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Microbiological characterization of *Gioddu*, an Italian fermented milk

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Abstract

Gioddu, also known as "*Miciuratu"*, "*Mezzoraddu"*, or "*Latte ischidu"* (literally meaning acidulous milk), is the sole variety of traditional Italian fermented milk. The aim of the present study was to elucidate the microbiota as well as the mycobiota occurring in artisan *Gioddu* samples collected from three Sardinian producers by combining the results of viable counting on selective culture media and, high-throughput sequencing. Physico-chemical parameters were also measured. The overall low pH values (3.80-4.22) detected in the analyzed *Gioddu* samples attested the strong acidifying activity carried out by lactic acid bacteria during fermentation. Viable counts revealed the presence of presumptive lactococci, presumptive lactobacilli, and non-*Saccharomyces* yeasts. A complex (kefir-like) microbiota of bacteria and yeasts was unveiled through sequencing. In more detail, the dominance of *Lactobacillus delbrueckii* was highlighted in all the samples, thus representing the key species in *Gioddu* fermentation together with *Streptococcus thermophilus*, whose presence suggested the establishment of a yogurt-like protocooperation. Unexpectedly, samples from two out of the three producers revealed the presence of *Lactobacillus kefiri* in all the three analyzed batches, thus representing an absolute novelty and suggesting the presence of bioactive compounds (e.g. exopolysaccharides) similar with those present in milk kefir beverage. Mycobiota population, studied for the very first time, revealed a more complex population where *Kluyveromyces marxianus*, *Galactomyces candidum* and *Geotrichum galactomyces* constituted the core mycobiota. Further research is needed to disclose the presence in *Gioddu* of probiotic cultures and bioactive compounds (e.g. exopolysaccharides, angiotensin-converting enzyme inhibitory peptides, antimicrobial compounds) with potential health-benefits for the consumers.

Keywords: yeast-lactic fermentation, metagenomic sequencing, *Lactobacillus delbrueckii*, *Lactobacillus kefiri*, *Kluyveromyces marxianus*.

1. Introduction

Milk represents a perishable food and its fermentation has been from long time one of the main strategies to extend its shelf life. The history of milk fermentation dates to Sumerians, Babylonians, Pharos and Indians although the exact origin of such a practice is difficult to be established (Tamime, 2002). At the present time, fermented milks are produced at both industrial and artisan scale through complex processes that are usually based on a combination of ancient techniques and modern knowledge (biochemistry, enzymology, microbiology, physics and engineering) (Tamime, 2002). The production of fermented milks is obtained by the conversion of lactose in milk to lactic acid using starter cultures obtained from previous manufactures (back-slopping) or by direct inoculum of selected microbial strains. The microorganisms involved in the biochemical modifications of milk include lactic acid bacteria (the main microorganisms involved in the production of lactic acid) or a combination of lactic acid bacteria and eumycetes (generally yeasts) (Aryana and Olson, 2017; Cardinali et al., 2016; 2017). It is noteworthy that many lactic acid bacteria strains produce exopolysaccharides thus enhancing the rheological properties of the fermented milk and improving health benefits to consumers (Rahbar Saadat et al., 2019). Moreover, fermented milk can be a natural source of probiotic microorganisms able to lower risk and therapy support for gastrointestinal diseases, enhance the immune responses and maintain urogenital health although the interaction among probiotics, diet and host is still to be fully clarified (Ceapa et al., 2013).

Based on their physicochemical and microbiological characteristics, fermented milks can be tentatively classified as: i) milk products produced by mesophilic (e.g. cultured buttermilk) or thermophilic (e.g. yogurt) lactic acid fermentation; ii) milk products fermented by lactic acid bacteria and yeasts (e.g. kefir and koumis); milk products fermented by lactic acid bacteria and moulds (Tamime, 2002).

Many fermented dairy products are manufactured all over Italy in accordance with ancient local traditions and are considered by consumers as a heritage of undisputed value. *Gioddu*, also known as "*Miciuratu"*, "*Mezzoraddu"*, or "*Latte ischidu"* (literally meaning acidulous milk) is an acid-alcoholic fermented beverage produced in Sardinia (Italy) using ovine or goat milk (Maoloni et al., 2019). It is included in the official list of Sardinian traditional products published by the Italian Ministry of Agriculture and Forestry (G.U. Repubblica Italiana no. 168, 22/07/2015 Suppl. Ord. no. 43). The preparation of *Gioddu* from boiled or pasteurized milk is usually carried out via back-slopping using *Gioddu* obtained from previous productions. It is characterized by a porcelain white colour with a creamy consistency (firmer than cow's yoghurt) and acidic taste. The typical aroma of *Gioddu* reflects that of the milk of the species of origin.

To the authors' knowledge, only a few studies dealing with the characterization of the microbiota occurring in Gioddu have been carried out (Arrizza et al., 1983; Maoloni et al., 2019; Ortu et al., 2007). To date, a few lactic acid bacteria species have been identified together with concurrent yeast species, which have very recently been detected through Polymerase Chain Reaction–Denaturing Gradient Gel Electrophoresis (PCR-DGGE) analysis of the microbial DNA extracted directly from Gioddu samples (Maoloni et al., 2019). Notwithstanding, the microbiology of *Gioddu* is still far from being disclosed, being such an ancient fermented milk a still unlisted source of microbial diversity. Indeed, the most recent study on *Gioddu* has been carried out with the exclusive use of a culture-independent technique (PCR-DGGE) that, although still useful for the study of complex microbial communities, is characterized by well-known biases that usually allow the detection of the sole dominant species (Cocolin et al., 2013; Garofalo et al., 2017). Therefore, the aim of the present study was to elucidate the bacteria and yeast species occurring in artisan *Gioddu* samples collected from three Sardinian producers by combining the results of viable counting on selective culture media and high-throughput sequencing. Physico-chemical parameters of *Gioddu* were also evaluated.

2. Materials and methods

2.1. Gioddu production

The *Gioddu* samples analyzed in the present study were collected from three artisanal producers located in the Sardinia Region. For each producer (producer A, producer B and producer C), three different production batches (batch 1, batch 2 and batch 3), realized between March and May 2019, were analyzed. The production of *Gioddu* was carried out in accordance with local tradition using ovine milk from daily milking. Briefly, ovine milk was collected approximately after 2 hours of milking, strained, and boiled for about 7 min. The milk was then cooled down to 30 °C and inoculated via back-slopping using 2% of a *Gioddu* manufacture of the previous day. During the 12 h fermentation, the temperature of 30 °C was maintained and the final products were stored at 5 °C. For each sample, 100 g were collected immediately after the storage at 5 °C. No commercial starter cultures were added to ovine milk prior to coagulation.

Gioddu samples were produced in accordance with Good Hygiene Practices (GHP), although the artisanal dairy plants involved in the study showed some limitations due to their reduced production capacities.

2.2. Determination of pH

pH values of the *Gioddu* samples were measured by pH meter equipped with a solid HI2031 electrode (Hanna Instrument, Padova, Italy). For each sample, the measurements were performed in duplicate and the results were expressed as mean value ± standard deviation.

2.3. Viable counting

One mL of each *Gioddu* sample was added with 9 mL of a sterile peptone water (0.1% peptone, w/v) and homogenized by Stomacher 400 Circulator apparatus (VWR International PBI, Milan, Italy) at 260 rpm for 3 min. The homogenates (dilution 10^{-1}) were additionally ten-fold diluted and subjected to viable counts of lactic acid bacteria (LAB) (lactobacilli and lactococci), total and non-*Saccharomyces* yeasts. The enumeration of lactobacilli and lactococci was performed on de Man Rogosa and Sharpe (MRS) Agar (WVR, International, Leuven, Belgium) and M17 agar (Merck KGaA, Darmstadt, Germany), respectively, using pour plate method. The agar plates were incubated at 37°C under anaerobic conditions (Gas-Pack system, Oxoid). Both growth media were supplemented with 250 mg/L of cycloheximide to inhibit yeast growth.

Total viable yeasts were enumerated on WL-nutrient agar (Oxoid) and non-*Saccharomyces* yeasts on Lysine agar (Oxoid) after incubation at 25°C for 72 h. Both media were supplemented with 100 mg/L of chloramphenicol to inhibit the bacterial growth. The results were expressed as colony forming units (CFU) per mL of sample ± standard deviations.

2.4. DNA extraction, library preparation, and sequencing.

Total DNA was extracted from the pellet of 1ml of the first decimal dilution prepared for the viable counts by using E.Z.N.A. soil DNA kit (Omega bio-tek, Norcross, GA, USA) following the manufacturer's instructions.

Microbiota were studied by amplifying the V3 and V4 region of the 16S rRNA using primers and condition described by Klindworth et al. (2013). Mycobiota were evaluated by amplifying the D1 domain of the 26S using primers and condition described by Mota-Gutierrez et al. (2019). Library preparation was performed according to the illumina metagenomic procedure. Sequencing was performed by MiSeq instrument (Illumina) with V3 chemistry and generated 250-bp paired-end reads, following the producer’s instructions.

2.5. Bioinformatics

After sequencing reads were assembled, quality filtered and processed by using the QIIME 1.9.0 software (Caporaso et al., 2010), and the pipeline described by Ferrocino et al. (2017) (for 16S) and from Mota-Gutierrez et al. (2019) for the 26S. Centroids sequences of each cluster were manually check by Basic Local Alignment Search Tool (BLAST) to confirm the taxonomic assignment. QIIME was used to rarefy the operational taxonomic unit (OTU) table at the lowest number of sequences per sample and to build the OTU table. The OTU table displays the higher taxonomy resolution reached when the taxonomy assignment was not able to reach the species level, genus or family name was displayed.

The 16S and the 26S sequences are available at the Sequence Read Archive of the NCBI (SRA accession number SRP217105).

2.6. Color measurement

Chroma Meter CR-200 (Minolta, Japan) was used to define colorimetric profile of *Gioddu* samples determining lightness (L), redness-greenness (a*: + red; - green), yellowness-blueness (b*: + yellow; - blue) coordinates according to CIELab color space system, using a D65 light source. Moreover, the chromaticity of *Gioddu* samples was characterized calculating Chroma (C) and Whiteness Indexes (WI) with the following equation: $\sqrt{}\ (a^{*2} + b^{*2})$ and $100 - \sqrt{}\ (100\text{-}L)^2 + a^{*2} + b^{*2}$, respectively. The colorimetric readings were performed in triplicate for each *Gioddu* sample. Ultra-High Temperature (U.H.T.) goat milk was used as control.

2.7. Statistical analysis

The one-way analysis of variance (ANOVA) of colorimetric parameters was performed including *Gioddu* Producer (GP) as main effect. The GP had three levels: producer A, producer B, and producer C. The Tukey-Kramer test ($P \leq 0.05$) was carried out to detect differences through multiple mean comparisons. The statistical analysis was performed using JMP software (version 11.0). In addition, pH and bacterial count data collected were subjected to one-way ANOVA using JMP software (version 11.0), and differences were considered non-significant at $P < 0.05$. As a measure of the association between microbiota and mycobiota, the Spearman’s rank correlation coefficient was obtained through the function psych and plotted through the corrplot package of R (FDR < 0.05).

3. Results

3.1. Determination of pH

The results of pH values detected in the analyzed *Gioddu* samples are reported in Table 1. In more detail, pH values of samples from Producer A were comprised between 4.1±0.1 (batch 2) and 4.3±0.3 (batch 3), whereas values between 3.9±0.1 (batch 1) and 4.0±0.1 (batch 2) were detected in samples from Producer B. Samples from Producer C showed pH values that ranged between 3.5±0.3 (batch 2) and 4.0±0.1 (batch 1). Producer A showed significantly higher overall pH mean value, whereas no significant differences were detected between overall pH mean values of samples from Producer B and Producer C.

3.2. Viable counting

The results of viable counts are reported in Table 1. In more detail, for samples from Producer A, counts of presumptive lactococci ranged between 4.9±0.1 (batch 1) and 5.7±1.3 (batch 2) log cfu mL^{-1}, whereas for samples from Producer B, counts between 4.5±0.5 (batch 2) and 5.5±0.2 (batch 1) log cfu mL^{-1} were detected, as for samples from Producer C, values ranged between 4.5±0.7 (batch 1) and 4.7±0.3 (batch 3) log cfu mL^{-1}. Producer A showed significantly highest overall mean value, whereas no significant differences were detected between overall mean values of Producer B and Producer C.

Regarding presumptive lactobacilli, samples from Producer A showed counts comprised between 4.7±0.2 (batch 1) and 4.9±0.2 (batch 3) log cfu mL^{-1}. The counts from Producer B ranged between 2.9±0.1 (batch 3) and 5.9±0.6 (batch 1) log cfu mL^{-1}, whereas samples from Producer C showed viable counts between 5.1±0.8 (batch 1) and 6.5±0.2 (batch 2) log cfu mL^{-1}. Producer C showed significantly highest overall mean value, whereas no significant differences were detected between overall mean values of Producer A and Producer B.

As for total yeasts, values comprised between 5.6±0.2 (batch 3) and 6.3±0.1 (batch 1) log cfu mL^{-1} were detected in samples from Producer A, whereas, in samples from Producer B, yeasts counts ranged between 6.9±0.1 (batch 3) and 7.3±0.1 (batch 2) log cfu mL^{-1}. Finally, values between 7.3±0.1 and 8.4±0.1 log cfu mL^{-1} were detected in samples from Producer C, this latter showing significantly highest overall mean value and Producer A the lowest.

Finally, non-*Saccharomyces* yeasts detected in samples from Producer A showed counts comprised between 5.6±0.1 (batch 3) and 6.5±0.2 (batch 1) log cfu mL^{-1}. Counts from Producer B ranged between 6.7±0.1 (batch 1) and 7.3±0.3 (batch 2) log cfu mL^{-1}, whereas samples from Producer C showed counts between 7.2±0.1 (batch 1) and 8.1±0.1 (batch 2) log cfu mL^{-1}. Producer C showed significantly highest overall mean value, whereas overall mean values of Producer A were the lowest.

3.3. Microbiota composition

The total number of paired sequences obtained from the samples reached 336,855 raw reads. After quality filtering, a total of 237,722 reads were used, with an average value of 26,413 ± 7,239 reads/sample, and a mean sequence length of 465 bp. Alpha diversity index showed a satisfactory coverage for all samples (> 98%) however it did not show different level of complexity based on the producers.

A simple microbiota composition was observed (Figure 1) with the dominance of *Lactobacillus delbrueckii* (higher than 85% in all the samples), while *Streptococcus thermophilus* was observed only in Producer A (14% of the relative abundance) and Producer C (7%). Taking into the account the minor microbial composition it was possible to observed the presence of *Lactobacillus kefiri* and *Lactococcus lactis* only in producer B and producer C (2.7 and 0.05% for the first one and 2% and 2.4% for the second ones respectively). *Pseudomonas fragi* was only detected in Producer B samples (1.4% of the relative abundance).

3.4. Mycobiota composition

The total number of paired sequences obtained from the samples reached 577,662 raw reads. After quality filtering, a total of 509,845 reads were used, with an average value of 56,649 ± 31,359 reads/sample, and a mean sequence length of 366 bp. Alpha diversity index showed a satisfactory coverage for all samples (> 99%) however it did not show different level of complexity based on the producers.

Taking into the account the mycobiota composition at the highest taxonomic level (Figure 2) we can observe that samples from producer B and producer C has a higest degree of similarity characterizad by the predominance of *Kluyveromyces marxianus* (83 and 52% of the relative abundance in producer B and producer C respectively), *Galactomyces candidum* (11 and 34%) and by the presence of *Geotrichum galactomyces* (4 and 12% in producer B and producer C respectively). Samples from producer A showed a high presence of *Pichia cactophila* (45%), *Glomus hyderabadensis* (16%) and *Saccharomyces cerevisiae* (7 %). The presence of several minor taxa belonging to *Alternaria*, *Cladosporium* and *Aerobasidium* was also observed.

3.5. Correlations between microbiota and mycobiota profile

Plotting the correlation between the microbiota and mycobiota (Figure 3, FDR<0.05), it was observed that the presence of *L. delbrueckii* exclude the presence of *Alternaria tenuissima* and *Aerobasidium* while the presence of the minor lactic acid bacteria (*L. lactis* and *L. zeae*) were associated with the prensence of *Galactomyces candidum* and *Geotrichum galactomyces.*

3.6. Color measurement

Results of color measurements carried out on *Gioddu* samples are reported in Supplementary Table 1. Ultra-High Temperature (U.H.T.) goat milk, used as control, showed the following colorimetric profile: L 90.52±0.01; a* -7.68±0.02; b* 9.63±0.01; C 12.83±0.90; WI 84.46±0.01. ANOVA results, reported in Supplementary Table 1, showed significant difference between producers only for Lightness (L) parameter. Goat milk, used as a reference control, resulted with a higher Lightness (L), redness (a*) and WI compared to the three different dairy products. *Gioddu* made by Producer B and Producer C highlighted significantly higher mean values for L than the fermented dairy products manufactured by the Producer A.

4. Discussion

Sardinia inhabitants live in a geographical hot spot of exceptional longevity known as the “Longevity Blue Zone” (Pes et al., 2015). As reviewed by Pes et al. (2015) it is likely that, besides genetically predisposition, such an enviable status could be based on the availability of high-quality foods including traditional fermented products of milk-origin. Indeed, it is known that dairy fermented products are natural carriers of beneficial microorganisms (and their metabolites) with potential health benefits for the consumers (Şanlier et al., 2019).

Regarding pH, the detected values were in accordance with those reported by Maoloni et al. (2019) in *Gioddu* samples; to the authors’ knowledge no other studies reporting *Gioddu* pH values are available in the scientific literature for further comparison of data. The detected values agreed with those reported for sheep milk traditionally fermented at 37°C that showed pH values of 3.99–3.73 (Nadelman et al., 2017); the detected values were also in accordance with pH values reported for *Dahi*, a traditional fermented milk produced in Bangladesh, that showed pH values of 3.9-4.0 (Nahidul-Islam et al., 2018). *Gioddu* pH values were lower than those reported for ovine milk kefir that is usually fermented to pH 4.6–4.7 (Tamime et al., 2011). The overall low pH values detected in the analyzed *Gioddu* samples attested the strong acidifying activity carried out by lactic acid bacteria along fermentation.

Regarding viable counts, the occurrence of both presumptive lactococci and lactobacilli was in accordance with the results reported by Arrizza et al. (1983) on the same dairy product, where both these two groups of lactic acid bacteria were concurrently detected. Lactic acid bacteria represent the most extensively studied microorganisms for milk fermentation. Species belonging to this group are well-adapted to the milk environment where they produce organic acids (e.g. lactic and/or acetic acid) and secondary metabolites with potential antimicrobial and/or bioactive properties (Widyastuti et al., 2014). The counts of lactic acid bacteria detected in the present study were generally lower than those reported for fermented milks by other authors, where average counts usually attested at > 8 log cfu mL^{-1} (Surono and Hosono, 2011). Interestingly, the relatively low lactic acid bacteria counts did not reflect in the low pH values (3.80-4.32) detected in the analyzed *Gioddu* samples at the end of fermentation, thus suggesting that among the detected species, strong acidifier were present, although at relatively low cell numbers. It is noteworthy that, regarding lactic acid bacteria, there are no growth media able to sufficiently select for the growth of the sole lactococci or lactobacilli, thus limiting the information obtained by classical methods. Hence, the use of culture-independent methods in combination with agar media appears particularly convenient (Vera et al., 2009).

As for the occurrence of eumycetes, to the authors' knowledge no previous data reporting the counts of yeast in *Gioddu* are available in the scientific literature for further comparison of data. Interestingly, the counts of total eumycetes (including *Saccharomyces* species) and non-*Saccharomyces* yeast were almost overlapping, thus confirming the results of metagenomic sequencing where *Saccharomyces* species were minority. The counts of yeasts in the analyzed *Gioddu* samples were almost in accordance with those reported by Kebede et al. (2007) in *Sethemi*, a South African spontaneously fermented milk, where counts of approximately 6 log cfu mL^{-1} were detected. Yeast counts detected in *Gioddu* were also comparable with those reported by Kim et al. (2018a) and Guzel-Seydim et al. (2005) in milk kefir beverage that showed values of 6 log cfu mL^{-1}, thus suggesting similarities between *Gioddu* and this well-known health-promoting beverage, although the relative proportion of each microbial group should also be considered.

To the authors' knowledge the sole recent study dealing with the microbial diversity of *Gioddu* reported the presence of major taxa detected in samples obtained from two producers located in Sardinia (Maoloni et al., 2020). Although the study by Maoloni et al. (2020) shed a first light on the occurrence of both bacterial and fungal species, neither data on the relative abundances of the detected microorganisms nor minority species were reported. Hence, the metataxonomic approach applied in the present allowed major and minor taxa to be detected for the first time. In more detail, *L. delbrueckii* was detected as dominant species in all the analyzed samples. The occurrence of this lactic acid bacterium species has already been reported by Maoloni et al. (2019) in *Gioddu* samples analyzed via PCR-DGGE. The presence of this species has recently been reported by Jiang et al. (2020) in traditional fermented yak milk where *L. delbrueckii* was detected with relative abundance up to 99%. Moreover, *L. delbrueckii* was also detected by Raveschot et al. (2020) as part of the major microbiota occurring in Mongolian traditional dairy products. The species *L. delbrueckii* includes lactobacilli that produces the D(-) isomer of lactic acid from lactose or from other carbohydrates. This milk-adapted species contributes to characterize the aroma of fermented milks due to the production of secondary compounds including acetaldehyde. As reported by Rizzello and De Angelis (2016), *L. delbrueckii* produces exopolysaccharides and bacteriocins that could influence the nutritional, safety and rheological aspects of the final product. Interestingly, Tang et al. (2020) recently reported that exopolysaccharides produced by *L. delbruecckii* ssp. *bulgaricus* could also exert prebiotic activity, although the functionality of these compounds should be confirmed through *in vivo* studies.

S. thermophilus has been detected in samples from Producer A and Producer C, thus suggesting the occurrence of a yogurt-like protocooperation between *S. thermophilus* and *L. delbrueckii*. *S. thermophilus* is a thermophilic aerotolerant-anaerobe bacterium with an optimal growth temperature of 42 °C. In fermented milks, *S. thermophilus* is responsible for the production of lactic acid, together with secondary products as acetaldehyde and diacetyl that characterize the aroma and texture of the end products (Uriot et al., 2017). During lactic acid fermentation, the production of formate and CO_2 by *S. thermophilus* stimulates *L. bulgaricus* growth that, in turn, releases peptides and amino acids from milk proteins, thus improving the growth of *S. thermophilus* (Sieuwerts et al., 2008). The presence of *S. thermophilus* in *Gioddu* has already been reported by Arrizza et al. (1983), although such lactic acid bacterium was not constantly detected in the core microbiota.

Of note the presence of *L. kefiri* in all the samples from the 3 batches collected from both producer B and Producer C. To the authors' knowledge, *L. kefiri* has usually been associated with kefir beverage, being part of the dominant microbiota of such health-promoting fermented milk (Garofalo et al., 2015; Slattery et al., 2019). Indeed, as reported by Sharifi et al. (2017) the consumption of milk kefir could exert antibacterial, antifungal, anti-allergic and anti-inflammatory effects. Moreover, bioactive compounds produced by kefir microbiota as polysaccharides and peptides could inhibit proliferation of colorectal, breast and lung tumor cells (Sharifi et al., 2017). In kefir beverage, *L. kefiri*, together with *L. kefiranofaciens*, *L. kefirgranum*, *L. parakefiri*, releases exopolysaccharides with potential antioxidant, antitumor, antimicrobial, and immunomodulating properties (Prado et al., 2015), such exopolysaccharides (EPS) production is boosted by the complex symbiosis with yeasts that occur in kefir beverage. As reviewed by Slattery et al. (2019), *L. kefiri* strains could exert inhibitory effect on the growth of a wide range of human foodborne pathogens as *Pseudomonas aeruginosa*, *Salmonella* Enteritidis, *Listeria monocytogenes*, *Bacillus cereus*, *Staphylococcus aureus*, and *Cronobacter sakazakii*, this latter with particular risk to infants (Kim et al., 2018b). Moreover, a significant *in vitro* and *in vivo* cholesterol reducing activity was also reported for *L. kefiri* strain DH5 by Kim et al. (2017). Based on such evidences, it could be supposed that analogous activities of *L. kefiri* might be exerted by strains detected in the microbiota of *Gioddu*, although further research is needed to verify viability and EPS production in the analyzed samples. In addition to this, it is also essential to isolate and identify this microorganism from *Gioddu*. Although found with low frequencies, the detection of *L. kefiri* in *Gioddu* represents an absolute novelty and suggests that this fermented milk could be the source of still unknown beneficial effects on the health of consumers.

As for *L. lactis*, detected with low abundances in samples from Producer B and Producer C, the presence of this lactic acid bacteria has already been reported in fermented milks (including buttermilk and sour cream) by different authors (Cavanagh et al., 2015; McNulty et al., 2011; Yang et al., 2013; Zhang et al., 2019). *L. lactis*, that represents one of the key lactic acid bacteria species in the dairy industry, produces lactic acid from lactose and flavour compounds from milk-proteins proteolysis (Cavanagh et al., 2015). Moreover, it is responsible for the production of EPS, these latter strongly related with texture development (van Hylckama Vlieg et al., 2006). Interestingly, a protocooperation between *L. lactis* and *Kluyveromyces lactis* in *Lben* product, a traditional Moroccan fermented milk, has been reported (Mangia et al., 2014).

Analogously to milk kefir, populations of eumycetes were detected in all the analyzed samples. In more detail, *K. marxianus* constituted the core mycobiota of samples from Producer B and Producer C, and a small fraction of yeasts detected in samples from Producer A. The detection of such yeast species is in accordance with preliminary studies carried out by Maoloni et al. (2019) in *Gioddu* samples through PCR-DGGE. Moreover, *K. marxianus* strains with potential probiotic features have recently been isolated by Fadda et al. (2017) from *Fiore Sardo*, a typical Sardinian hard cheese. *K. marxianus* has also been detected in homemade Chinese koumiss (Ni et al., 2007), in different French cheeses produced with raw milk (Callon et al., 2006), in African artisanal yoghurt and in traditionally fermented milks (Maïworé et al., 2019). *K. marxianus* is a dairy yeast that represents a sister species to *Kluyveromyces lactis* and is phylogenetically related to *S. cerevisiae* (Lane and Morrissey, 2010). *K. marxianus* is capable to assimilate lactose through β-galactosidase that hydrolyses this sugar to glucose and galactose, thus using these sugars as carbon source (Lane and Morrissey, 2010). This probiotic yeast could decrease cholesterol level, exert antifungal, antibacterial, anti-inflammatory activity producing pro-inflammatory cytokines, thus reducing local and systemic inflammations (Pacini and Ruggiero, 2017; Şanlidere Aloğlu et al., 2016; Xie et al., 2015). Moreover, Rahbar Saadat et al. (2020) recently described the inhibitory role of the EPSs produced by *K. marxianus* on colorectal cancer.

As for *G. candidum*, such filamentous yeast-like fungus, that represents the teleomorphic state of *Geotrichum candidum*, has already been detected in *Gioddu* by Maoloni et al. (2019) through PCR-DGGE. *G. candidum* has recently been isolated by Maïworé et al. (2019) in African fermented milks where it predominated among the detected mycobiota. Interestingly, species belonging to the genus *Galactomyces* were recenlty detected by Araújo-Rodrigues et al. (2019) in *Serpa* PDO cheese, a raw ewes' milk cheese coagulated with extracts of *Cynara cardunculus* L., and in traditional fried cottage cheese (Grygier et al., 2020). It is noteworthy that members of the genus *Galactomyces* showed probiotic potential (Oliveira et al., 2017) as well as the capability to release bioactive peptides (e.g. angiotensin-converting enzyme inhibitory peptides) from milk proteins (Ahtesh et al., 2018).

Regarding *G. galactomyces*, to the authors' knowledge no previous reports on the presence of such species in fermented milks or cheeses are available in the scientific literature for further comparison of data. To the *Geotrichum* species belongs acid-tolerant yeast-like fungi that are commonly detected in many mold-ripened, smear-ripened, and acid-coagulated cheeses (Eliskases-Lechner et al., 2011). Species belonging to the genus *Geotrichum* (e.g. *Geotrichum candidum)* have already been detected in *Armada*, a traditional Spanish cheese from goat milk, showing proteolytic and/or lipolytic capacity, thus conferring a strong "goat aroma" to the end product (Sacristán et al., 2012).

Pichia cactophila stronlgy characterized the mycobiota of *Gioddu* samples collected from Producer A. The presence of such yeast species in *Gioddu* has already been reported by Maoloni et al. (2019), moreover, strains of *P. cactophila* have been isolated by Aponte et al. (2010) in Mozzarella cheese produced with water buffalo milk. *Pichia* species have also been detected in artisanal *Fiore Sardo* cheese (Fadda et al., 2004) and in African artisanal yoghurts and traditional fermented milks (Maïworé et al., 2019), thus confirming the adaptation of such yeast genus to the dairy environment. Notwithstanding, the detection of *P. cactophila* in dairy products is rare, being this yeast commonly associated with necrotic stems of cacti (Moraes et al., 2005). Hence, it is likely that the occurrence of *P. cactophila* in the analyzed samples could be derived from environmental contamination.

Glomus hyderabadensis was detected in samples from Producer A and Producer B. To the authors' knowledge, this study reports the first detection of *G. hyderabadensis* in *Gioddu* and more generally in dairy products. This mycorrhizal fungus is commonly associated with rhizosphere soils; no reports on the occurrence of *Glomus* species in the food environment are actually available in the scientific literature (Rani et al., 2004). It is likely that the occurence of *G. hyderabadensis* in *Gioddu* could be related to environmental contamination of the dairy environment.

Finally, *S. cerevisiae* was detected in samples from producer A and, at very low frequencies, in samples from Producer B. The presence of such a yeast species has already been reported by several authors in milk kefir grains (Diosma et al., 2014; Gao et al., 2012; Garofalo et al., 2015; Zhou et al., 2009). Although unable to ferment lactose, *S. cerevisiae* is able to ferment galactose and could advantage by the presence of concurrent microbial species that release such a sugar through *β*-galactosidase activity. Moreover, *S. cerevisiae* can grow on lactate produced by lactic acid bacteria, as already described in kefir (Sieuwerts et al., 2018), thus explaining its presence in the analyzed *Gioddu* samples.

Regarding colorimetric parameters (L, a*, b*) of *Gioddu*, the WI, expressing the preferences of consumers for white colors, is frequently used for the evaluation of many dairy products (Ghasemlou et al., 2011; Gul et al., 2018) since it is a synthetic expression of lightness perception and color coordinates into a single term (Pathare et al., 2013). However, as referred by Vargas et al. (2008), the WI can be affected by several physical-chemical parameters of milk as fat globules dimension, casein ratio, and casein micelles aggregations in the end product. Therefore, the mean values observed in the present study resulted very uniform and could be affected by the properties of the raw milk used to obtain *Gioddu*.

Conclusions

Based on the results of the metataxonomic approach, applied for the first time to *Gioddu*, the analyzed samples were characterized by a complex (kefir-like) microbiota of bacteria and yeasts where previously undetected species were found. Specifically, *L. delbrueckii* was detected at very high levels in all the samples, thus representing the key species in *Gioddu* fermentation together with *S. thermophilus*, whose presence suggested the establishment of a yogurt-like protocooperation. Unexpectedly, samples from two out of the three producers revealed the presence of *L. kefiri* in all the three analyzed batches, thus representing an absolute novelty and suggesting the presence of bioactive compounds (e.g. EPS) similar with those present in milk kefir beverage. Eumycetes population, studied for the first time in this fermented milk product, revealed a more complex mycobiota where potentially probiotic species, including *K. marxianus*, were detected. The data overall collected opened new research horizons aimed at discovering the presence in *Gioddu* of probiotic cultures that, through isolation, could be exploited in other fermented food products. Moreover, the presence of bioactive compounds (e.g. EPS, angiotensin-converting enzyme inhibitory peptides, antimicrobial compounds, etc.) with potential health-benefits for the consumers can also be hypothesized. Further research is needed to establish the stability of the microbial consortium throughout the back-slopping process.

Of note, the presence of *Pseudomonas* and eumycetes as *P. cactophila*, *G. hyderabadensis*, *Alternaria*, *Cladosporium* and *Aerobasidium* suggested the need for an improvement of hygiene practices during *Gioddu* manufacturing.

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Conflict of interest

The authors declare that they have no conflict of interest.

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FIGURE LEGENDS

Figure 1. Relative abundance of the microbiota detected in *Gioddu* samples. Only OTUs which showed an incidence above 0.2% in at least 2 samples are shown.

Figure 2. Relative abundance of the mycobiota detected in *Gioddu* samples. Only OTUs which showed an incidence above 0.5% in at least 2 samples are shown

Figure 3. Correlation plot showing Spearman's correlation between microbiota and mycobiota composition. Only significance associations are shown (FDR< 0.05). The intensity of the colors represents the degree of correlation, where the color blue represents a positive degree of correlation and red a negative correlation.

Table 1. Viable counts (log cfu/mL) and pH values of *Gioddu* samples collected from the three producers (A, B, and C).

	Producer A				**Producer B**				**Producer C**			
	Batch 1	Batch 2	Batch 3	Overall mean	Batch 1	Batch 2	Batch 3	Overall mean	Batch 1	Batch 2	Batch 3	Overall mean
Presumptive lactococci	4.9±0.1	5.7±1.3	5.5±0.8	5.4±0.83^{a}	5.5±0.2	4.5±0.5	4.9±0.6	4.9±0.6^{b}	4.5±0.7	4.6±0.9	4.7±0.3	4.6±0.6^{b}
Presumptive lactobacilli	4.7±0.2	4.6±0.8	4.9±0.2	4.8±0.48^{b}	5.9±0.6	5.4±0.1	2.9±0.1	4.7±1.4^{b}	5.1±0.8	6.5±0.2	5.8±0.7	5.8±0.8^{a}
Total yeasts	6.3±0.1	5.8±0.2	5.6±0.2	5.9±0.40^{c}	6.8±0.1	7.3±0.1	6.9±0.1	7.1±0.2^{b}	7.3±0.1	8.4±0.1	7.8±0.2	7.8±0.5^{a}
Non-*Saccharomyces* yeasts	6.5±0.2	5.8±0.1	5.6±0.1	5.9±0.41^{c}	6.7±0.1	7.3±0.3	6.9±0.3	6.9±0.2^{b}	7.2±0.1	8.1±0.1	7.7±0.2	7.7±0.1^{a}
pH	4.3±0.1	4.1±0.1	4.3±0.3	4.2±0.13^{a}	3.9±0.1	4.0±0.1	3.9±0.1	3.9±0.1^{b}	4.0±0.1	3.5±0.3	3.8±0.1	3.8±0.1^{b}

Means ± standard deviations of triplicate independent experiments are shown.
Within each row, overall means with different superscript letters are significantly different ($P < 0.05$).
cfu, colony forming units.

Highlights

Microbiota of *Gioddu* samples collected from three Sardinian producers was analyzed

Lactococci, lactobacilli, and non-*Saccharomyces* yeasts were detected

The dominance of *Lactobacillus delbrueckii* was highlighted in all the samples

The presence of *Lactobacillus kefiri* was revealed in the majority of samples

Kluyveromyces, *Galactomyces*, and *Geotrichum* constituted the core mycobiota

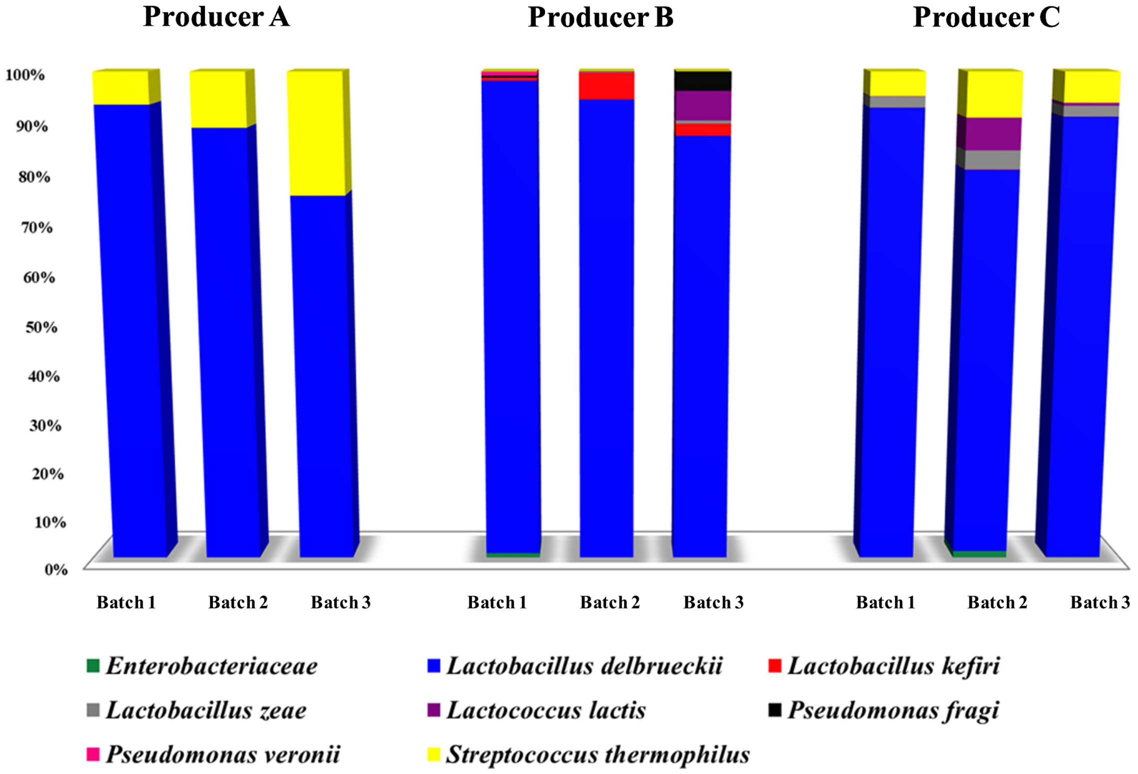
Producer A
Producer B
Producer C
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Batch 1
Batch 2
Batch 3
Batch 1
Batch 2
Batch 3
Batch 1
Batch 2
Batch 3
Enterobacteriaceae
Lactobacillus delbrueckii
Lactobacillus kefiri
Lactobacillus zeae
Lactococcus lactis
Pseudomonas fragi
Pseudomonas veronii
Streptococcus thermophilus

Figure 1

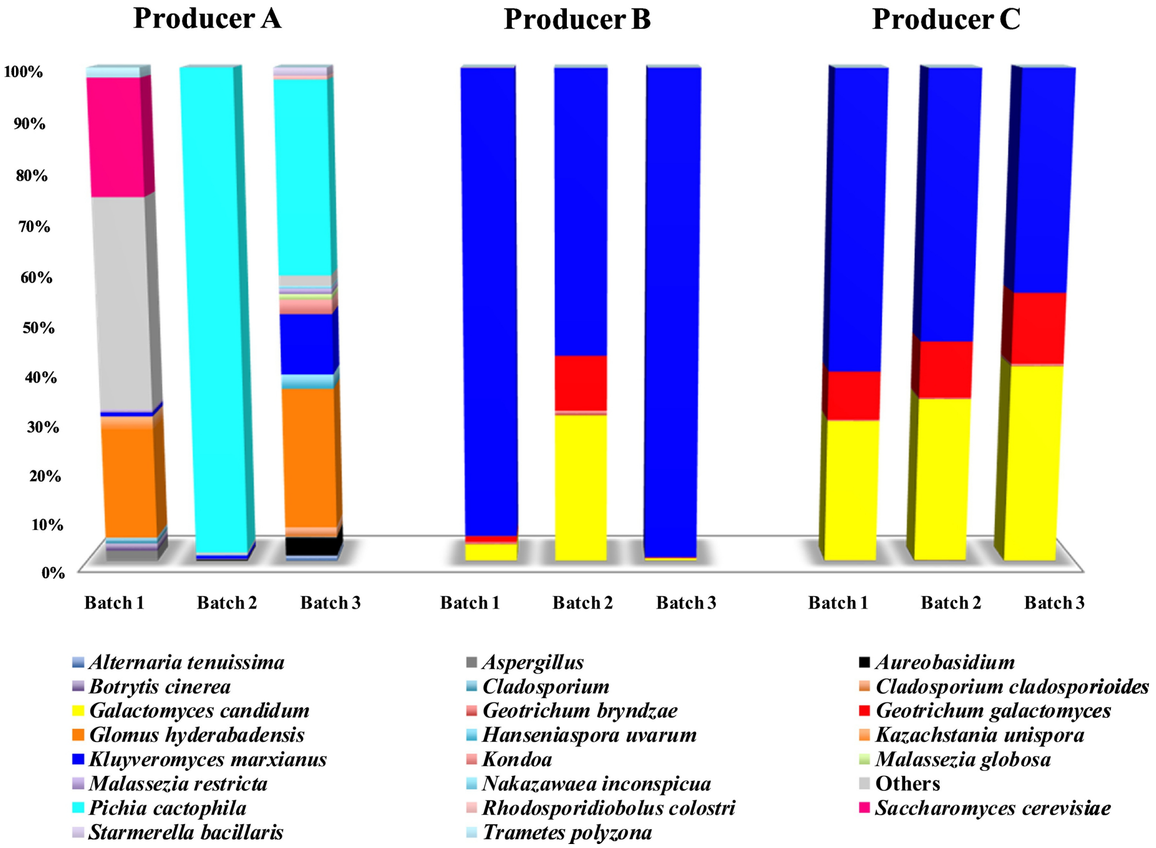
Producer A
Producer B
Producer C
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Batch 1
Batch 2
Batch 3
Batch 1
Batch 2
Batch 3
Batch 1
Batch 2
Batch 3
Alternaria tenuissima
Aspergillus
Aureobasidium
Botrytis cinerea
Cladosporium
Cladosporium cladosporioides
Galactomyces candidum
Geotrichum bryndzae
Geotrichum galactomyces
Glomus hyderabadensis
Hanseniaspora uvarum
Kazachstania unispora
Kluyveromyces marxianus
Kondoa
Malassezia globosa
Malassezia restricta
Nakazawaea inconspicua
Others
Pichia cactophila
Rhodosporidiobolus colostri
Saccharomyces cerevisiae
Starmerella bacillaris
Trametes polyzona

Figure 2

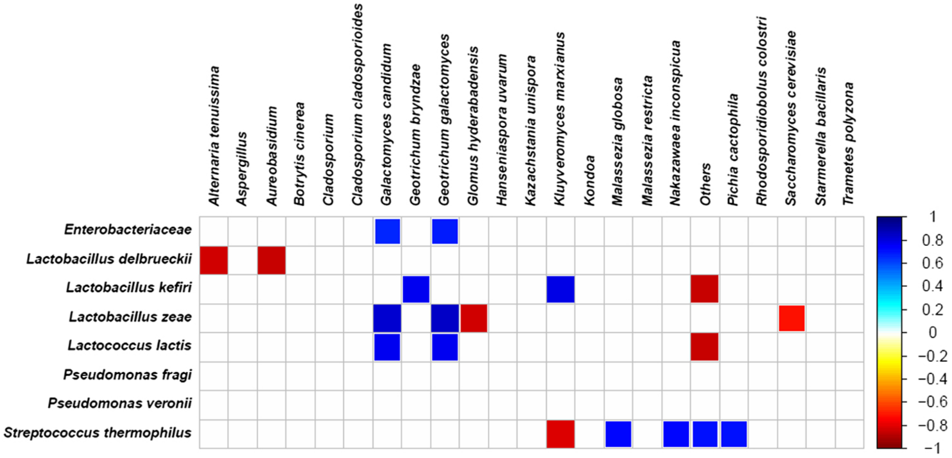
Alternaria tenuissima
Aspergillus
Aureobasidium
Botrytis cinerea
Cladosporium
Cladosporium cladosporioides
Galactomyces candidum
Geotrichum bryndzae
Geotrichum galactomyces
Glomus hyderabadensis
Hanseniaspora uvarum
Kazachstania unispora
Kluyveromyces marxianus
Kondoa
Malassezia globosa
Malassezia restricta
Nakazawaea inconspicua
Others
Pichia cactophila
Rhodosporidiobolus colostri
Saccharomyces cerevisiae
Starmerella bacillaris
Trametes polyzona
Enterobacteriaceae
Lactobacillus delbrueckii
Lactobacillus kefiri
Lactobacillus zeae
Lactococcus lactis
Pseudomonas fragi
Pseudomonas veronii
Streptococcus thermophilus
1
0.8
0.6
0.4
0.2
0
−0.2
−0.4
−0.6
−0.8
−1

Figure 3