four isolates closely related to the *L. plantarum* group were obtained. Members of this non-starter lactic acid bacteria group have previously been recovered from farmhouse goat's milk cheeses (Lavilla-Lerma et al., 2013). In the present study, only one out of the four isolates exhibited lipase activity, in agreement with observations reported by Uppada et al. (2017). All isolates within the *L. plantarum* group showed protease activity, which may enhance and intensify cheese flavor through the release of volatile compounds and free amino acids (Şahingil et al., 2026).

In the present study, only one closest relative to *C. alimentarius* was unambiguously identified. This species has already been isolated by Esen and Çetin (2021) from ripened traditional middle east surk cheese, a skim-milk cheese consumed in the eastern Mediterranean, covering regions of Turkey, Syria, Lebanon, and the broader Middle East. The *C. alimentarius* isolate in this study showed lipase activity, consistent with the findings of Di Cagno et al. (2006), who reported esterase activity for the same microorganism when used as an adjunct culture in miniature Caciotta cheese. Those authors also documented aminopeptidase activity (Di Cagno et al., 2006), which aligns with the protease activity observed in the isolate examined here. No EPS production was detected for this isolate. Notably, the *C. alimentarius* culture examined was the only one to test positive for the *hdcA* gene, which excludes its potential use as an adjunct culture for safety reasons.

Finally, two isolates closely related to *L. paracasei* subsp. *tolerans* were recovered from brine. *L. paracasei* has previously been reported as one of the predominant lactic acid bacteria in traditional fermented dairy products of Central Asia, such as shubat, chal, and khoormog (Konuspayeva et al., 2023). Both isolates exhibited lipase activity, although information on the lipolytic potential of *L. paracasei* subsp. *tolerans* is limited, making direct comparisons difficult. Nonetheless, this activity may contribute positively to cheese sensory qualities. Regarding protease, both isolates tested positive for this enzymatic activity, consistent with the high proteolytic capacity reported for *L. paracasei* subsp. *tolerans* in yogurt by Özdemir (2023). No EPS production was detected in all the isolates.

With regard to *hdcA* detection, apart from the *C. alimentarius* isolate, none of the cultures obtained from environmental, milk, cheese, or brine samples tested positive for this gene, thereby supporting their suitability for use as potential starter or adjunct cultures.

4. Conclusions

This study provides the first integrated, season-resolved characterization of the physico-chemical traits, morpho-textural properties, and, most importantly, the complex microbial ecology of a traditional Macedonian raw goat's milk brined cheese.

Overall, the physico-chemical results highlighted clear seasonal differences, with autumn-produced cheeses showing higher salt content, lower a_w, and increased protein concentration compared with spring cheeses. These variations likely reflect season-dependent changes in milk composition and processing conditions, confirming the relevance of seasonality as a key factor influencing the chemical matrix of traditional brined goat's milk cheese.

Morpho-textural analyses revealed a clear seasonal pattern, with cohesiveness and springiness being significantly affected by the production period. These differences are likely associated with season-dependent variations in the cheese matrix and ripening dynamics, further supporting the influence of seasonality on the textural identity of traditional brined goat's milk cheese.

By combining culture-dependent methods, high-resolution metataxonomics, and an extensive phenotypic assessment of isolates, the work reveals a microbial diversity that had not been previously documented for this product. Spring cheeses exhibited a microbial profile enriched in Carnobacteriaceae, *Enterococcus*, *Serratia*, and *T. halophilus*, whereas the autumn samples were instead marked by the predominance of *Companilactobacillus* and *Lactococcus*, together with several members of the Enterobacteriaceae family. The clear separation between spring and autumn microbiota, as well as the emergence of unusual dairy-associated taxa such as Amphibacillaceae, *T. halophilus*, and *Companilactobacillus*, highlights a new ecological landscape shaped by artisanal practices, raw milk variability, and local environmental conditions.

A key innovative contribution of this study is the comprehensive isolation and characterization of 134 lactic acid bacteria from milk, cheese, brine, and the dairy environment. Evaluating their proteolytic and lipolytic activities, exopolysaccharide production, and, critically, their safety profile through *hdcA* gene screening provided fundamental insights into the functional roles these microorganisms play in acidification, ripening, and flavor development.

A key finding of this study is the remarkable diversity and persistence of *Enterococcus* species across milk, cheese, brine, and the dairy environment. Multiple taxa including *E. faecium*, *E. faecalis*, *E. durans*, *E. lactis*, *E. italicus*, and *E. mundtii* formed a stable component of the microbial ecosystem, reflecting both raw milk ecology and continuous transfer from artisanal processing surfaces. Despite their ambivalent reputation, these isolates displayed relevant technological traits.

Identifying autochthonous strains with desirable technological properties and no histamine-forming potential offers a robust foundation for future selection of pro-technological microorganisms that can valorize and partially standardize traditional products while preserving their territorial identity.

Overall, the findings demonstrate that a full understanding of artisanal cheese microbiomes requires integrating ecological and culture-based approaches, and they reaffirm the central importance of isolate-level characterization in transforming microbial biodiversity into practical knowledge for improving quality, safety, and valorization of traditional dairy products.

However, the lack of a complete macronutrient profile represents limitation that should be addressed in future studies. Further research integrating extended compositional analyses, multi-season and multi-dairy surveys, and functional genomics approaches would be valuable to better elucidate the mechanisms driving seasonal variability and to support the optimization and valorization of traditional artisanal cheeses.

CRediT authorship contribution statement

Hava Miftari: Investigation, Formal analysis. **Daniela Nikolovska Nedelkoska:** Writing – original draft, Resources, Formal analysis, Conceptualization. **Giorgia Rampanti:** Writing – original draft, Investigation, Formal analysis. **Joanna Harasym:** Investigation. **Ilario Ferrocino:** Formal analysis. **Ismail Ferati:** Investigation. **Federica Cardinali:** Investigation, Formal analysis. **Agnieszka Orkusz:** Investigation. **Vesna Milanović:** Resources, Investigation, Formal analysis. **Irene Franciosa:** Formal analysis. **Cristiana Garofalo:** Resources, Formal analysis. **Lucia Aquilanti:** Resources, Formal analysis. **Andrea Osimani:** Writing – review & editing, Writing – original draft, Supervision, Resources, Conceptualization.

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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2026.118806.

Data availability

Data will be made available on request.

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