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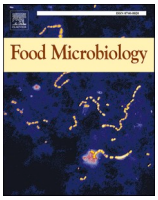

Comparative study of the bacterial community of organic and conventional cow's milk

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ABSTRACT

Agricultural practises such as conventional and organic farming can potentially affect the microbial communities in milk. In the present study, the bacterial diversity of milk was investigated using high-throughput sequencing on ten organic and ten conventional farms in the Azores, a region where milk production is largely based on year-round grazing systems. The microbiota of milk from both production systems was dominated by *Bacillota*, *Pseudomonadota*, *Actinomycetota* and *Bacteroidota*. The organic milk showed greater heterogeneity between farms, as reflected in the dispersion of diversity indices and the large variation in the relative abundances of the dominant genera. In contrast, conventionally produced milk showed a high degree of similarity within each season. In the conventional production system, the season also had a strong influence on the bacterial community, but this effect was not observed in the organic milk. The LEfSe analysis identified the genus *Iamia* as significantly ($p < 0.05$) more abundant in organic milk, but depending on the season, several other genera were identified that distinguished organic milk from conventionally produced milk. Of these, *Bacillus*, *Iamia* and *Nocardioides* were associated with the soil microbiota in organic farming.

1. Introduction

At the core of conventional agriculture is the technological model based on the intensive use of mechanization, highly soluble mineral fertilizers, and plant protection products such as pesticides, which pose potential risks to the environment and consumers (Adl et al., 2011). If used improperly, they lead to soil, water, and air pollution, generate additional resistance to pests, reduce soil biodiversity and contribute to the increase in greenhouse gases (Tscharntke et al., 2012). The environmental impacts caused by conventional agriculture, combined with consumer awareness of animal welfare and demand for safer and healthier food, have led to a rethinking of agricultural policy and the adoption of more environmentally and animal-friendly practices such as organic farming (Rosati and Aumaitre, 2004; Torjusen et al., 2004).

In contrast to conventional agriculture, the use of artificial fertilizers, pesticides, herbicides, and antibiotics is banned or restricted in organic farming (Erisman et al., 2016; Gomes et al., 2020; Gomiero et al., 2011). These restrictions in organic farming are likely to alter the microbial communities in the soil and farm environment, potentially affecting the microbiota detected in raw milk.

The microbial quality of raw milk is of critical importance to the dairy industry as it can provide a desirable microbiota for the production of various dairy foods and influence the organoleptic properties of these products (Breitenwieser et al., 2020). Conversely, undesirable organisms can enter the milk and cause food spoilage (Kousta et al., 2010; Radoslava et al., 2020). In particular, the presence of spore-forming microorganisms and thermoduric enzymes produced by Gram-negative psychrotrophs not destroyed during pasteurization, can lead to major quality problems in milk and dairy products (Porcellato et al., 2021; Quigley et al., 2013). In addition, there is growing evidence of the importance of the dairy microbiota in maintaining a favorable gut microbiome and promoting health (Aslam et al., 2020). However, the understanding of the effects of different agricultural practices such as conventional and organic farming on the food microbiota is still insufficient (Gomes et al., 2020; Quigley et al., 2013). To our knowledge, only one study in the Netherlands has compared the microbiota of dairy cows from conventional and organic farming (Gomes et al., 2020). Also, there are few studies on the microbiota diversity of milk produced in pasture systems and no study has been conducted comparing the milk microbiota of organic and conventional pasture systems. Therefore, this study investigated the bacterial communities of milk from pasture-based systems using Next Generation Sequencing (NGS) methods and compared

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the microbiota of organic and conventional milk produced in two seasons (winter and spring).

2. Materials and methods

2.1. Farm characterization

In this study, all dairy farms were located on the island of Terceira, Azores, Portugal. The archipelago of the Azores has exceptional edaphoclimatic conditions for the growth of grass (temperatures of 11–16 °C in winter and 15–20 °C in spring), so that milk production is largely based on year-round grazing systems (Silva et al., 2019). A total of 40 milk samples were collected from 20 dairy farms with pure Holstein Friesian cows. Ten of these farms were certified for organic milk production, while the other 10 followed the conventional milk production system. On each farm, samples were taken from the milk tank in March (end of winter – lower grass growth) and June (spring – peak of grass growth). The organic farms were mainly located in the south-east quadrant of the island, while the conventional farms were randomly distributed across the island. The feeding of cows on all organic farms was based on maximizing the use of pasture, i.e. fresh grass from pasture makes up the majority of the diet on all farms throughout the year. All organic farms use dry fodder or straw from the pastures of producers certified for the organic production method. None of these farms used silage or feed supplements, and three farms fed organic concentrates only very sporadically (less than 40 kg/cow/month). On 9 farms, milking took place twice a day, in the early morning and late afternoon, and on one farm only once, in the afternoon. The size of the herds on the organic farms ranged from 8 to 25 cows, while it was more heterogeneous on the conventional dairy farms, i.e. on 8 farms it varied between 25 and 100 cows and on the other two farms it was over 100 animals. On the conventional farms, the animals were fed on pasture for a large part of the year, especially in spring and summer. In winter, the animals were fed concentrates and maize silage, as the availability of fresh grass was lower.

2.2. Sampling collection and DNA extraction

Raw milk samples (approx. 50 mL) were aseptically collected from the cooling tank of ten conventional and ten organic dairy farms. To further avoid confounding impacts and allowing to treat farms as replicate in analysis, we ensured that in each sampling time (March and June), all milk samples (from conventional and organic production) were taken within the same week. All samples arrived at the laboratory within 24 h of collection and were immediately used for DNA extraction. Microorganisms in milk were concentrated by centrifugation (50 mL) at 7000×*g* for 10 min (Beckman J2-HS centrifuge). The supernatant was discarded, and the pellet was washed twice with TE buffer (Tris-EDTA: 2 M Tris HCl + 0.5 M EDTA, pH 8.0) before being resuspended in 1 mL of TE buffer. Total genomic DNA was extracted using the UltraClean® extraction kit Microbial DNA Isolation Kit (MoBio, Carlsbad, CA) according to the manufacturer procedure. The quantity and quality of extracted DNA was evaluated by measuring absorbance at 260 and 280 nm (LVis Plate, Fluorstar Omega, BMG Labtech). The quality of the extracted DNA was confirmed via 1.5% agarose (w/v) gel electrophoresis.

2.3. Sample preparation and high-throughput sequencing

Samples were prepared for Illumina Sequencing by 16 S rRNA gene amplification of the bacterial community. The DNA was amplified for the hypervariable V3–V4 region with specific primers and further reamplified in a limited-cycle PCR reaction to add sequencing adapters and dual indexes. First PCR reactions were performed for each sample using KAPA HiFi HotStart PCR Kit according to manufacturer suggestions, 0.3 μM of each PCR primer: forward primer Bakt_341 F 5′–CCTACGGGNGGCWGCAG-3′ and reverse primer Bakt_805 R 5′–GACTACHVGGGTATCTAATCC-3’ (Herlemann et al., 2011; Klindworth et al., 2013) and 2.5 μL of DNA in a total volume of 25 μL. The PCR conditions involved a 3 min denaturation at 95 °C, followed by 30 cycles of 98 °C for 20 s, 55 °C for 30 s and 72 °C for 30 s and a final extension at 72 °C for 5 min. Second PCR reactions added indexes and sequencing adapters to both ends of the amplified target region according to manufacturer's recommendations (Illumina, 2013). Negative PCR controls were included for all amplification procedures. PCR products were then one-step purified and normalized using SequalPrep 96-well plate kit (ThermoFisher Scientific, Waltham, USA) (Comeau et al., 2017), pooled and pair-end sequenced in the Illumina MiSeq® sequencer with the Miseq Reagent kit v3 (600 cycles), according to manufacturer's instructions (Illumina, San Diego, CA, USA) at Genoinseq (Cantanhede, Portugal).

2.4. Bioinformatics and statistical analysis

Raw reads were extracted from Illumina MiSeq® System in fastq format. The QIIME2 package version 2020.2.0 (Bolyen et al., 2019) was used for amplicon sequence variants (ASV) generation, and taxonomic identification. Denoising was carried out with DADA2 (Callahan et al., 2017). DADA2 within Qiime2 was used to denoise and create ASVs. DADA2 detected and corrected sequencing errors, detected and removed chimeric sequences based on the consensus method, and filtered out phiX reads. After denoising, taxonomic assignments were determined for ASVs with the q2 feature-classifier plugin (Bokulich et al., 2018) against the SILVA database version 138 (Quast et al., 2012). Sequence data was processed at Genoinseq (Cantanhede, Portugal).

Rarefaction curves and biodiversity indices (Taxa, Chao1, Dominance, Equitability, Shannon index and Simpson index) were determined with the Past program (Hammer & Harper, v. 4.11). The amplicon sequence variants (ASVs) profiles of each sample were normalized (total sum normalization, TSS) and compared with the Bray–Curtis distance metric. To display community composition differences between farm systems and season, principal coordinate analysis (PCoA) was performed with Bray–Curtis distance matrices for ASV. To assess differentially abundant taxonomic genus most likely to explain differences between agriculture systems the discriminant linear analysis (LDA) of effect size (LEfSe) was performed using $\alpha = 0.05$ and LDA threshold of 2.0 (Segata et al., 2011), and the Galaxy program (http://huttenhower.sph.harvard.edu/galaxy/). The Kruskal-Wallis H test (for $\alpha = 0.05$) was also applied for comparisons of diversity indices and ASVs at the genus level, for the different groups (conventional vs. organic milk) and for the different seasons of the year (winter vs. spring). Statistical analysis was performed using the IBM SPSS Statistics software (v. 25, IBM Corporation).

3. Results and discussion

Culture-independent methods are increasingly used as they allow a more in-depth assessment of microbial communities in environmental and food samples and provide information on microorganisms that are difficult to culture or uncultivable. Although NGS methods have revealed the complexity of milk microbiota, these methods are inherently subject to bias as they may over- or underestimate some bacterial groups. Milk, in particular, is a challenging sample due to its physical and chemical properties. Difficulties include high quality DNA recovery that is representative of the entire microbial population, and the amplification of DNA from dead cells. Despite all these limitations, analyses of the microbial communities in milk using the NGS methodology are very useful to detect shifts in the bacterial population. In the present study, bacterial communities were compared between milk samples collected and treated under the same conditions to ensure an appropriate comparison between organic and conventional milk production systems.

Based on 97% similarity, a total of 3484 amplicon sequence variants (ASVs) were identified, of which 3467 ASVs were assigned to the *Bacteria* domain and 17 to the *Archaea* domain. The average value of reads per sample was 8459, varying between the minimum value of 2631 reads/sample and the maximum value of 16,580 reads/sample.

3.1. Alpha diversity

Alpha diversity of milk samples from both production types (conventional/organic) in the two seasons (winter/spring) were assessed using different alpha diversity indices: Taxa number, Individuals, equitability, Shannon index, Simpson index, and Chao1 (Fig. 1). No significant differences ($p > 0.05$) were found for the different biodiversity indices between the production types (conventional *vs.* organic) of milk produced in both seasons (winter and spring). As for the effect of season, significant differences ($p < 0.05$) were found in the number of individuals and evenness between the two seasons (winter and spring) for conventional milk. For bacterial richness, expressed by the number of individuals, the conventional milk produced in spring had higher values ($p < 0.05$) than the milk produced in winter (Fig. 1). The winter-produced milk also had a higher value for equitability ($p < 0.05$), indicating a more even distribution of species diversity and consequently a lower number of dominant species. In the organic milk samples, there was no influence of season ($p > 0.05$) on any of the biodiversity indices. This result can be explained by the fact that the cows on the organic farms were on pasture all year round and, contrary to those on conventional farms, were only provided with limited supplement feed (organic) in winter. In contrast, the conventional farms showed greater differences in feeding between winter and spring. While the cows consume almost exclusively grass in spring, when pasture was plentiful, more silage (grass and maize) as grass supplement was used in winter, as the conventional production method is also more intensive. Accordingly, the biodiversity indices of conventional and organic milk were similar in spring, as the feeding systems were similar and based on grazing. It can also be stressed that organic milk had a greater dispersion of diversity indices (Taxa, Simpson, Shannon, Equitability and Chao1

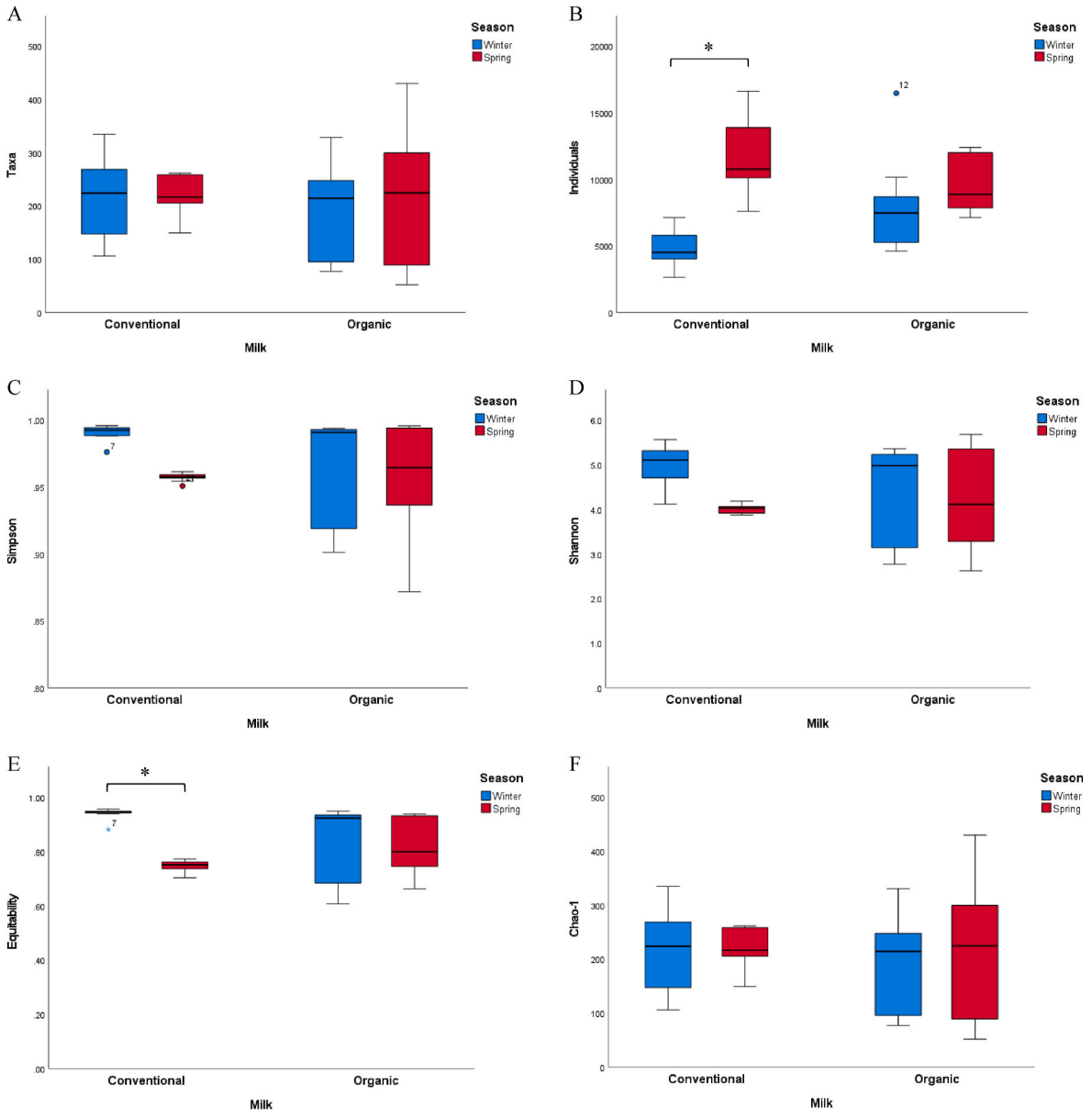

Fig. 1. Alpha diversity of organic and conventional milk produced in winter and spring. A) Number of different taxa, B) Number of individuals, C) Simpson index D) Shannon index, E) Equitability, F) Chao-1. *$p < 0.05$.

indices) between samples (farms). These results can be justified by a greater variability of the microbiota on organic farms, as they are not exposed to the influence of conditioning factors such as the use of pesticides and chemical soil fertilizers, which can select a more resistant microbiota, leading to greater uniformity between samples.

To evaluate the distribution of the 3484 ASVs among the different groups, a Venn diagram was constructed (Fig. 2). The diagram indicates that 108 ASVs were common to all groups and correspond to 55.3% of all reads. The conventionally produced milk showed the highest number of exclusive ASVs in winter (1051) and the lowest exclusive ASVs in spring (252, 1.2% of the total reads). In organic milk, 914 exclusive ASVs were observed in winter, while 752 exclusive ASVs were found in spring. In addition, a higher number of ASVs were common of organic milk in winter and spring (63 ASVs), compared to the ASVs shared by the two seasons of conventional milk (4 ASVs). Furthermore, the number of ASVs shared by the conventional and organic milk in winter was lower (31 ASVs) than the number of ASVs shared by the two production systems in spring (74 ASVs).

The microbiota of fresh grass is very different from the microbiota of silage, and although there is no direct transfer of the dietary microbiota to the milk produced, there is scientific evidence of the occurrence of bacterial transfer related to the environment in which the animals live (Gomes et al., 2020; Keshri et al., 2018; Nguyen et al., 2020; Tannenbaum et al., 2020). The ambient air, cow lying areas, pasture, and the overall environment of the animal's living, is depending on the season and production system and can thus influence the microbiota of the milk. These transfers occur mainly through contact between teats and air, litter, feces, soil and feed, with bacteria migrating into the teat canal and subsequently appearing in the milk (Celano et al., 2022; Nguyen et al., 2020).

3.2. Taxonomic composition of bacterial communities

Thirty phyla were identified, of which three belong to the *Archaea* domain and twenty-seven phyla to the *Bacteria* domain. *Bacillota* (25–94%) dominated the bacterial community in both conventional and organic milk. It is noteworthy that the lowest and highest abundances of *Bacillota* were observed in organic milk (Fig. 3A). In winter, the core microbiota of conventional milk (M1 to M10, Fig. 3) included 4 dominant taxa – *Bacillota* (46–61%), *Actinomycetota* (14–33%), *Bacteroidota* (5–15%) and *Pseudomonadota* (5–14%). In winter-produced organic milk (M11 to M20, Fig. 3) lower relative abundances of *Actinomycetota* (6–17%) and *Bacteroidota* (0–15%) were observed, while *Pseudomonadota* exhibited higher abundances (5–56%) and *Bacillota* accounted for a wider range of abundances (25–89%). In spring, the core bacterial community of conventional milk (M21 to M30, Fig. 3) changed to a higher proportion of *Bacillota* (66–71%) and *Pseudomonadota* (20–26%) and a lower proportion of *Actinomycetota* (3–6%) and *Bacteroidota* (2–4%), while organic milk (M31 to M40, Fig. 3) showed a wider range of *Bacillota* (43–93%), *Pseudomonadota* (0–40%), *Actinomycetota* (1–5%) and *Bacteroidota* (1–28%).

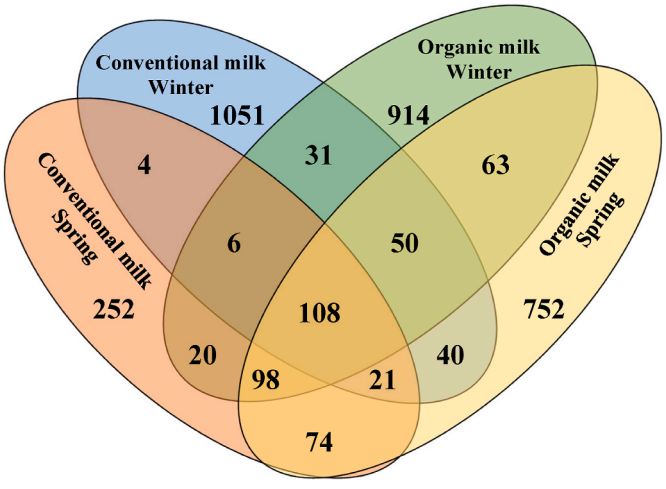

Fig. 2. Venn diagram showing the occurrence of the 3484 unique and common ASVs in organic and conventional milk in winter and spring.

The high abundances of these taxa are consistent with other studies showing that the core microbiota in raw milk is dominated by *Bacillota*, followed by *Pseudomonadota* and *Actinomycetota* (Kaczorowski et al., 2022). In Ireland, Quigley et al. (2013) reported that *Bacillota* accounted for 80% of the bacterial community in raw milk. Similar results were also reported from the Italian region of Puglia, where the core microbiota of raw milk was dominated by *Bacillota* and *Pseudomonadota* (Celano et al., 2022).

At the genus level, the results obtained showed a wide variation among the milk samples with more than 400 genera identified. Fig. 3B shows the relative abundances of the sequences identified at the genus level that contributed more than 5% to the total abundance in at least one sample of each group. The large differences in the relative abundances of the genera observed in the milk samples are consistent with other studies (Celano et al., 2022; Sun et al., 2022; Yuan et al., 2022).

The genus *Lactococcus* dominated the bacterial community of milk with 36% of the total sequences (ASVs) and was the dominant genus in most milk samples in both winter and spring. However, in the milk samples from conventional production in spring (M21 to M30), their relative abundance was more uniform (56%–64%). The highest proportion of bacteria of the genus *Lactococcus* was found in the milk from organic production in the winter samples M11, M12 and M16 (79%–89%). Within this genus, the species *Lactococcus raffinolactis* was identified in organic milk samples in both seasons and in conventional spring milk (Supp. Fig. 1). Some species of *Lactococcus* have probiotic properties and are commonly used in the production of fermented milk products to convert lactose into lactic acid. However, this species is not currently used by the dairy industry, mainly because of its lack of caseinolytic activity (Boucher et al., 2003; Jung et al., 2020).

The genus *Acinetobacter* was observed in spring in considerable abundance (16–23%) in different samples of organic milk (M35, M36, M38 and M39) and conventional milk (M21 - M30). In sample M15 (organic winter milk), the genus *Pseudomonas* was more abundant compared to the other samples. Similar results were also obtained by other authors who found higher proportions of sequences from different genera of *Pseudomonadota* (such as *Acinetobacter* and *Pseudomonas*), probably due to environmental contamination (Aziz et al., 2022; Yuan et al., 2022). Some studies also report that there is a difference in the composition of the milk microbiota on farms where cold storage is used. While milk from farms without refrigeration is dominated by Gram-positive bacteria (*Staphylococcus*, *Macrococcus*, *Corynebacterium*, *Lactococcus*, *Lacticaseibacillus* and *Enterococcus*), higher proportions of Gram-negative bacteria such as *Pseudomonas*, *Acinetobacter* and *Chryseobacterium* may occur on farms that have some form of refrigerated storage (Balthazar et al., 2022; Ouamba et al., 2022; Vithanage et al., 2016).

Various factors such as soil type, nutrition and other management practices can influence milk quality and thus the milk microbiota. In the present study, the composition of the soil and milk was not analyzed. However, in terms of soil type, all farms were located on the volcanic island of Terceira in the Azores (400.3 km^2), which has a similar soil type. The microbial composition of raw milk is also influenced by other factors, such as microorganisms in the teat canal, on the teat skin surface, in the ambient air, in the feed and by other environmental factors, such as farm conditions, water quality and hygiene of milking equipment (Celano et al., 2022). The production system of the respective farm (outdoor *vs.* stable) also influences the microbiota of raw milk. For example, when teats are exposed to different environments (pasture/-stable), different bacteria settle by adhering to the teat surface. Some

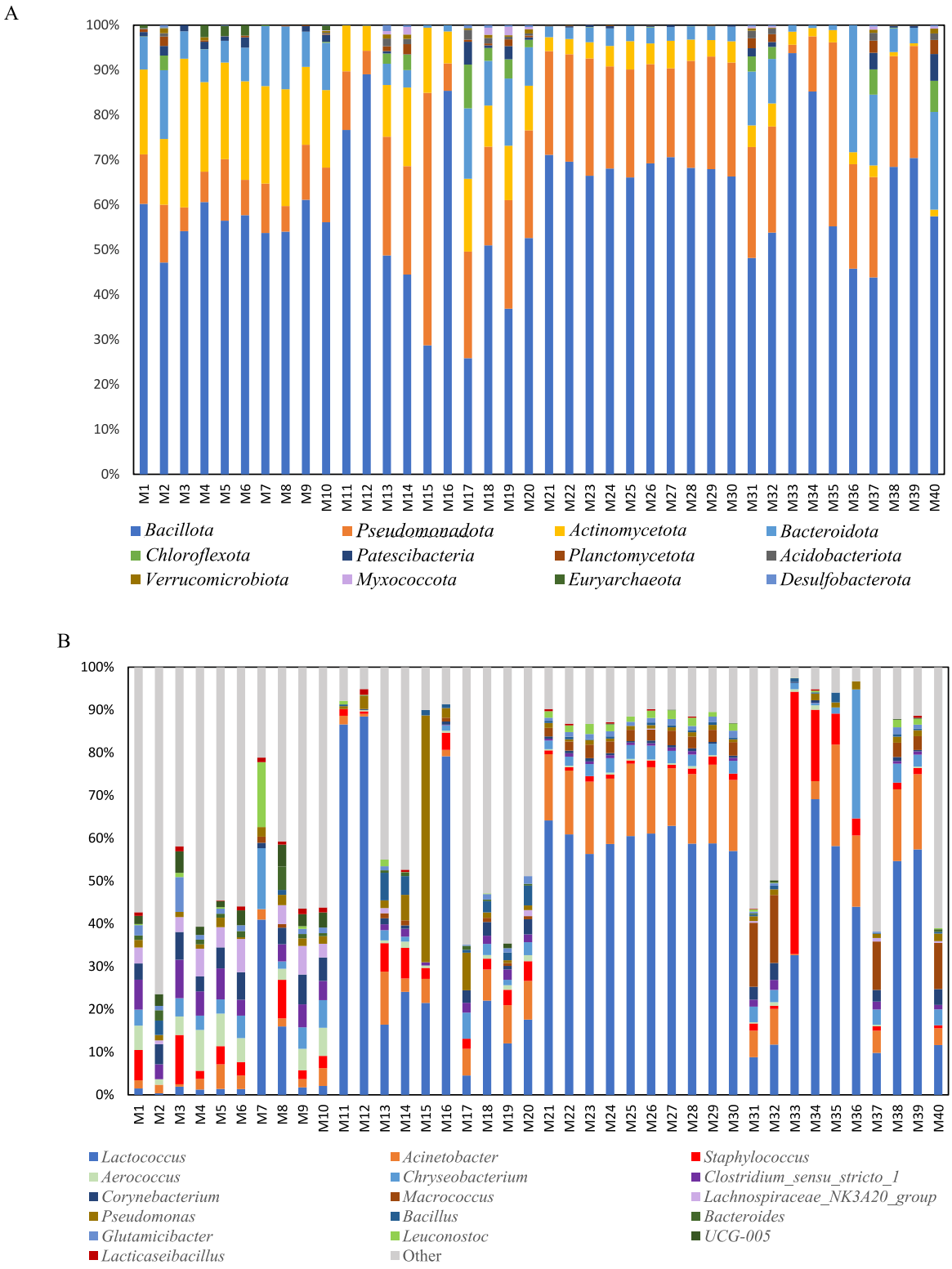

Fig. 3. Relative abundance (%) of identified ASVs at (A) phylum and (B) genus level in conventional winter milk (M1 to M10), organic winter milk (M11 to M20), conventional spring milk (M21 to M30) and organic spring milk (M31 to M40).

bacteria may even migrate into the teat canal and appear later in the milk, even if the teats are disinfected before milking (Celano et al., 2022; Doyle et al., 2017; Ouamba et al., 2022; Parente et al., 2020). Therefore, the high presence of bacteria of the genus *Lactococcus* in raw milk is probably due to the transfer of the microbiota via the teat (Balthazar et al., 2022; Frétin et al., 2018; Ouamba et al., 2022; Parente et al., 2020). In addition, several bacteria from the environment were also detected in this study, such as *Acinetobacter*, *Chryseobacterium* and *Enhydrobacter*. These have also been identified in other studies on the milk microbiota (Dalmasso et al., 2016; De Pasquale et al., 2014).

3.3. Beta diversity of bacterial communities

To illustrate the differences in bacterial communities in conventionally and organically produced milk samples in two seasons, microbial community profiles were compared using principal coordinate analysis (PCoA). Fig. 4 shows the PCoA analysis of the identified genus-specific ASVs. The two PCoA axes accounted for 61% of the total variability, with PCoA 1 and PCoA 2 describing 47% and 14% of the variability, respectively. Comparing the profiles of bacterial communities in conventional milk samples between the two seasons, two different groups were obtained (Fig. 4, groups A and B). Thus, a high similarity of the bacterial community in samples of conventionally produced milk can be observed both in spring (group A) and in winter (group B). On the other hand, there is almost no difference between the two seasons in the organic milk samples, so that a scattering of these samples can be observed in the PCoA diagram.

To confirm the differences between the samples observed in the PCoA analysis, a cluster analysis at genus level was carried out for the samples from winter (Fig. 5A) and spring (Fig. 5B) of the two production systems. In this analysis, the differentiation of the groups was confirmed, with a clear separation of the cluster of conventionally produced milk in winter, which had the highest degree of dissimilarity to the other clusters. Two clusters were also observed, one containing only samples of organic winter milk and the other a sample of conventional spring milk with four samples of organic winter milk, which show a very high degree of similarity.

To determine the genera that most likely explain the differences between organic milk and conventional milk, a linear discriminant analysis (LEfSe) was performed (Segata et al., 2011). Fig. 6 show the results of LEfSe applied to the genera that occur with a frequency greater than 2% in at least one sample and were identified as significantly differentiating variables ($p < 0.05$) in the samples collected in winter and spring. Independent of the season, the genus *Iama* was the differentiating genus of organic milk, while the genera *Ruminococcus* and *Glutamicibacter* differentiated the conventional milk (Fig. 6A). In the samples collected in winter, several genera (26) were identified as significant differentiators ($p < 0.05$) between the two production systems. In the organic milk, *Lactococcus* was one of the differentiating genera (Fig. 6B). This genus is characterized by containing bacteria with technological and beneficial potential. Other genera in the dominant microbiota that distinguished the organic winter milk were the genera *Bacillus* and *Nocardioides*, normally associated with the teat surface microbiota, *Bacteroidetes*, associated with the gastrointestinal tract microbiota, and *Streptococcus*, showing technological potential (Parente et al., 2020). Within the genus *Streptococcus*, the species *Streptococcus parauberis* had a higher incidence of ASVs in both conventional and organic milk produced in spring (Supp. Fig. 1). In addition, *Streptococcus porcinus*, *Streptococcus dysgalactiae* and *Streptococcus pluranimalium* were also identified mostly in milk produced in winter. The presence of these bacterial species in milk may indicate the presence of subclinical infections in the udder of cows (Gursul and Ozdemir, 2022; Oliver et al., 2011; Pan et al., 2018; Skaar et al., 1994; Timoney, 2022).

In conventional milk produced in winter, among the genera that distinguish it from organic milk, the genus *Lacticaseibacillus* stands out, as it is associated with silages consumed in the conventional system (Guan et al., 2018; Jaipolsaen et al., 2022). Within this genus, *Lacticaseibacillus paracasei* was the most abundant species, especially in conventional winter milk (Suppl. Fig. 1). The genera that positively discriminate milk from the conventional system also included the genera *Aerococcus* and *Corynebacterium* associated with the teat surface microbiota, the *Lachnospiraceae* group NK3A20, *Clostridium* and *Ruminococcus* associated with the gastrointestinal tract microbiota, and psychrotrophs such as *Chryseobacterium* (Parente et al., 2020). The latter genus includes several pathogenic species that play a role in human diseases and are also considered food spoilers because they are psychrotrophic, produce proteolytic enzymes and biogenic amines, and are resistant to disinfectants (Mwanza et al., 2022).

Fewer genera distinguishing between organically and conventionally produced milk were found in milk produced in spring, confirming the previous results (Fig. 6B). In conventionally produced milk, 5 characteristic genera were identified, including the dominant genus *Lactococcus*. The abundance of this genus remains the same in the organic milk in both seasons, while it changes significantly in the conventional winter milk between the two seasons (Supp. Fig. 2). In spring, the abundance of this genus was more homogeneous in the conventional milk, exceeding 50%. Although some organic farms had a high incidence

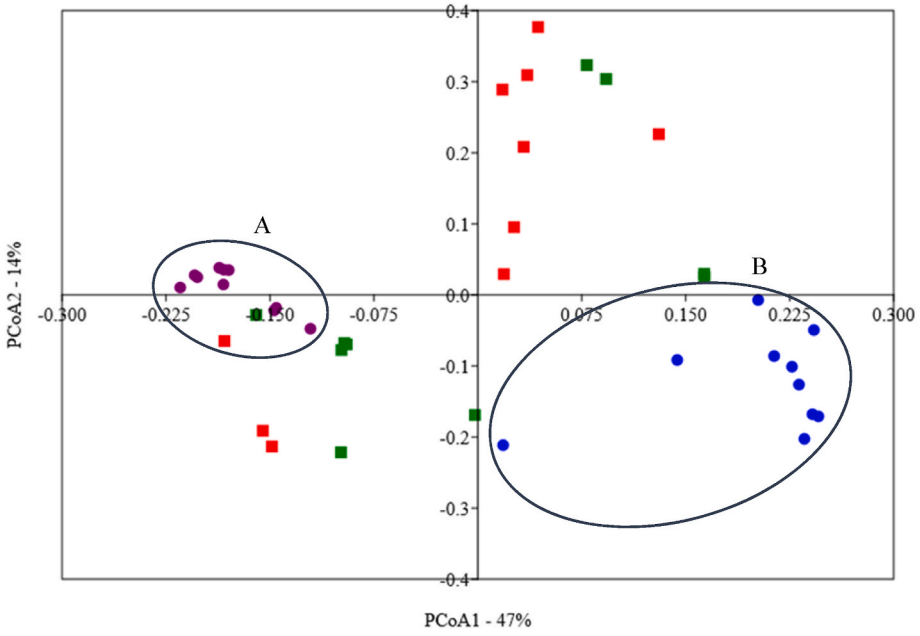

Fig. 4. Principal coordinate analysis (PCoA) of ASVs identified at genus level in milk samples from conventional and organic production in two seasons (winter and spring). The samples of conventionally produced milk are represented by circles (blue: winter, purple: spring) and the samples of organic milk by squares (red: winter, green: spring). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

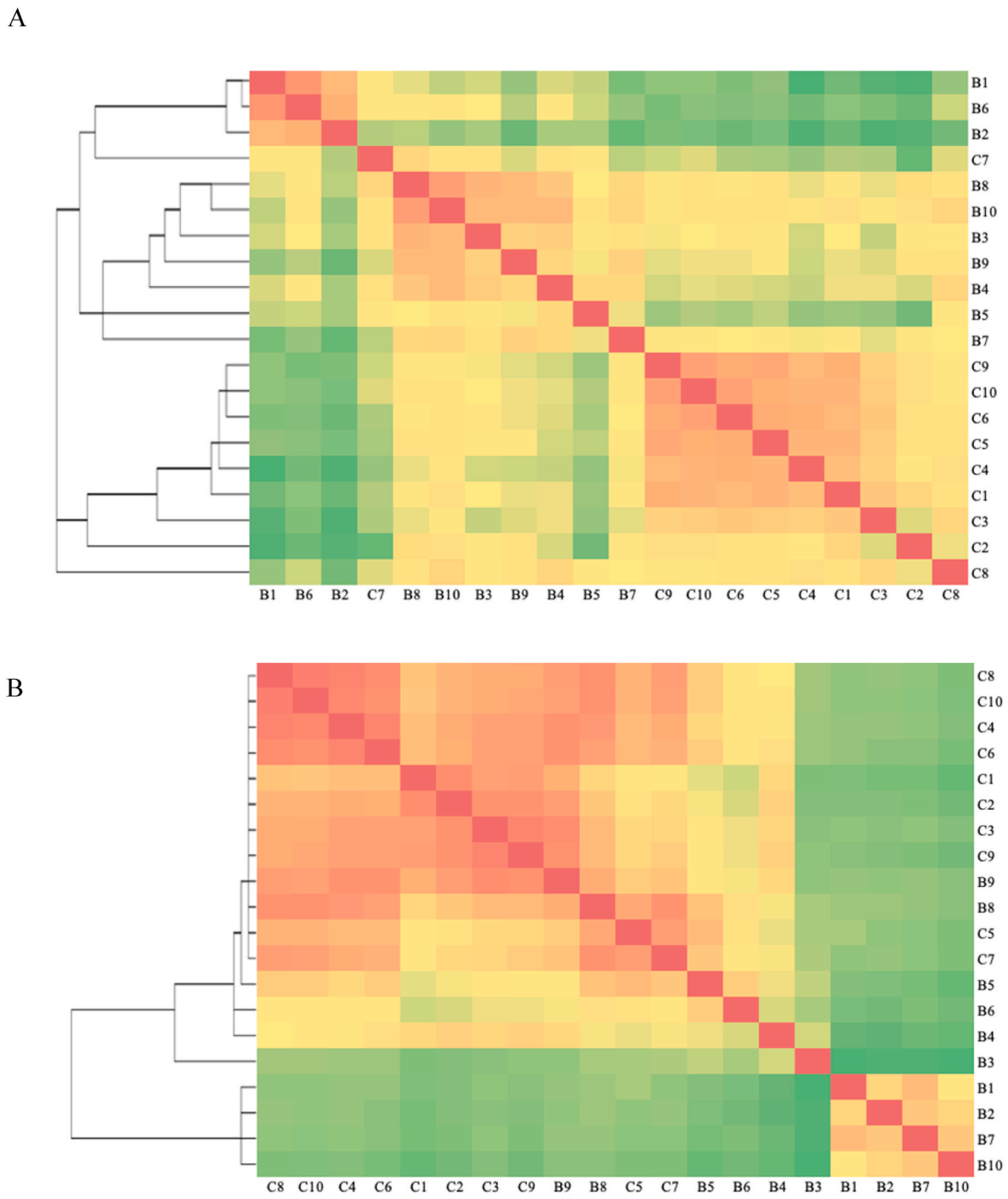

Fig. 5. Dendrogram and heat map with the Bray-Curtis dissimilarity matrix of bacterial genera in samples of A) winter milk and B) spring milk in organic (B1–B10) and conventional (C1–C10) modes. Only ASVs with a frequency of 0.1% in at least one sample were considered. Color coding from green to red, where red stands for high similarity and green for low similarity of ASVs in the sample. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

of this species (50–60%), the values on other farms are much lower, reflecting the greater heterogeneity of this production method. The genera *Escherichia* and *Shigella* are closely related (they are referred to as *Escherichia/Shigella*) and have been linked to the incidence of mastitis (Parente et al., 2020). The genus *Leuconostoc*, which also differs positively from traditional spring production, includes species with technological potential, namely for the organoleptic quality of artisanal cheese (Coelho et al., 2023).

As for the genera (8) that positively discriminate spring milk in organic production, the genus *Staphylococcus* is described in numerous studies as dominant in cow's milk (Parente et al., 2020). Within this genus, only two species were identified – *Staphylococcus vitulinus* and *Staphylococcus equorum*, but these species were more abundant in the milk produced during the winter (Suppl. Fig. 1). These species have been isolated from traditional cheeses and sausages and were used as starters in meat products (Fettahoğlu et al., 2023).

Two genera also appear in LEfSe as discriminators of spring-produced organic milk: *Christensenellaceae* R-7 group and *Iamia* (Fig. 6). The genus *Christensenellaceae* R-7 group (cluster containing isolate R-7) is also associated with the human gastrointestinal system.

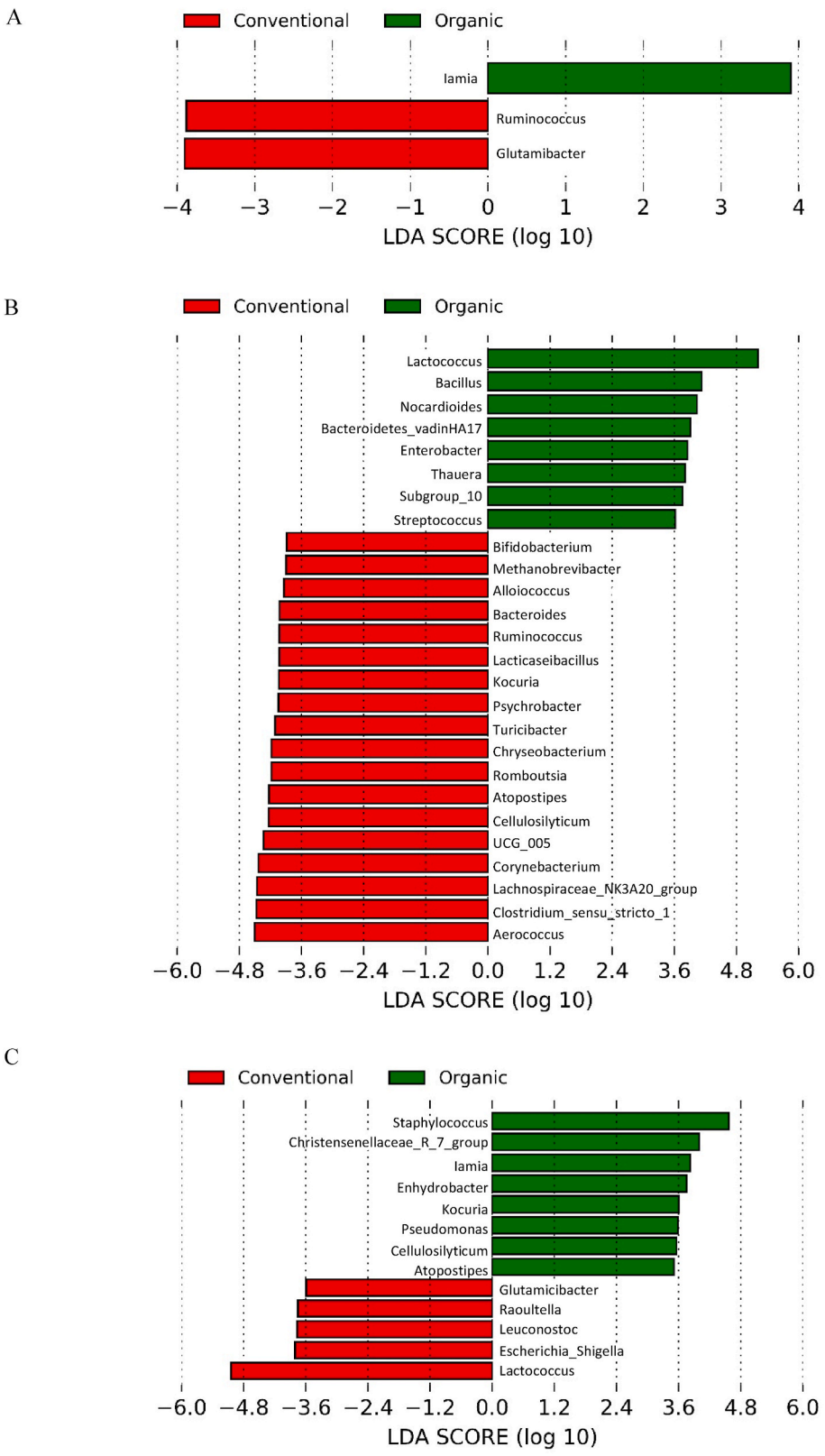

Fig. 6. Bacterial genera discriminating between milk samples from organic and conventional production produced in A) both seasons, B) winter, and C) spring, calculated by linear discriminant analysis (LEfSe). Only genera occurring with a frequency of more than 2% in at least one sample were included in the analysis.

Several studies have found an inverse relationship between the relative abundance of this genus in the human gut and body mass index (Tavella et al., 2021; Waters and Ley, 2019). Other studies have also linked the presence of bacteria from the *Christensenellaceae* R-7 group in the human gastrointestinal tract to a healthy metabolic profile (Alcazar et al., 2022).

The genus *Iamia* is associated with the marine environment, although it has also been isolated from soil. Both the genus *Iamia* and *Nocardioides*

(Supp. Figs. 2K and L) - characteristic features of organic farming in spring and winter, respectively - have been associated with a positive effect on the rhizosphere microbiome (Borowik et al., 2022) and with the reduction of pesticides in the soil (Lu and Lu, 2018), probably due to the absence of pesticides in organic farming. Although the composition of the soil microbial community was not investigated in the present study, other studies have shown that the soil microbiota is influenced by the farming systems (use of inorganic fertilizers and pesticides compared to organic fertilizers and no use of pesticides). In a comparative study of soil microbiota between conventional and organic farming systems, the genera *Bacillus*, *Iamia* and *Nocardioides* were found in greater abundance in soils from organic farming (Armalytė et al., 2019). In the present study, these genera were also found as characteristic features of milk in organic production systems, indicating a probable transfer of these bacteria from soil to milk.

4. Conclusions

This is the first study conducted by the NGS to gain a better understanding of the microbial community of cow's milk produced in the Azores (year-round grazing). It compared cow's milk produced in organic and conventional farming in two seasons (winter and spring). The results showed that the cow's milk microbiota was dominated by the phyla *Bacillota*, *Pseudomonadota*, *Actinomycetota* and *Bacteroidota*. At the genus level, several genera were found in milk, including *Lactococcus*, *Acinetobacter*, *Staphylococcus*, *Aerococcus*, *Chryseobacterium*, *Clostridium*, *Corynebacterium*, *Macrococcus*, *Pseudomonas*, *Bacillus*, *Bacteroides*, *Lacticaseibacillus* and *Leuconostoc*. Both organic and conventional milk showed similar biodiversity indices in spring when these production types are also very similar, based on pasture. However, in the conventional farms, the influence of the season on some biodiversity indices was observed, with a lower number of individuals and dominant species in the milk produced in winter when the abundance of grass is lower.

Organic milk also showed greater heterogeneity between farms, which was reflected in the spread of diversity indices and in the large differences in the relative frequencies of the dominant genera. In this production system, no clear separation of the bacterial community was observed between the milk produced in the two seasons probably due to year-round grazing. Some genera were also identified as characteristic of the organic production system. These include the genera *Iamia, Bacillus* and *Nocardioides*, which are associated with the soil microbiota in organic production systems, and the genus *Christensenellaceae* R-7 group, belonging to the family *Christensenellaceae*, which may have a positive effect on health. Overall, these results illustrate the extent to which agricultural practises such as organic farming can influence the indigenous microbiota of the milk produced. Further studies are needed to assess the potential impact of organic milk microbiota on regional products such as raw milk cheeses with a protected designation of origin (PDO).

CRediT authorship contribution statement

Nuno M.L. Paiva: Writing – original draft, Investigation, Formal analysis. **Susana C. Ribeiro:** Methodology, Investigation, Formal analysis. **Henrique J.D. Rosa:** Writing – review & editing, Formal analysis. **Célia C.G. Silva:** Writing – review & editing, Supervision, Methodology, Funding acquisition, 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.

No conflict of interest exists.

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Appendix A. Supplementary data

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References

Adl, S., Iron, D., Kolokolnikov, T., 2011. A threshold area ratio of organic to conventional agriculture causes recurrent pathogen outbreaks in organic agriculture. Sci. Total Environ. 409, 2192–2197.

Alcazar, M., Escribano, J., Ferré, N., Closa-Monasterolo, R., Selma-Royo, M., Feliu, A., Castillejo, G., Luque, V., Feliu-Rovira, A., Muñoz-Hernando, J., 2022. Gut microbiota is associated with metabolic health in children with obesity. Clin. Nutr. 41, 1680–1688.

Armalytė, J., Skerniškytė, J., Bakienė, E., Krasauskas, R., Šiugždinienė, R., Kareivienė, V., Kerzienė, S., Klimienė, I., Sužiedėlienė, E., Ružauskas, M., 2019. Microbial diversity and antimicrobial resistance profile in microbiota from soils of conventional and organic farming systems. Front. Microbiol. 10, 892.

Aslam, H., Marx, W., Rocks, T., Loughman, A., Chandrasekaran, V., Ruusunen, A., Dawson, S.L., West, M., Mullarkey, E., Pasco, J.A., 2020. The effects of dairy and dairy derivatives on the gut microbiota: a systematic literature review. Gut Microbes 12, 1799533.

Aziz, S.A.A.A., Mahmoud, R., Mohamed, M.B.E.D., 2022. Control of biofilm-producing *Pseudomonas aeruginosa* isolated from dairy farm using Virokill silver nano-based disinfectant as an alternative approach. Sci. Rep. 12, 9452.

Balthazar, C.F., Cabral, L., Guimarães, J.T., Noronha, M.F., Cappato, L.P., Cruz, A.G., Sant'Ana, A.S., 2022. Conventional and ohmic heating pasteurization of fresh and thawed sheep milk: energy consumption and assessment of bacterial microbiota during refrigerated storage. Innov. Food Sci. Emerg. Technol. 76, 102947.

Bokulich, N.A., Kaehler, B.D., Rideout, J.R., Dillon, M., Bolyen, E., Knight, R., Huttley, G. A., Gregory Caporaso, J., 2018. Optimizing taxonomic classification of marker-gene amplicon sequences with QIIME 2's q2-feature-classifier plugin. Microbiome 6, 1–17.

Bolyen, E., Rideout, J.R., Dillon, M.R., Bokulich, N.A., Abnet, C.C., Al-Ghalith, G.A., Alexander, H., Alm, E.J., Arumugam, M., Asnicar, F., 2019. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nat. Biotechnol. 37, 852–857.

Borowik, A., Wyszkowska, J., Kucharski, J., 2022. Bacteria and soil enzymes supporting the valorization of forested soils. Materials 15, 3287.

Boucher, I., Vadeboncoeur, C., Moineau, S., 2003. Characterization of genes involved in the metabolism of α-galactosides by *Lactococcus raffinolactis*. Appl. Environ. Microbiol. 69, 4049–4056.

Breitenwieser, F., Doll, E.V., Clavel, T., Scherer, S., Wenning, M., 2020. Complementary use of cultivation and high-throughput amplicon sequencing reveals high biodiversity within raw milk microbiota. Front. Microbiol. 11, 1557.

Callahan, B.J., McMurdie, P.J., Holmes, S.P., 2017. Exact sequence variants should replace operational taxonomic units in marker-gene data analysis. ISME J 11, 2639–2643.

Celano, G., Calasso, M., Costantino, G., Vacca, M., Ressa, A., Nikoloudaki, O., De Palo, P., Calabrese, F.M., Gobbetti, M., De Angelis, M., 2022. Effect of seasonality on microbiological variability of raw cow milk from apulian dairy farms in Italy. Microbiol. Spectr. 10.

Coelho, M.C., Malcata, F.X., Silva, C.C., 2023. Distinct bacterial communities in são jorge cheese with protected designation of origin (PDO). Foods 12, 990.

Comeau, A.M., Douglas, G.M., Langille, M.G., 2017. Microbiome helper: a custom and streamlined workflow for microbiome research. MSystems 2.

Dalmasso, A., Civera, T., Pattono, D., Cardazzo, B., Bottero, M.T., 2016. Characterization of microbiota in Plaisentif cheese by high-throughput sequencing. LWT 69, 490–496.

De Pasquale, I., Di Cagno, R., Buchin, S., De Angelis, M., Gobbetti, M., 2014. Microbial ecology dynamics reveal a succession in the core microbiota involved in the ripening of pasta filata Caciocavallo Pugliese cheese. Appl. Environ. Microbiol. 80, 6243–6255.

Doyle, C.J., Gleeson, D., O'Toole, P.W., Cotter, P.D., 2017. Impacts of seasonal housing and teat preparation on raw milk microbiota: a high-throughput sequencing study. Appl. Environ. Microbiol. 83.

Erisman, J.W., Eekeren, N.v., Wit, J.d., Koopmans, C., Cuijpers, W., Oerlemans, N., Koks, B.J., 2016. Agriculture and biodiversity: a better balance benefits both. AIMS Agric. Food. 1, 157–174.

Fettahoğlu, K., Kaya, M., Kaban, G., 2023. Evaluation of autochthonous coagulase—negative staphylococci as starter cultures for the production of pastirma. Foods 12, 2856.

Frétin, M., Martin, B., Rifa, E., Isabelle, V.-M., Pomiès, D., Ferlay, A., Montel, M.-C., Delbès, C., 2018. Bacterial community assembly from cow teat skin to ripened cheeses is influenced by grazing systems. Sci. Rep. 8, 200.

Gomes, S.I., van Bodegom, P.M., van Agtmaal, M., Soudzilovskaia, N.A., Bestman, M., Duijm, E., Speksnijder, A., van Eekeren, N., 2020. Microbiota in dung and milk differ between organic and conventional dairy farms. Front. Microbiol. 11, 1746.
Gomiero, T., Pimentel, D., Paoletti, M.G., 2011. Environmental impact of different agricultural management practices: conventional vs. organic agriculture. Crit. Rev. Plant Sci. 30, 95–124.
Guan, H., Yan, Y., Li, X., Li, X., Shuai, Y., Feng, G., Ran, Q., Cai, Y., Li, Y., Zhang, X., 2018. Microbial communities and natural fermentation of corn silages prepared with farm bunker-silo in Southwest China. Bioresour. Technol. 265, 282–290.
Gursul, N.C., Ozdemir, E., 2022. *Streptococcus porcinus* endocarditis: the first reported case in humans. Eskisehir Medical J 3, 1–4.
Herlemann, D.P., Labrenz, M., Jürgens, K., Bertilsson, S., Waniek, J.J., Andersson, A.F., 2011. Transitions in bacterial communities along the 2000 km salinity gradient of the Baltic Sea. ISME J 5, 1571–1579.
Illumina, 2013. 16S Metagenomic Sequencing Library Preparation. Preparing 16S Ribosomal RNA Gene Amplicons for the Illumina MiSeq System. Metagenomic Sequencing Library Preparation Manual.
Jaipolsaen, N., Sangsritavong, S., Uengwetwanit, T., Angthong, P., Plengvidhya, V., Rungrassamee, W., Yammuenart, S., 2022. Comparison of the effects of microbial inoculants on fermentation quality and microbiota in napier grass (*Pennisetum purpureum*) and Corn (*Zea mays* L.) Silage. Front. Microbiol. 12, 784535.
Jung, M.Y., Lee, C., Seo, M.J., Roh, S.W., Lee, S.H., 2020. Characterization of a potential probiotic bacterium *Lactococcus raffinolactis* WiKim0068 isolated from fermented vegetable using genomic and in vitro analyses. BMC Microbiol. 20, 1–10.
Kaczorowski, Ł., Powierska-Czarny, J., Wolko, Ł., Piotrowska-Cyplik, A., Cyplik, P., Czarny, J., 2022. The influence of bacteria causing subclinical mastitis on the structure of the cow's milk microbiome. Molecules 27, 1829.
Keshri, J., Chen, Y., Pinto, R., Kroupitski, Y., Weinberg, Z.G., Sela, S., 2018. Microbiome dynamics during ensiling of corn with and without *Lactobacillus plantarum* inoculant. Appl. Microbiol. Biotechnol. 102, 4025–4037.
Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., Glöckner, F.O., 2013. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. Nucleic Acids Res 41 e1-e1.
Kousta, M., Mataragas, M., Skandamis, P., Drosinos, E.H., 2010. Prevalence and sources of cheese contamination with pathogens at farm and processing levels. Food Control 21, 805–815.
Lu, X.-M., Lu, P.-Z., 2018. Response of microbial communities to pesticide residues in soil restored with *Azolla imbricata*. Appl. Microbiol. Biotechnol. 102, 475–484.
Mwanza, E.P., Hugo, A., Charimba, G., Hugo, C.J., 2022. Pathogenic potential and control of *Chryseobacterium* species from clinical, fish, food and environmental sources. Microorganisms 10, 895.
Nguyen, T.T., Wu, H., Nishino, N., 2020. An investigation of seasonal variations in the microbiota of milk, feces, bedding, and airborne dust. Asian-australas. J. Anim. Sci. 33, 1858.
Oliver, S.P., Murinda, S.E., Jayarao, B.M., 2011. Impact of antibiotic use in adult dairy cows on antimicrobial resistance of veterinary and human pathogens: a comprehensive review. Foodborne Pathog. Dis. 8, 337–355.
Ouamba, A.J., Gagnon, M., LaPointe, G., Chouinard, P.Y., Roy, D., 2022. Graduate Student Literature Review: farm management practices: potential microbial sources that determine the microbiota of raw bovine milk. J. Dairy Sci. 105, 7276–7287.
Pan, Y., An, H., Fu, T., Zhao, S., Zhang, C., Xiao, G., Zhang, J., Zhao, X., Hu, G., 2018. Characterization of *Streptococcus pluranimalium* from a cattle with mastitis by whole genome sequencing and functional validation. BMC Microbiol 18, 1–9.
Parente, E., Ricciardi, A., Zotta, T., 2020. The microbiota of dairy milk: a review. Int. Dairy J. 107, 104714.
Porcellato, D., Smistad, M., Bombelli, A., Abdelghani, A., Jørgensen, H.J., Skeie, S.B., 2021. Longitudinal study of the bulk tank milk microbiota reveals major temporal shifts in composition. Front. Microbiol. 12, 616429.
Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., Glöckner, F.O., 2012. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res 41, D590–D596.
Quigley, L., O'Sullivan, O., Stanton, C., Beresford, T.P., Ross, R.P., Fitzgerald, G.F., Cotter, P.D., 2013. The complex microbiota of raw milk. FEMS Microbiol. Rev. 37, 664–698.
Radoslava, S.R., Nemanja, Z., Branko, V., 2020. Occurrence and characterization of enterotoxigenic Staphylococci isolated from soft cheeses in Serbia. Acta Vet. 70, 238–254.
Rosati, A., Aumaitre, A., 2004. Organic dairy farming in Europe. Livest. Prod. Sci. 90, 41–51.
Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W.S., Huttenhower, C., 2011. Metagenomic biomarker discovery and explanation. Genome Biol 12, 1–18.
Silva, C.C., Silva, S.P., Prates, J.A., Bessa, R.J., Rosa, H.J., Rego, O.A., 2019. Physicochemical traits and sensory quality of commercial butter produced in the Azores. Int. Dairy J. 88, 10–17.
Skaar, I., Gaustad, P., Tønjum, T., Holm, B., Stenwig, H., 1994. Streptococcus phocae sp. nov., a new species isolated from clinical specimens from seals. Int. J. Syst. Evol. Microbiol. 44, 646–650.
Sun, L., Lundh, Å., Höjer, A., Bernes, G., Nilsson, D., Johansson, M., Hetta, M., Gustafsson, A.H., Saedén, K.H., Dicksved, J., 2022. Milking system and premilking routines have a strong effect on the microbial community in bulk tank milk. J. Dairy Sci. 105, 123–139.
Tannenbaum, I., Kaur, J., Mann, R., Sawbridge, T., Rodoni, B., Spangenberg, G., 2020. Profiling the *Lolium perenne* microbiome: from seed to seed. Phytobiomes J 4, 281–289.
Tavella, T., Rampelli, S., Guidarelli, G., Bazzocchi, A., Gasperini, C., Pujos-Guillot, E., Comte, B., Barone, M., Biagi, E., Candela, M., 2021. Elevated gut microbiome abundance of *Christensenellaceae, Porphyromonadaceae* and *Rikenellaceae* is associated with reduced visceral adipose tissue and healthier metabolic profile in Italian elderly. Gut Microbes 13, 1880221.
Timoney, J.F., 2022. Streptococcus. In: Gyles, C.L., Prescott, J.F., Songer, J.G., Thoen, C. O. (Eds.), Pathogenesis of Bacterial Infections in Animals, fourth ed. Blackwell Publishing, pp. 565–587.
Torjusen, H., Sangstad, L., O'Doherty Jensen, K., Kjærnes, U., 2004. European Consumers' Conceptions of Organic Food: A Review of Available Research, Professional Report No. 4-2004. National Institute for Consumer Research, Oslo, Norway, p. 147.
Tscharntke, T., Clough, Y., Wanger, T.C., Jackson, L., Motzke, I., Perfecto, I., Vandermeer, J., Whitbread, A., 2012. Global food security, biodiversity conservation and the future of agricultural intensification. Biol. Conserv. 151, 53–59.
Vithanage, N.R., Dissanayake, M., Bolge, G., Palombo, E.A., Yeager, T.R., Datta, N., 2016. Biodiversity of culturable psychrotrophic microbiota in raw milk attributable to refrigeration conditions, seasonality and their spoilage potential. Int. Dairy J. 57, 80–90.
Waters, J.L., Ley, R.E., 2019. The human gut bacteria *Christensenellaceae* are widespread, heritable, and associated with health. BMC Biol 17, 1–11.
Yuan, H., Han, S., Zhang, S., Xue, Y., Zhang, Y., Lu, H., Wang, S., 2022. Microbial properties of raw milk throughout the year and their relationships to quality parameters. Foods 11, 3077.