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Influence of the Mefite D'Ansanto Valley on the chemical characteristics and microbial consortia of raw sheep milk for the production of Pecorino Carmasciano cheese

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ABSTRACT

Pecorino Carmasciano cheese is produced from the milk of sheep that graze in a unique environment called Mefite d'Ansanto, a non-volcanic site located in the Ansanto Valley, in the province of Avellino (Campania, Italy). In order to provide new insights into the determinants of the distinctive quality of ewe milk used for the production of Pecorino Carmasciano cheese, we examined the chemical characteristics of the milk and evaluated the correlation with the bacterial composition, analysed using a high-throughput sequencing approach. The milk was analysed and compared with that belonging to sheep from other provinces of the Campania region (Caserta and Salerno). Milk from sheep grazing in the Ansanto Valley (Avellino province) especially showed significantly higher concentrations of proteins, iron, potassium and zinc compared to the other sheep milk studied (from Caserta and Salerno provinces). The pipelines used for metagenomic classification highlighted the predominant *phyla* in milk from the different provinces, with Proteobacteria as the dominant *phylum*, followed by Firmicutes and Bacteroides. Metagenomic analysis revealed however a greater diversity of families, genera and species in the milk from Avellino than in those from Caserta and Salerno. The statistical analysis of the data acquired on raw milk samples (physico-chemical, microbiological and amino acid composition) showed that the presence of metals had an impact on the higher number of species detected in milk from Avellino. Moreover, peculiar bacterial species (*Anoxybacillus kestanbolensis*, *Petrobacter succinatimandens* and *Thermoanaerobacterium saccharolyticum*), appeared related to the specific area of the Mefite.

1. Introduction

Over the past 20 years, several researchers have studied the characteristics of a particular pecorino cheese, named "Carmasciano" from its production area. In fact, Carmasciano is a small district located in Alta Irpinia, in the province of Avellino (Italy), and the interest in this cheese (Coppola et al., 2006; Marrone et al., 2014; Mercogliano et al., 2010; Ricciardi et al., 2014) is linked to the fact that sheep graze largely on Mefite D'Ansanto Valley, considered as the largest non-volcanic environment on the Earth featured by natural emission of low temperature CO_2 rich gases, followed by minor amounts of non-atmospheric N_2, H_2S and CH_4 (Chiodini et al., 2010). Pecorino Carmasciano cheese, made from ewe raw milk, is characterised by strong flavour and taste due to the use of lamb past chyimosin, and by a slight piquant flavour that appears during ripening.

Due to the peculiar geomorphology of Mefite, several authors have tried to find specific markers that can correlate the characteristics of this particular site with ewe milk and cheese produced here. Cozzolino et al. (2021) assessed the presence of more than 80 volatile organic compounds (VOCs) in samples of Pecorino Carmasciano, but there were no sulphur compounds among them, and statistical analysis revealed no significant differences in VOCs compared to other Italian pecorino

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cheeses. Mercogliano et al. (2010) evaluated the production of vasoactive amines in Pecorino Carmasciano due to the activity of microbial amino acid decarboxylase (Colautti et al., 2024), but they found the typical changes occurring during the ripening of other pecorino cheeses. Marrone et al. (2014) studied the fatty acid profile and cholesterol content of Pecorino Carmasciano during the ripening. They concluded that the relatively high level of polyunsaturated fatty acids, the ratio of n-6 to n-3 fatty acids, and the low cholesterol content, together with a good presence of conjugated linoleic acid, make Pecorino Carmasciano a case for consideration by the Council of the European Union's Regulation for the awarding of a Protected Designation of Origin (PDO).

With regard to the microbial composition of Carmasciano milk and cheese, so far mainly culture-dependent approaches have been used. Coppola et al. (2006) reported the presence of both non-lactic and lactic acid bacteria (LAB), with the highest counts registered in cheeses between 3 and 21 d of ripening for all searched microbial groups. Ricciardi et al. (2014) used a PCR-DGGE based approach for the identification of predominant LAB strains after simulated digestion of Pecorino Carmasciano cheese, concluding that 1 g of fresh Carmasciano cheese can supply the human colon with approximately the same amount of vital LAB as a probiotic drink.

The investigation of microbial populations using High Throughput Sequencing (HTS) is often chosen for its potential to show how the microbiome of milk and cheese can influence the ripening process and the sensory characteristics of cheese, suggesting its application as an additional tool to identify the key microbial species that contribute to PDO designation (Kamilari et al., 2019). For instance, De Pasquale et al. (2016) correlated VOCs with the metabolically active microbioma in three Italian PDO ewes milk cheeses (Fiore Sardo, Pecorino Siciliano and Pecorino Toscano) using pyrosequencing of the 16S rRNA targeting RNA. In the work by Gonçalves Dos Santos et al. (2017), authors studied the fungal communities of Serpa, an artisanal PDO pecorino cheese from Portugal. The HTS analysis of fungal communities in both PDO and non-PDO Serpa cheeses highlighted that production practices and the environment have a significant influence on the final yeast population.

To the best of our knowledge, to date no research investigated the microbiome of milk used to produce Pecorino Carmasciano cheese by animal grazing on Mefite D'Ansanto. Instead, a recent study (De Castro et al., 2024) profiled the diversity of microorganisms residing in the Mefite D'Ansanto through an HTS study of 16S rRNA and ITS2, showing that biogeochemical characteristics of this unique environment, such as pH, temperature, salts, minerals and organic compounds, significantly influence the composition of microbial communities.

In this study, we obtained new information on factors affecting Pecorino Carmasciano tipicity by investigating some chemical features of ewe milk for its production, and the possible relationship between these features and bacterial composition, studied through amplicons sequencing of the 16S rRNA region using an HTS approach.

2. Materials and methods

2.1. Milk sample collection

Ewe milk samples were obtained from 90 animals, 30 from each of three different farms in the Campania region located in Avellino (AV), Caserta (CE) and Salerno (SA) provinces. The flocks were all composed of different breed animals (Sarda, Lacaune, and, to a minor extent, Bagnolese and Laticauda). To minimise differences, the ewes from which the milk was collected were all of the Lacaune breed (which was the prevalent one), of the same age, free of disease and lambed for about two months. Milk was collected in June from pasture-fed animals, and the pasture in the province of Avellino was situated predominantly in the area of Mefite D'Ansanto. The bulk milk from each farm was transferred into sterile containers and sent to the laboratory of Food Microbiology - University of Molise - in refrigerated conditions. This procedure was repeated twice more within a week, for a total of three milk samples per farm.

2.2. Physico-chemical and microbiological analyses of milk

Fat, lactose, protein, urea and dry matter in milk were determined by mid infrared spectrometry (Method ISO 9622:2013, IDF 141:2013). Total microbial count at 30 °C for 72 h (Method ISO 4833–1:2013 and ISO 7218:2007/Amd 1:2013), somatic cells (Method ISO 13366–2:2006, IDF 148–2:2006), acidity (ISTSAN Report 96/34, p. 229), freezing point (Method ISO 5764|IDF 108:2009) and antibiotics (Method AOAC DSM 930704 1993) were also determined.

2.3. Amino acid analysis

All the chemicals were purchased from Merck (Merck KGaA, Darmstadt, Germany). A 24-h hydrolysis of the proteins with 6 mol/L HCl was performed, followed by cooling, filtration, removal of the concentrated acid with a rotary evaporator, and resuspension of the sample in 0.1 mol/L HCl. Amino acid determination was performed with High-Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD) ICS6000 (Thermo Fisher Scientific, Italy). A Rheodyne injector (Cotati, CA, USA) with a 25 μL loop was used for sample injection. Separation was performed with a 250 mm × 2 mm Aminopac PA 10 analytical column with a particle size of 8.5 μm (Dionex, Thermo Fisher Scientific, Italy). The chromatographic run was performed at a flow rate of 0.25 mL/min, using a water gradient mobile phase, 0.25 mol/L NaOH and 1.0 mol/L CH_3COONa. Instrument control, data collection and total quantification were performed using Chromeleon chromatographic software (Dionex, Thermo Fisher Scientific, Italy). Results were expressed as g/100g protein.

2.4. Detection of mineral content in milk

Calcium (Ca), Iron (Fe), Manganese (Mn), Nickel (Ni), Potassium (K), Copper (Cu), Sodium (Na), Strontium (Sr) and Zinc (Zn) were determined according to the method ISO 21424:2018 on milk samples. Analysis was performed by Inductively Coupled Plasma (ICP)-MS using an Agilent 7800 ICP-MS equipped with autosampler and Integrated Sample Introduction System. A multi-element stock standard solution (Multi-Element Standard) was purchased from the manufacturer. Briefly, aliquots of 0.5 g of each sample were added with 2 mL concentrated HNO_3 into cleaned digestion tube (Pyrex 50 mL culture tubes). For digestion, tubes were left overnight at room temperature (25 °C), followed by a digestion at 120 °C until the liquid dried out. Two additional mL of HNO_3 was added and heated to dryness, this step was performed until samples resulted clear. Additional digestion was done by adding 1 mL solution of HNO_3 and $HClO_4$ (50:50) and leaving the tubes for 2 h at 180 °C. The temperature was increased to 220 °C and the solution was heated to dryness. Tubes were then cooled to room temperature (25 °C), and the ash obtained was dissolved in 1 mL concentrated HCl and 10 mL of 5% HNO_3. Samples were injected into ICP-MS (Agilent 7800) to determine the mineral content. Minerals were expressed in ppm (mg/Kg). Analyses were repeated in triplicate for each sample.

2.5. Metagenomic analysis

Total genomic DNA from the samples was extracted using the "Stool DNA Isolation Kit" (Norgen, Biotek Corp., Thorold, ON, Canada) according to the manufacturer's instructions. DNA, extracted from 200 μL of homogenised sample, was standardised at a concentration of 10 ng/μl for metagenomic sequencing. Bacterial communities were accessed by sequencing the V3-V4 region of 16S rRNA amplified with universal primers 341F (5′-CCTACGGGNBGCASCAG-3′) and 805R (5′-GACTACNVGGGTATCTAATCC-3′) (Herlemann et al., 2011; Klindworth et al., 2013; Takahashi et al., 2014). Amplicons were sequenced using the Illumina MiSeq Paired-End (PE) 300 platform (Illumina, San Diego, CA, USA) performed through an external service (Biodiversa s. r.l., Rovereto, Italy). Raw PE reads were analysed using the QIIME2 (v. 2020.8) pipeline and DADA2 (v. 1.16). For the quality control of reads, the quality score value of 20 was used as a threshold and the low-quality regions of sequences were removed. ASVs were classified using the DADA2 and QIIME2 naïve Bayes classifier (qiime feature-classifier fit--classifier-naive-bayes), trained on the GreenGenes database (v. 13_8) (McDonald et al., 2012).

2.6. Statistical analysis

All the data are expressed as the mean ± standard deviation (SD) obtained from three distinctive trials. Data were elaborated with SPSS (IBM SPSS Statistics, Version 2019) by using ANOVA coupled with the Tukey HSD post-hoc test. The significance level for the statistical tests was set to an an of 0.05. Principal Component Analysis (PCA) was performed on microbiological, chemical and physico-chemical data to discriminate the variables between the different milk samples. For this purpose, the FactoMineR and factoextra R (v4.3.2) packages were used. The corrr R package was used to calculate the Pearson's correlation coefficient (r) between variables. The unique microbial species for the sheep milk samples were analysed with R software and represented using the Evenn platform (Yang et al., 2024).

3. Results and discussion

3.1. Physico-chemical characteristics of milk

The values obtained from the composition of sheep milk (Table 1) showed the good quality of the three milks under analysis, with parameters in agreement with those recorded by other authors who have studied sheep milk from Campania region (Landi et al., 2021), even if fat and protein content detected in our study resulted higher than those reported in other works (Serrapica et al., 2020). These differences are, however, to be considered normal, since milk characteristics vary considerably depending on several factors, such as season, lactation, feed, management system and breed. Specifically, ewes selected for this study were mainly of Lacaune breed in the three flocks located in AV, CE and SA provinces of the Campania region; this choice was made to reduce the variability within milk samples, selecting animals of the same age, free of disease and lambed for about two months. For this reason, the different milks had a similar composition, reflecting the chemical and microbiological features recorded in Lacaune sheep milk (Pulina et al., 2005; Castillo et al., 2008), with little, physiological, variation among samples. Significant differences among all samples were found for Urea and Somatic Cells (SC), both likely affected by the ewe diet (Pulina et al., 2006) attributable to the different pastures. Differences were also highlighted for milk protein, the amount of which can be influenced by the dietary energy concentration and milk yield (Pulina et al., 2005, 2006), and for acidity, which is mainly influenced by hygienic and climatic conditions, especially temperature (Pavić et al., 2002). Values recorded were, however, in the ranges usually detected in sheep milk. The absence of antibiotic underlined the good health status of the animals and, as a consequence, the good healthy properties of milk produced in Campania region.

Table 1

Chemical and microbiological characteristics of ewe milk from Avellino (M_Av), Caserta (M_Ce) and Salerno (M_Sa) provinces. Values are reported as average of three determinations and ±standard deviation (SD). Letters are significant differeces ($p < 0.05$) within each row.

	M_Av	M_Ce	M_Sa
UREA (mg/100 mL)	37.70 ± 0.36^{a}	43.07 ± 0.66^{b}	40.73 ± 0.15^{c}
Fat (%w/w)	7.15 ± 0.15^{a}	7.15 ± 0.13^{a}	7.20 ± 0.10^{a}
Proteins (%w/w)	5.72 ± 0.12^{b}	$5.34 \pm 0.12^{a.b}$	5.15 ± 0.11^{a}
Lactose (%w/w)	4.26 ± 0.04^{a}	4.41 ± 0.09^{a}	4.32 ± 0.10^{a}
Dry matter (%w/w)	10.77 ± 0.20^{a}	10.45 ± 0.11^{a}	10.17 ± 0.13^{a}
Acidity (°SH)	7.97 ± 0.12^{a}	8.53 ± 0.08^{b}	8.51 ± 0.10^{b}
Somatic cells (cells/ml)	448400 ± 3143^{b}	672666 ± 4163^{c}	213900 ± 16703^{a}
Total microbial count (Log CFU/ml)	7.84 ± 0.45^{a}	8.40 ± 0.25^{a}	7.98 ± 0.17^{a}
Freezing point (°C)	-0.55 ± 0.01^{a}	-0.55 ± 0.01^{a}	-0.56 ± 0.01^{a}
Antibiotics (presence/ absence)	absence	absence	absence

The amino acids in the milk samples are reported in Table 2. No statistical differences were found for Lysine (Lys), Glutamine (Gln), L-Arginine (Arg), Threonine (Thr), Glycine (Gly) and Serine (Ser) in the three analysed milk samples, whereas quantitative differences were found for the other amino acids. Glutamic acid (Glu) was the most abundant one (about 19.12%, 19.27% and 19.38% in M_Av, M_Ce and M_Sa samples, respectively) followed by, Proline (Pro) (about 10.12%, 9.63% and 9.73% in M_Av, M_Ce and M_Sa samples, respectively), Leucine (Leu) (9.35%, 9.12% and 9.16% in M_Av, M_Ce and M_Sa samples, respectively) and Lys (about 7.73%, 7.75% and 7.76% in M_Av, M_Ce and M_Sa, respectively). In all cases, values detected in our study were in line with those reported by other authors for ewe milk (Eluu et al., 2024; Hejtmánková et al., 2012; Landi et al., 2021; Milan et al., 2020).

Noteworthy, sulphur-containing amino acids Methionine (Met) and Cystine (Cys) were lower in milk M_Av (about 2.4% and 0.6%, respectively) than in the other samples (M_Ce and M_Sa). This datum is particularly intriguing considering the environment (Mefite D'Ansanto) in which sheep from the province of Avellino graze. In fact, SO_2 gas emissions characterise the site (giving it a persistent sulphur smell), and together with CO_2 emissions, are able to impose a strong selection pressure on all organisms inhabiting it (Di Iorio et al., 2023). In this context, it could be of interest the evaluation of Met and Cys in wool and skin of sheep grazing on the Mefite, since Pisulewski and Buttery (1985) showed a compartmentalization of these amino acids within individual tissues, especially the skin, with effects on wool growth (Liu & Masters, 2000).

The presence of metals in ewe milk samples is reported in Table 3. Only Ca, Fe, K, Na and Zn were detected in all samples, whereas Mn, Cu and Ni were detected in traces (quantification level <0.05 mg/kg). In all mammals, Mn, Cu and Ni are considered as micro-minerals that are required in small quantities for proper body functions. Data reported in the literature highlighted a significant variability of the content of these

Table 2

Amino acid composition (g/100g protein) of ewe milk from Avellino (M_Av), Caserta (M_Ce) and Salerno (M_Sa) provinces. Values are reported as average of three determinations and ± standard deviation (SD). Letters are significant differeces ($p < 0.05$) within each row.

Amino acid	M_Av	M_Ce	M_Sa
Lys	7.726 ± 0.024^{a}	7.746 ± 0.033^{a}	7.759 ± 0.024^{a}
Gln	1.156 ± 0.042^{a}	1.151 ± 0.023^{a}	1.179 ± 0.076^{a}
Arg	3.504 ± 0.010^{a}	3.493 ± 0.011^{a}	3.497 ± 0.009^{a}
Ala	4.740 ± 0.087^{b}	4.393 ± 0.045^{a}	$4.527 \pm 0.036^{a,b}$
Thr	4.715 ± 0.060^{a}	4.514 ± 0.061^{a}	4.525 ± 0.053^{a}
Gly	1.766 ± 0.012^{a}	1.755 ± 0.033^{a}	1.793 ± 0.022^{a}
Val	6.965 ± 0.052^{b}	6.373 ± 0.075^{a}	6.546 ± 0.064^{a}
Ser	5.569 ± 0.116^{a}	5.985 ± 0.122^{a}	5.974 ± 0.112^{a}
Pro	10.124 ± 0.029^{b}	9.629 ± 0.029^{a}	9.734 ± 0.029^{a}
Ile	4.954 ± 0.012^{c}	4.481 ± 0.017^{a}	4.777 ± 0.012^{b}
Leu	9.352 ± 0.023^{b}	9.123 ± 0.019^{a}	9.160 ± 0.014^{a}
Met	2.379 ± 0.017^{a}	2.553 ± 0.017^{b}	2.507 ± 0.018^{b}
His	2.553 ± 0.068^{a}	2.842 ± 0.044^{b}	2.585 ± 0.051^{a}
Phe	4.465 ± 0.023^{b}	4.142 ± 0.023^{a}	4.224 ± 0.024^{a}
Glu	19.116 ± 0.010^{a}	$19.272 \pm 0,017^{b}$	19.377 ± 0.015^{c}
Asp	7.401 ± 0.043^{a}	7.654 ± 0.026^{b}	7.514 ± 0.010^{a}
Cys	0.613 ± 0.012^{a}	0.678 ± 0.011^{b}	0.709 ± 0.013^{b}
Tyr	4.938 ± 0.022^{a}	5.619 ± 0.026^{c}	5.444 ± 0.019^{b}

Table 3
Presence of minerals in ewe milk from Avellino (M_Av), Caserta (M_Ce) and Salerno (M_Sa) provinces. Values (mg/Kg) are reported as average of three determinations and ± standard deviation (SD). Letters are significant differeces ($p < 0.05$) within each row.

	M_Av	M_Ce	M_Sa
Ca	1816.39 ± 33.20^{a}	1792.92 ± 34.58^{a}	2020.50 ± 27.05^{b}
Fe	1.17 ± 0.08^{b}	0.75 ± 0.05^{a}	0.54 ± 0.06^{a}
Mn	Trace	Trace	Trace
Ni	Trace	Trace	Trace
K	2112.41 ± 25.01^{c}	1402.34 ± 27.70^{a}	1523.73 ± 23.20^{b}
Cu	Trace	Trace	Trace
Na	467.43 ± 12.14^{a}	768.76 ± 13.69^{c}	603.68 ± 14.30^{b}
Sr	Trace	Trace	2.11 ± 0.09
Zn	8.36 ± 0.30^{b}	5.81 ± 0.41^{a}	3.97 ± 0.70^{a}

minerals in sheep milk, depending on region, physiological state and type of feed (Caraba & Caraba, 2023; Park et al., 2007). Higher values than those recognised as normal in ewe milk (Antunović et al., 2023; Moniello et al., 2005) are generally considered an indicator of environmental pollution (Sharma et al., 2024).

Mean concentration of Sr in milk from the Salerno province (M_Sa) was 2.11 mg/kg, while traces were detected in M_Av and M_Ce. The presence of this element especially in M_Sa is likely related to the geographical place of sampling, as other authors have found its presence in sheep milk with marked variability depending on the sampling location (Amalfitano et al., 2024; Miedico et al., 2016).

Milk from Avellino province (M_Av) contained the highest amount of K, Zn and Fe. Plants are a rich source of K, so its deficiency is rare in herbivores (Calhoun & Shelton, 1983). In fact, K value was in the normal range (about 0.15% in milk) in samples M_Ce and M_Sa, whereas a significantly higher value was detected in the sample M_Av. Zn content in milk is also related to plant bioavailability, which in turn depends on soil content (Martino et al., 2019). Our data suggest that all the three locations where sheep routinely graze (provinces of AV, CE and SA) do not suffer from soil Zn deficiency. Noteworhy, M_Av had a Zn content significantly higher than that detected in samples M_Ce and M_Sa. In this regard, De Castro et al. (2024) described the metal content at the Mefite D'Ansanto site, showing that the highest concentration is that of Alluminium, followed by Fe, but a discrete presence of Zn has also been described. Studies concerning similar environments also reported elevated enrichments in metals, including Zn and Fe (Nuccio, 2016).

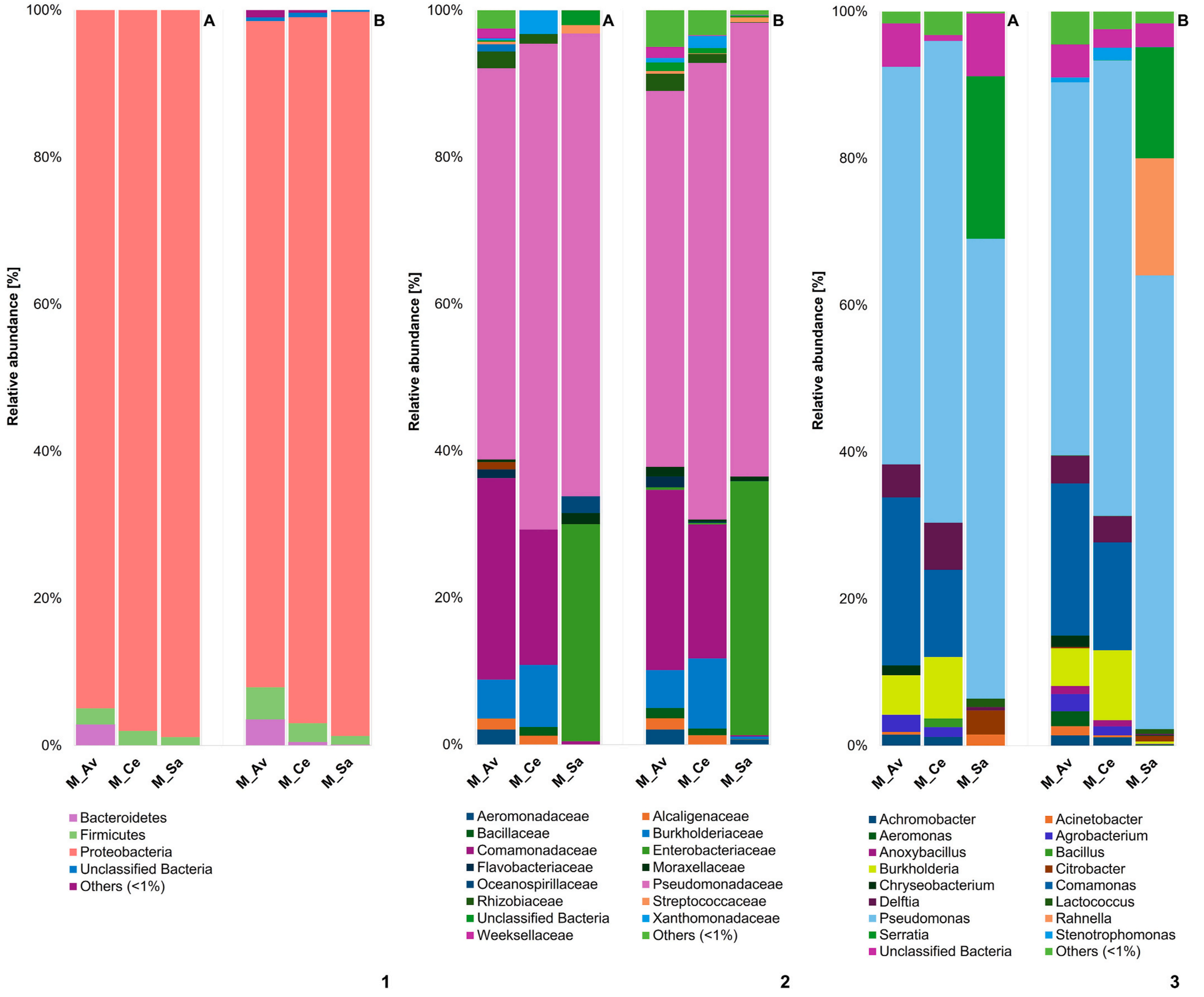

Fig. 1. Distribution of relative abundance of taxa at phylum (Panel 1), Family (Panel 2) and genus (Panel 3) level. Taxonomic analysis was performed with DADA2 (A) and QIIME2 (B). The group "Others (<1%)" indicates a relative abundance <1%.

With regard to the latter mineral, analyses conducted on the blood plasma of grazing or fed sheep have shown that Fe deficiency is uncommon (Hidiroglou, 1979). However, milk may have a low Fe content even in healthy animals (Moniello et al., 2005). In our study, the Fe values found in M_Av were not only higher than those found in samples M_Ce and M_Sa, but also than those reported in sheep milk analysed by other authors (Raynal-Ljutovac et al., 2008; Siddiqui et al., 2023; Yabrir et al., 2014). This finding is particularly interesting given the concomitant high Fe concentration found in the Mefite soil (De Castro et al., 2024), given that most of the nutrients needed by the animals come from the plants via the soil (Shukla et al., 2016) or directly from ingestion of soil by grazing sheep (Stewart et al., 2021), thus influencing the composition of the milk.

3.2. NGS analysis

The taxonomic analysis of sheep milk samples was carried out with two different pipelines to avoid problems of methodological bias and to obtain as many unique species as possible. As shown in Fig. 1, the analysis with QIIME2 returned more *phyla*, classes, orders, families and genera than DADA2 (16 *phyla*, 23 classes, 35 orders, 64 families and 83 genera detected with QIIME2 *vs* 3 *phyla*, 8 classes, 14 orders, 18 families and 20 genera detected with DADA2). Specifically, QIIME2 facilitated the identification of a greater number of bacterial classes, orders, and genera compared to DADA2. This phenomenon may be due to the inclusion of additional steps and analytical methods within the QIIME2 pipeline, despite the potential overlap in denoising results between the direct application of DADA2 and its integration within the QIIME framework. Data analysis showed that Proteobacteria were the predominant *phylum* in all sheep milk samples, followed by Firmicutes and Bacteroides. Proteobacteria showed average relative abundance (ARA) ranging from 94.95% to 98.85% using DADA2 and from 90.95% to 98.48% using QIIME2. Similarly, Firmicutes showed ARA ranging from 1.15% to 2.19% and 1.17%–4.38% using DADA2 and QIIME2 respectively. Bacteroides were detected only in M_Av with DADA2 whereas ranged between 0.12% and 3.54% in all samples with QIIME2.

The data obtained are consistent with those reported in the literature, according to which Proteobacteria is the dominant *phylum* in raw milk (Endres et al., 2021; Fuka et al., 2013; Guo et al., 2023; Yan et al., 2022; Yuan et al., 2022). With regard to the results at the family and genus level, QIIME2 performed again better than DADA2. In particular, a total of 56, 44 and 24 families were identified in M_Av, M_Ce and M_Sa, respectively, and 60, 47 and 23 genera in M_Av, M_Ce and M_Sa, respectively.

In a large study comprising 142 samples of raw milk from China, *Pseudomonas*, *Acinetobacter*, *Streptococcus* and *Lactobacillus* were detected as the most common genera using 16S rRNA amplicon sequencing (Yuan et al., 2022). Indeed, in our study *Acinetobacter*, *Streptococcus* and *Lactobacillus* were detected with very low abundance, whereas *Pseudomonas* spp. was present with abundance between 48.65% and 57.72% in all the samples. In the study of Santamarina-García et al. (2024), *Escherichia* dominated in raw sheep milk, followed by *Enterococcus* and *Lactococcus*, while Esteban-Blanco et al. (2020) showed *Staphylococcus*, *Cutibacterium*, *Corynebacterium Streptococcus*, *Massilia* and *Bacillus* as the predominant genera in Churra sheep milk.

At the species level, only 5 species had an ARA above 1% at least in one milk sample (Fig. 2, Panel 1). Other 29 species were present below 1% in all the milk samples (Fig. 2, Panel 2), collectively representing about 3% in the M_Av sample (pipeline QIIME2) and about 1% in the M_Ce and M_Sa samples (both pipelines DADA2 and QIIME2).

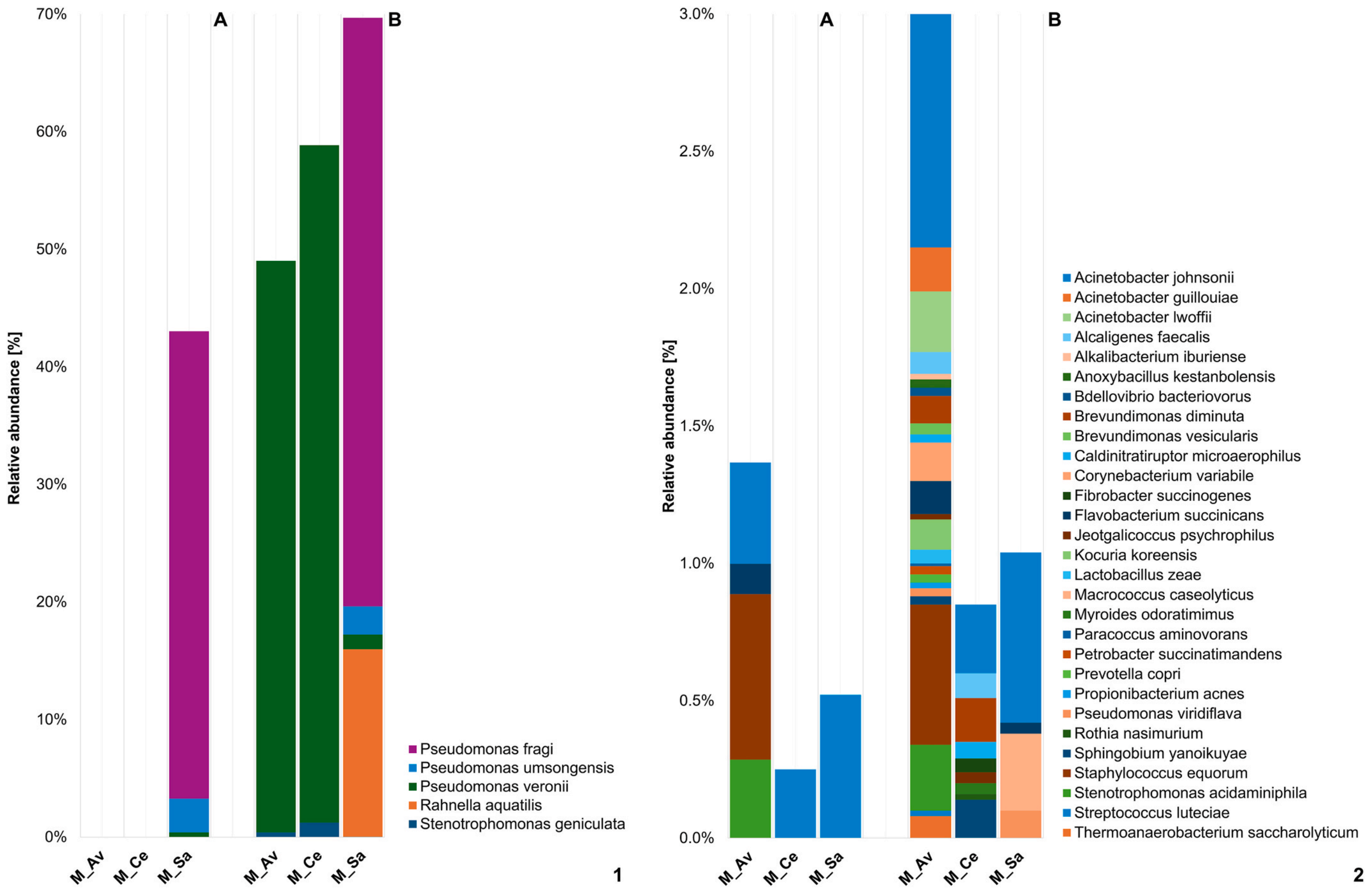

Fig. 2. Distribution of relative abundance (Panel 1, ARA >1% in at least one sample; Panel 2, ARA <1%) of taxa at species level. Taxonomic analysis was performed with DADA2 (A) and QIIME2 (B).

Specifically, based on the QIIME2 pipeline, the predominant species in the M_Av and M_Ce samples was *Pseudomonas veronii* (ARA of 48.62 and 57.62%, respectively). *Pseudomonas fragi* dominated in the sample M_Sa (50.07%) followed by *Rahanella aquatilis* (15.95%), while *Pseudomonas veronii* accounted only for 1.25%. Furthermore, *Stenotrophomonas geniculata* was observed in sample M_Ce with an ARA >1% (1.23%), while lower ARA were detected in samples M_Av and M_Sa. *Pseudomonas umsongensis* showed ARA >1% only in the sample M_Sa (2.39%). With regard to the analysis performed with DADA2, fewer species were detected than with QIIME2, and those in common had lower ARA, except for *Pseudomonas umsongensis* in the sample M_Sa (ARA of 2.86% and 2.39% as detected with DADA2 and QIIME2, respectively).

Considering the species with ARA <1%, (Fig. 2, Panel 2), it is evident that the M_Av sample harboured a complex microbial ecosystem, composed of a wider range of specific bacteria than the other milk samples analysed in this study.

The Venn diagram shown in Fig. 3 helps to clarify the data: 27 of the 34 species assigned in the three milk samples belonged to M_Av, while a lower number, i.e. 11 and 9, belonged to the M_Ce and M_Sa samples. Overall, only 3 bacterial species (*Stenotrophomonas geniculata, Pseudomonas veronii* and *Acinetobacter johnsonii*) were common to all 3 milk samples, whereas 2 (*Pseudomonas viridiflava* and *Flavobacterium succinicans*) and 5 (*Sphingobium yanoikuyae, Jeotgalicoccus psychrophilus*, *Caldinitratiruptor microaerophilus*, *Brevundimonas diminuta* and *Alcaligenes faecalis*) were common between M_Av and M_Sa and M_Av and M_Ce, respectively. The major part of the species described above are normal inhabitants of raw sheep milk or related dairy and environmental sources (Esteban-Blanco et al., 2020; Santamarina-García et al., 2024). Other species have been found to be differentially associated with plants and soil. Indeed, certain plant characteristics can positively or negatively influence the bacterial community in the rhizosphere (Schlatter et al., 2015; Zengin et al., 2024), thus influencing the microbiota of the milk of grazing animals. The same effect can be attributed to soil structural properties (Seaton et al., 2022). For example,

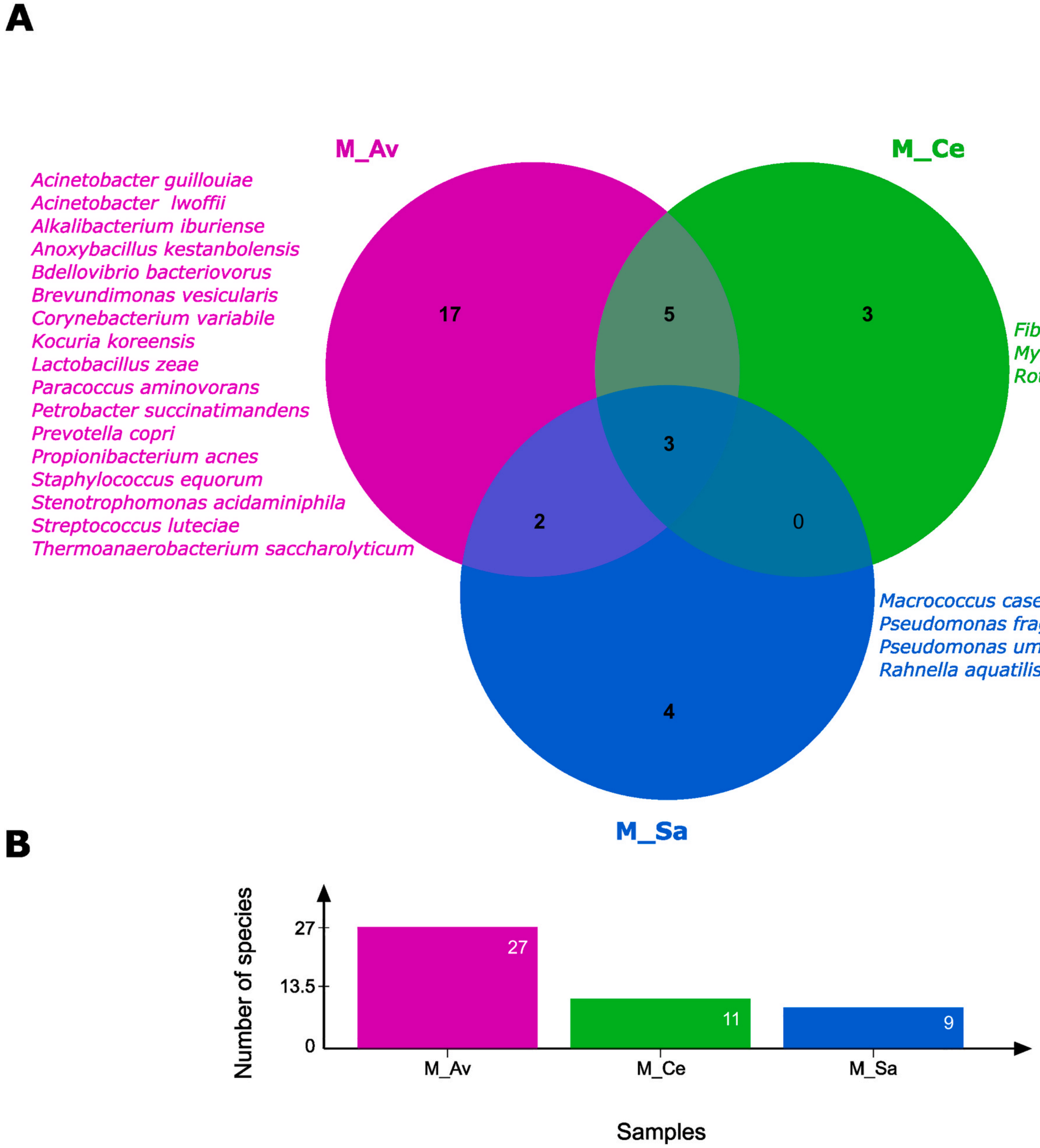

Fig. 3. A) Venn diagrams based on the shared bacterial communities among milk samples from Avellino (M_Av), Caserta (M_Ce), and Salerno (M_Sa) provinces. B) Aboundance of bacterial species identified in each milk sample.

Rahanella aquatilis, found only in sample M_Sa, is a phosphate-solubilising bacterium associated with the rhizosphere, especially in areas with (excessive) soil fertilisation with phosphorous (Kim et al., 1997), so its presence in the milk sample from the Salerno province could be due to particular physico-chemical properties of the soil where sheep graze.

In sample M_Ce, peculiar species were *Fibrobacter succinogenes*, previously isolated from sheep rumen microbiome (Christodoulou et al., 2023), *Myroides odoratimimus*, an opportunistic pathogen commonly found in the environment and in milk (Oleghe et al., 2020), and *Rothia nasimurium*, commonly found as a commensal bacterium in the upper respiratory tract and gut of humans and other animals (Guo et al., 2021).

In ewe milk from the Avellino province (M_Av) most of the species annotated are commonly inhabitants of the dairy environment. Other species have different habitats, such as *Alkalibacterium iburiense*, an obligately alkaliphilic bacterium that exists ubiquitously (Nakajima et al., 2005). Still others are linked to very particular habitats, such as the Mefite D'Ansanto. For instance, *Anoxybacillus kestanbolensis* is a moderately thermophilic bacterium isolated from extreme environments (Valenzuela et al., 2024) and also found in milk of different species (horse, yak, cow), but no information is available on the milk sampling environment (Luo et al., 2024; Parente et al., 2020). *Petrobacter succinatimandens* is a thermophilic heterotrophic denitrifying bacterium active in thermal environments (Wang et al., 2023), sporadically isolated in dairy products but not correlated to milk sampling environment (Lo et al., 2016). *Thermoanaerobacterium saccharolyticum* is an anaerobic thermophilic bacterium which grows in a temperature range between 45 °C and 78 °C, originally found in thermal acid springs and hydrothermal vent (Lee et al., 1993; Hung et al., 2011; Fiamenghi et al., 2024), also reported as a spoilage microorganism in the food sector (Mtimet et al., 2016). However, these three species have never before been detected together in ewe milk or analysed in relation to the sampling environment of milk.

3.3. Data analysis

From the data reported so far, each ewe milk sample from the different provinces of the Campania region had peculiar chemical and microbiological characteristics. In light of these observations, we used a statistical approach by selecting all variables considered in this study (excluding those with no significant differences) to understand their impact on the richness of bacterial species in the milk samples. As shown in Fig. 4, the number of species, Zn, Fe and proteins were close on the same positive side of the PC1 axis, higligting a high correlation, while Sr and Ca were on the opposite side. The other variables showed a weaker correlation with the number of species.

The correlation between the number of bacteria in raw milk and protein content has already been analysed by other authors (Boix-Amorós et al., 2016), who highlighted that only certain bacteria are negatively correlated with protein content. Moreover, numerous studies considered the relationships between metals in raw ewe milk and the environment, especially to monitor the pollution status and the presence of toxic elements (Miedico et al., 2016). On the other hand, the correlation of metals with the number of bacterial species in ewe milk was not contemplated until now. In this connection, our findings suggest that some elements are highly related to the bacterial species abundance in ewe milk. Specifically, the correlogram shown in Fig. 5 higlighted the strong contribution of Zn and Fe to the presence of different bacterial species. All these traits (presence of Zn and Fe in high concentrations, high number of bacterial species) characterised the milk sample from the province of Avellino, where sheep graze in a particular environment, the Mefite D'Ansanto, characterised in turn by a particular soil richness of the aforementioned metals, especially Fe. K, also found in high concentration in M_Av, had a minor impact on the bacterial species, followed by Ca and Na. Sr, detected mainly in the sample M_Sa, negatively correlated with the number of species.

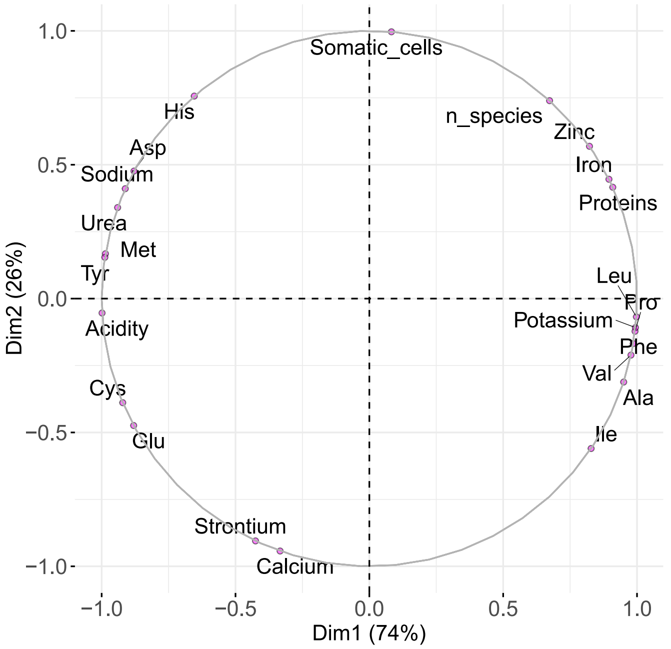

Fig. 4. PCA of the physico-chemical and microbiological characteristics of ewe milk from Avellino (M_Av), Caserta (M_Ce), and Salerno (M_Sa) provinces.

These data suggest that the bioavailability of some micronutrients more than others may enrich the bacterial composition of sheep milk. This aspect appears even more interesting considering the presence in the M_Av sample of three thermophilic-extremophilic bacteria, such as *Anoxybacillus kestanbolensis*, *Petrobacter succinatimandens* and *Thermoanaerobacterium saccharolyticum*, never before been detected togheter in sheep milk.

4. Conclusion

This study highlighted several differences in chemical and bacterial composition of ewe milk samples from different animals grazing in three provinces of the Campania region. In particular, the milk sample from the province of Avellino resulted particularly rich in Fe, K and Zn, and a correlation between Fe and Zn content and numbers of different bacterial species was detected. The same reachness in metals characterises the soil of the Mefite D'Ansanto Valley, where sheep are taken to pasture. A specific microbiome was individuated for each milk, mainly composed by typical genera and species belonging to the dairy environment. Moreover, in the milk sample from Avellino province, some species, such as *Anoxybacillus kestanbolensis*, *Petrobacter succinatimandens* and *Thermoanaerobacterium saccharolyticum*, appeared related to the specific area, that is, the Mefite. The combination of these distinctive traits is particularly interesting as this milk is used for the production of Pecorino Carmasciano, a typical Campanian cheese awaiting the award of PDO. In conclusion, results reported in this research highlighted that the peculiar characteristics of Mefite can influence the chemical and microbiological composition of milk. Of course, further studies are needed, as numerous variables may contribute to the definition of the chemical and microbiological features of milk from this specific area. It is desirable to examine in depth the causes of the differentiation due to the place of sampling, for example by analyzing water and environmental matrices (soils and grass), to obtain a more detailed information about the origin of minerals and bacteria. Furthermore, it could be of interest the evaluation of certain amino acids, such as Met and Cys, in wool and skin of sheep grazing on the Mefite to ascertain their possible increase in concentration.

Finally, considering that an important factor of variability may be the year of sampling, which in turn is correlated to the quality of the

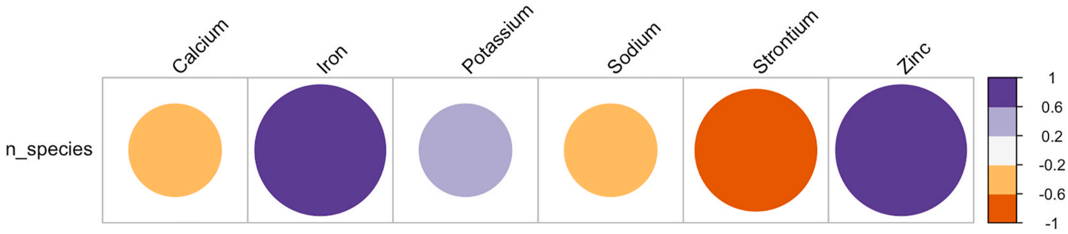

Fig. 5. Correlogram representing the Pearson's correlation between the number of species and the metals in milk samples. The size of the circles indicates the strength of the correlation, and the color indicates positive or negative correlation.

vegetation due to annual fluctuations in climatic conditions, it may be interesting to evaluate the durability of the results obtained in this study with regard to the composition of milk from sheep grazing in the area of Mefite D'Ansanto.

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CRediT authorship contribution statement

Diletta Bagnoli: Writing – original draft, Formal analysis, Data curation. **Gianfranco Pannella:** Visualization, Software, Data curation. **Franca Vergalito:** Writing – review & editing, Methodology, Formal analysis. **Francesca Coppola:** Software, Methodology, Data curation. **Maria Cristina Messia:** Methodology, Data curation. **Mariantonietta Succi:** Writing – original draft, Supervision, Project administration.

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.

Data availability

Data will be made available on request.

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