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Communication

Microbial Populations of Fresh and Cold Stored Donkey Milk by High-Throughput Sequencing Provide Indication for A Correct Management of This High-Value Product

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Abstract: Donkey milk is receiving increasing interest due to its attractive nutrient and functional properties (but also cosmetic), which make it a suitable food for sensitive consumers, such as infants with allergies, the immunocompromised, and elderly people. Our study aims to provide further information on the microbial variability of donkey milk under cold storage conditions. Therefore, we analysed by high-throughput sequencing the bacterial communities in unpasteurized donkey milk just milked, and after three days of conservation at 4 °C, respectively. Results showed that fresh donkey milk was characterized by a high incidence of spoilage Gram-negative bacteria mainly belonging to *Pseudomonas* spp. A composition lower than 5% of lactic acid bacteria was found in fresh milk samples, with *Lactococcus* spp. being the most abundant. The occurrence of microbial species belonging to risk group 2 was found in fresh milk. After three days of cold storage, the bacterial biodiversity of donkey milk was strongly reduced, since about 93% of the bacterial communities were identified as different species of psychrotrophic *Pseudomonas*. In conclusion, we report a preliminary description of the microbial diversity of donkey milk by using a metagenomic approach and encouraging a correct exploitation of this high-value niche product.

Keywords: donkey milk; *Pseudomonas*; psychrotrophic bacteria; high-throughput sequencing

1. Introduction

Donkey milk is a minor dairy production which is gaining growing interest and international acceptance mostly for human consumption, but also for the production of beauty products [1]. The current trends in the donkey industry in Europe show an increase of donkey breeds reared in Italy [2]. Moreover, the revaluation of equine milk for human consumption could contribute to the rural eco-sustainable development for the micro-economies of those areas threatened by marginalization [3]. Today, donkey milk is mainly marketed as raw, pasteurized, or freeze-dried [2,4]. Moreover, in the last few years, donkey milk has been proposed as an ingredient for the production of functional fermented beverages [5], and cheeses [6].

Due to its physico-chemical composition and nutritional quality similar to human milk, donkey milk is mainly used for feeding infants who suffer from a cows' milk protein allergy, or immunocompromised

elderly people [7,8]. However, due to the small production and target market of the consumers, potential safety hazards, including the bacterial quality of raw donkey milk, should be subjected to stricter regulation [9]. Indeed, due to its high nutritional content, milk can support a rich microbiota, including beneficial, spoilage, and pathogenic microorganisms [10].

Over the last few years, next-generation high-throughput sequencing (HTS) has been extensively used for the determination of microbial communities in milk and dairy products, in order to identify microorganisms that are difficult to culture or present at low concentration [11,12]. However, few studies aimed to elucidate the composition of the microbiota of non-cow milk and the corresponding products [13]. To date, knowledge on the microbiota of donkey milk is limited to only a few studies, mainly addressed by culture-dependent tools [14,15] and, only recently, by a metagenomic approach [16].

It is well known that cold storage could encourage the growth of spoilage psychrotrophic bacteria with detrimental effects on the quality of raw milk and its processing [17,18]. In the last few years, some studies evaluated the impact of refrigeration and storage on raw cow milk bacterial communities by using molecular tools [19,20]. However, no information is available on the progress of the microbial population in unpasteurized donkey milk stored at refrigerated conditions, even if recent studies suggest the possible exploitation of unpasteurized donkey milk to produce cheeses [6].

Therefore, in order to increase the knowledge of the microbial diversity associated with donkey milk, in this short communication we reported on the bacterial communities in fresh raw donkey milk, and of the same matrix after three days of cold storage.

2. Materials and Methods

2.1. Milk Sampling

Milk was sampled in the spring season from 10 healthy jennies, at the mid-period of lactation routinely milked by a mechanical milker, bred on a farm located in Apulian region (Martina Franca, Italy). Milk samples were collected in sterile 500 mL polypropylene centrifuge bottles with a seal cap (ProLab Supply, Miami Lake, US), and transported to our laboratory at refrigerated conditions. Three aliquots of the sample were immediately processed, while three aliquots were stored at 4 °C for three days before extracting microbial DNA.

2.2. Microbiological Analysis by Culture-Dependent Methods

Samples of fresh and cold-stored milk were submitted to serial decimal dilution by using a sterile saline solution (8.6 g L^{-1} NaCl) and spread onto PCA Petri dishes (Oxoid, Basingstoke Hampshire, UK). Colonies were enumerated after incubation at 25 °C for 48 h in aerobic conditions.

2.3. DNA Extraction

In order to isolate high-quality genomic DNA, samples were first submitted to a treatment to solubilize caseins, as reported by Fernández de Palencia et al. [21]. Milk samples of 50 mL were acidified with HCl 0.1M to achieve a pH of 6.5. Then, trisodium citrate (Sigma Aldrich, St Louis, MO, USA) was added at a concentration of 1% (w/v), and samples incubated at 4 °C for 15 min by manually shacking each 5 min. Finally, microbial cells were recovered by centrifugation (10,000× *g*, 10 min) and resuspended into 2 mL of sterile saline solution (8.6 g L^{-1} NaCl). Then, the extraction of genomic DNA was performed by using the Power Food Microbial DNA Isolation kit (Mio Bio Laboratories, Carlsbad, CA, USA), according to the manufacturer's instructions. The DNA concentration was quantified by using a BioTek Eon spectrophotometer (BioTek, VT, USA) and its integrity checked by visualization on 1.2% agarose gel. Samples were stored at −80 °C.

2.4. Amplicon Library Preparation and Pyrosequencing

Sequencing was performed on a MiSeq platform (Illumina, San Diego, CA) at LifeSequencing (Paterna, Spain). The bacterial composition was detected by amplification of the 16S rRNA hypervariable region V3-V4 [22].

2.5. Bioinformatics and Statistical Analysis

Forward and reverse sequences obtained from the MiSeq sequencing platform were merged using Pear 0.9.6 server tools [23]. Then, a quality filter was applied to delete sequences with poor quality. Bases in extreme positions with a Phred score < Q20 were removed, as well as sequences with a quality lower than a threshold of 20. The primers from the sequences obtained in the sequencing step were trimmed for the amplification primers in order to reduce the bias in the annotation step with Cutadapt 1.8.1 software tools [24]. Finally, sequences shorter than 300 bp were deleted, while Uchime algorithm was applied to remove chimera sequences [25]. Finally, to reduce the complexity of the annotation, sequences were clustered at a 97% similarity by using Cd-hit tools [26].

3. Results and Discussion

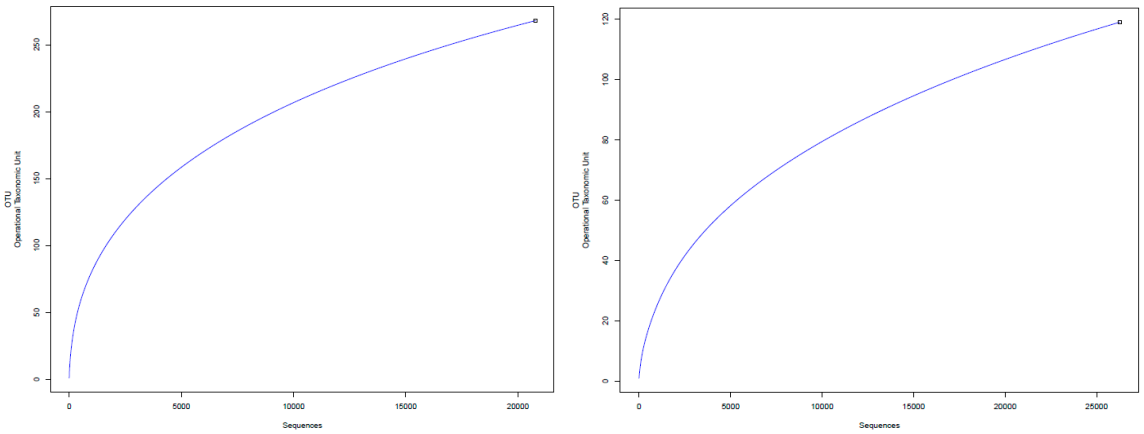
3.1. Sequences Analysis

In the present work, the microbial communities of non-pasteurized donkey milk were analysed by high-throughput pyrosequencing. Partial 16S rRNA gene sequencing was obtained from DNA extracted after milking, and after three days of cold storage at 4 °C. After quality control, a total of 49,624 high-quality 16S rRNA gene sequences with an average length of about 450 bp were recovered, which were clustered at genera taxonomic level in 387 OTUs. The Shannon index was calculated based on 3% genetic distance for the samples. Reads distribution and Shannon values are shown in Table 1.

Table 1. Sample information, sequence abundance and quality, observed diversity richness (OTUs), and microbial diversity index (Shannon) for 16S rRNA amplicons of fresh donkey milk and cold-stored.

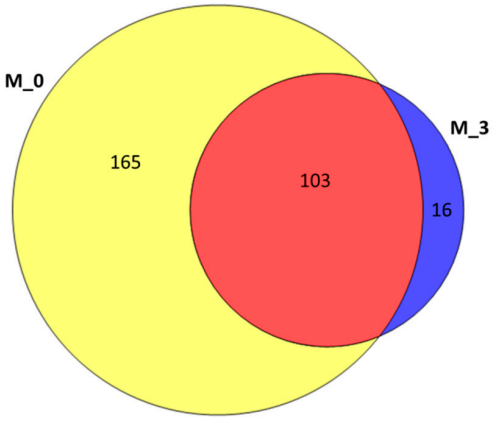
Sample	Sequences	Av. Length	Total Mb	Av. Quality	OTUs	Shannon Value
M_{t0}–16S	22,483	443.12	9.96	35.69	268	2.580
M_{t3}–16S	27,141	446.46	12.12	36.07	119	0.478

Rarefaction analysis at genera taxonomic level indicated a satisfactory coverage of the microbial diversity within the samples analysed (Figure 1A,B).



(**A**) (**B**)

Figure 1. *Cont.*



(C)

Figure 1. Rarefaction curves of bacterial population at genera taxonomic level for fresh (**A**) and cold stored (**B**) milk samples. Venn diagram (**C**) of the number of unique OTUs in fresh (M_0/yellow) and cold stored milk (M_3/blue). Shared OTUs are in red.

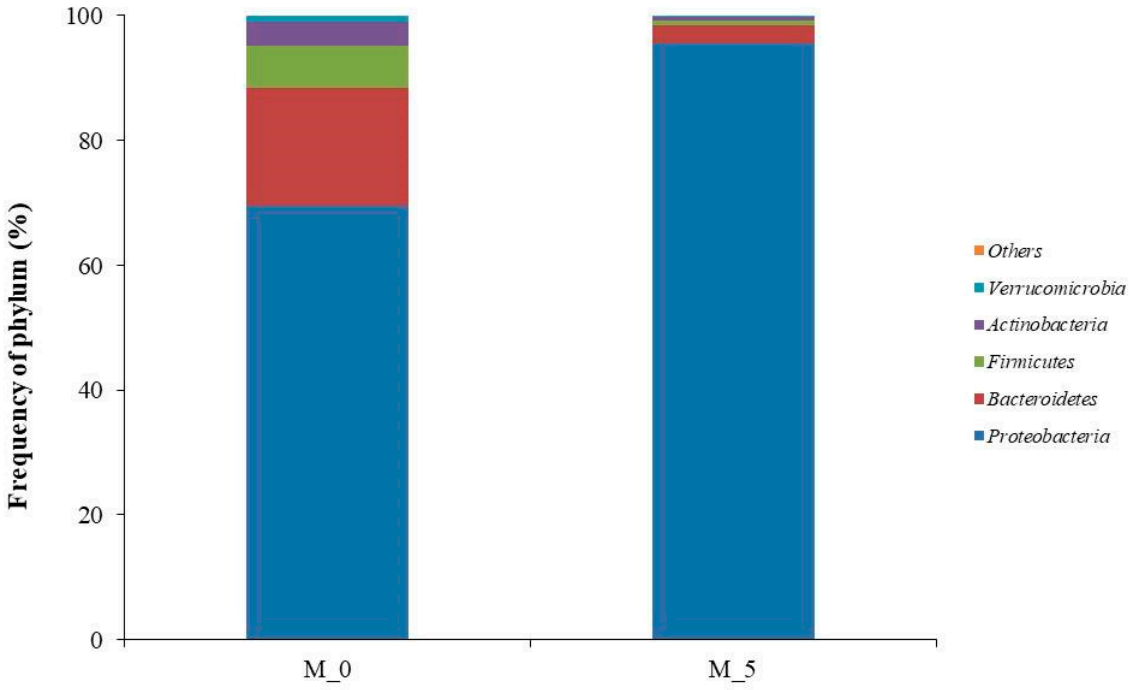
At genera taxonomic level, the number of OTUs and Shannon index for bacterial populations were about two- and five-fold higher in fresh milk than in cold-stored milk. A total of 103 OTUs were found to be shared by the two samples, which was about 87% of the OTUs in stored milk (Figure 1C), indicating that only a few bacterial populations of fresh milk could be detected after three days of cold storage. In contrast, Porcellato et al. [20] reported that the bacterial composition of pasteurized milk samples during storage at 4 °C remained stable throughout the product shelf life, while storage at 8 °C significantly increased the abundance of OTUs, indicating the importance of prompt treatment to increase the microbial stability of milk.

3.2. Analysis of Bacterial Diversity

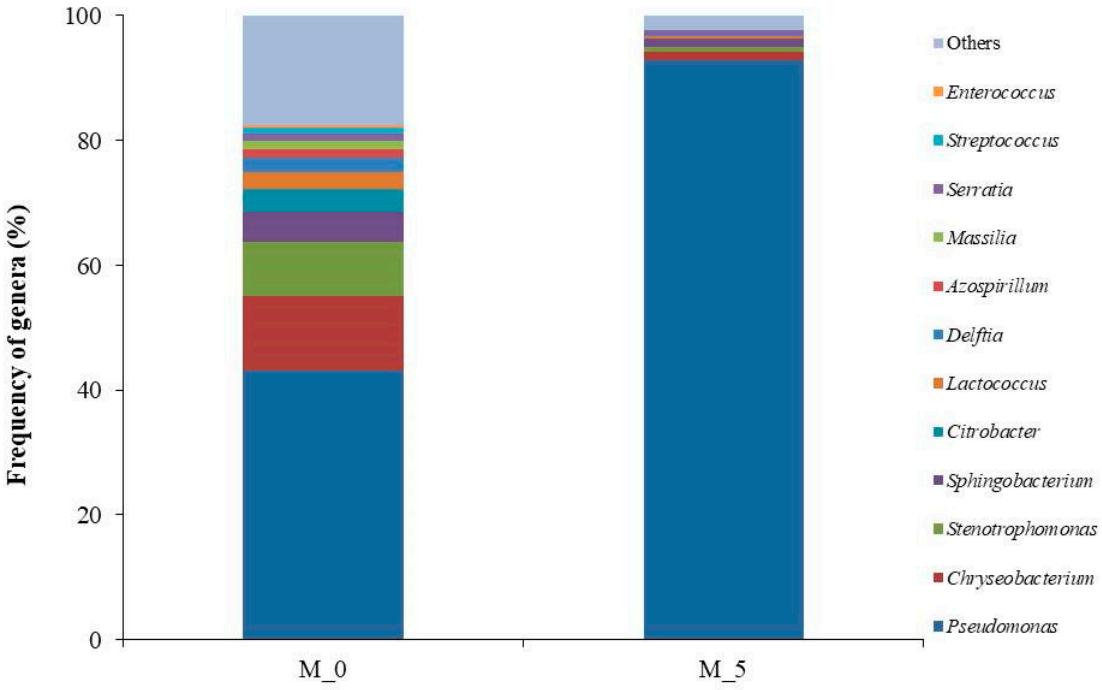
Relative abundance of bacterial communities at phylum, genus, and species level are shown in Figure 2. A total of five identified phyla were found in fresh milk, being *Proteobacteria* dominant (about 70%), followed by *Bacteroidetes* (less than 20%), and to a lesser extent by *Firmicutes*, *Actinobacteria*, and *Verrucomicrobia* (Figure 2A).

In a recent work, the bacterial composition of donkey milk from different farms was analysed by high-throughput sequencing, revealing the occurrence of the same phyla [16]. These authors found a higher concentration of *Proteobacteria* (more than 85%) and a lower composition in *Bacteroidetes* (less than 1%) in almost all samples [16], suggesting that the microbial milk composition could be strictly related to the different breeding conditions.

In the present work, a high incidence of Gram-negative psychrotrophic bacterial population was found in fresh milk, with *Pseudomonas* spp. being the most representative (more than 45%). The remaining bacteria were associated with species belonging to the genera *Chryseobacterium*, *Stenotrophomonas*, *Sphingobacterium*, *Citrobacter*, and *Delftia*, and to a lesser extent by *Azospirillum*, *Massilia*, and *Serratia* (Figure 2B). These results partially agree with the previous metagenomic analysis, which reported a dominance of *Pseudomonas* spp., and the occurrence at different levels of further Gram-negative bacteria belonging to the genera *Ralstonia*, *Acinetobacter*, and *Cupriavidus* [16].

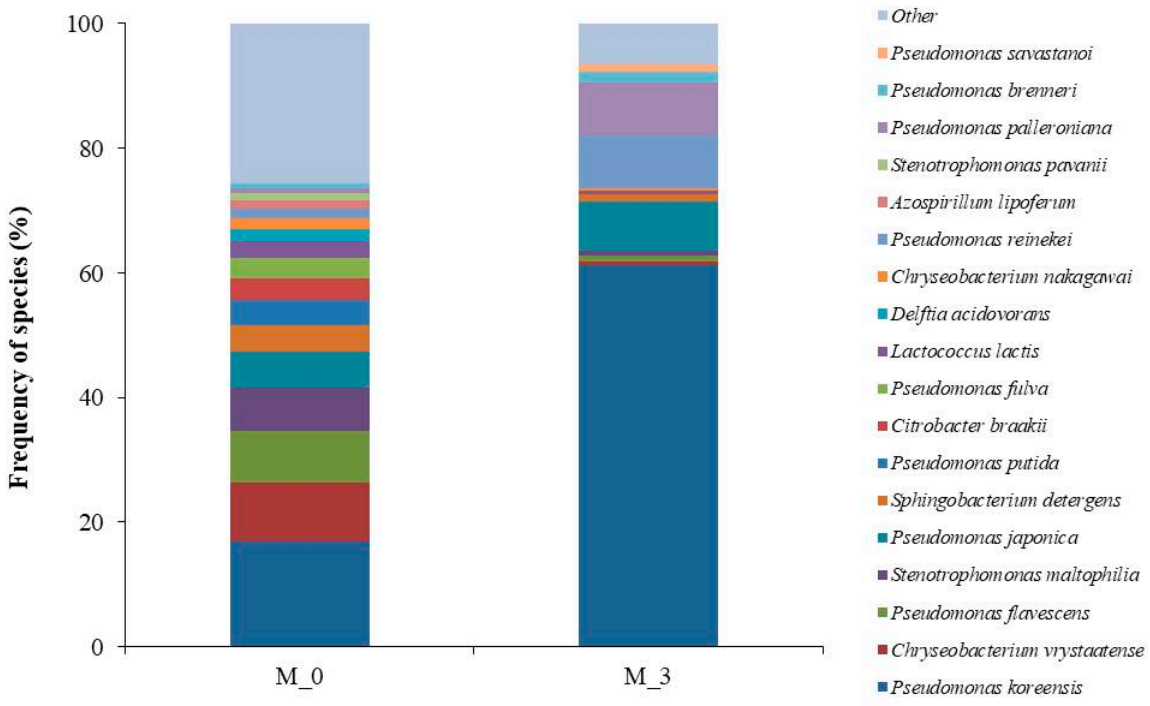


(A)



(B)

Figure 2. *Cont.*



(**C**)

Figure 2. Relative abundance of bacteria communities at phylum (**A**), genus (**B**), and species (**C**) level in fresh (M_0) and three-days cold stored (M_3) donkey milk. Phyla and genera occurring at <0.5% abundance, and species occurring at <1% are defined as "Others".

It is widely reported that Gram-negative psychrotrophic bacteria in raw milk are of special concern to the dairy industry because they can produce extracellular enzymes, mainly proteases and lipases, that contribute to the spoilage of dairy products [27,28]. Worldwide, species of *Pseudomonas* are the most common contaminants isolated from cold raw milk [16], while other genera, including *Chryseobacterium*, *Citrobacter*, and *Serratia*, have been reported as critical post-pasteurization spoilers [29–31]. Although Gram-negative bacteria are frequently identified in the milk of healthy animals, their abundance could be linked to mastitis and poor health conditions of the livestock, thus reducing the quality of raw milk [32,33]. However, *Salmonella* and *Staphylococcus aureus*, the main etiological agents for mastitis, were not found in our samples, and neither were the main foodborne pathogens in milk and the dairy environment (i.e., *Campylobacter jejuni*, Shiga-toxin producing *Escherichia coli*, *Listeria monocytogenes*, *Yersinia enterocolitica*, and *Salmonella* spp.). Nonetheless, the genera detected in fresh donkey milk included potential pathogenic species. For example, *Pseudomonas putida*, *Stenotrophomonas maltophilia*, and *Citrobacter braakii*, which together accounted for about 15% of the microbial population, are classified as risk group 2 biological agents, and reported as opportunistic human pathogens [34–36]. Furthermore, it is interesting to underline that in our samples we detected a level of *Serratia* spp. lower than other genera rarely documented in milk or dairy ecosystems. Besides, *Sphingobacterium* spp. has been isolated from different habitats, including raw cow and yak milk [37–39]. *Massilia* spp. was found in goat milk and fermented yak milk [12,40], while *Azospirillum* was only recently described as an inhabitant of fermented bovine products [41].

Concerning the protechnological microbes, in our samples of fresh milk, we detected reads for the lactic acid bacteria (LAB) lower than 5%, with *Lactococcus* spp. (2.7%) being the most abundant, followed by species of the genera *Streptococcus* spp. (1%), and *Enterococcus* spp. (0.5%) (Figure 2B). These results are consistent with those reported in donkey [14,16], and goat milk [12,42] but significantly lower than the LAB composition reported in conventional cow milk. Indeed, *Streptococcacea*, mainly belonging to *Lactococcus* spp., and to a minor extent to *Streptococcus* spp., was the most represented population

(about 57%) in raw cow milk [43]. However, different frequencies of LAB could be attributable to several factors, including diet, environment, season, and health status, as well as the method employed for identification [42].

Raw milk is often refrigerated for up to three or four days until it is processed. A recent review reported, in association with this matrix, the presence of *Bacillus*, *Clostridium*, *Corynebacterium*, *Micrococcus*, *Streptococcus*, *Staphylococcus*, *Microbacterium*, *Lactococcus*, and *Lactobacillus* (Gram-positive), and *Pseudomonas*, *Aeromonas*, *Alcaligenes*, *Achromobacter*, *Acinetobacter*, *Flavobacterium*, *Chryseobacterium*, and *Enterobacteriaceae* (Gram-negative) [31]. Therefore, we analysed the same samples after three days of storage at refrigeration temperature. In these samples, the bacterial biodiversity of donkey milk was strongly reduced, since about 93% of the bacterial communities were identified as different species of psychrotrophic *Pseudomonas*. In particular, *Pseudomonas koreensis* was found to be the dominant species, increasing its level from 17% in fresh milk to 61% after storage. In this last sample, *Pseudomonas reinekei*, *Pseudomonas palleroniana*, and *Pseudomonas japonica* were found at a concentration of about 8%. All the other Gram-negative genera decreased to about 4% of the total bacterial communities (Figure 2B,C). Microbiological analysis using culture-dependent methods showed a slight increase of the total aerobic microorganisms from 3.1×10^4 cfu mL^{-1} to 3.9×10^4 cfu mL^{-1}. These results are in agreement with those found by Zhang et al. [44] in donkey milk samples after 4 days of storage at 4 °C. Thus, the observed reduction of some taxonomic groups during cold storage could reflect a higher capability of certain *Pseudomonas* spp. to grow under refrigeration conditions, more than a reduction of viable cells.

It is well known that during cold storage of non-pasteurized milk, psychrotrophic microorganisms are the dominant microflora [45]. In particular, *Pseudomonas* spp. was found as the predominant microbiota in raw milk samples after a few days of refrigeration [38,46,47]. Moreover, although different treatments for the microbiological control could differently modulate the dominant bacterial populations in milk, cold-tolerant bacteria, such as *Pseudomonas*, *Stenotrophomonas*, and *Delftia* were persistent after submitting milk to different treatments for the microbial control [48]. The spoilage potential of psychrotrophic bacteria isolated from raw milk could be increased by the thermo-stability of their proteolytic and lipolytic enzymes [28], and by the ability to produce volatile organic compounds responsible of undesirable aromatic attributes [49]. Therefore, the metabolic activity of psychrotrophic bacteria results in a variety of defects that negatively affect the suitability of such milk for further processing. However, the low casein content of donkey milk does not allow caseification, thus reducing its processing availability. Nonetheless, in the last few years, donkey milk has been proposed to produce functional fermented beverages [5], and technological attempts at producing cheese from donkey milk have been suggested with the addition of bovine chymosin, camel chymosin, goat milk, or calf rennet [6,50–52].

In many European countries, raw milk can be sold at the farm directly to the consumer, and unpasteurized raw matrix is used for cheese production. However, EFSA stigmatized that, as the consumption of raw drinking milk poses public health risks [53].

Conte and Panebianco [9] provide a comprehensive background of the potential hazards associated with raw donkey milk consumption, suggesting the need to enhance the current scientific knowledge, to allow a suitable risk assessment along the whole donkey milk chain. Moreover, the authors solicited competent authorities to carry out more stringent official controls, with particular attention given to the level of primary production, as well as improving the traceability system in the donkey milk chain.

This is in agreement with other recent studies, which revealed that the microbiological quality of raw donkey milk was not optimal, highlighting the importance of raw milk management, particularly with the need for animal hygiene management, and good dairy farming practices on donkey farms to improve handling procedures [4,54].

Therefore, a timely treatment of the raw sample would be recommended to avoid the proliferation of an unwanted microflora, which could irreparably compromise the use of donkey milk for human consumption, and for any technological transformations. Also, in light of suggested cheese production from donkey raw milk [21,55], our findings suggested precautions to be adopted to assure the quality

and the safety of these dairy products, including the possible risks associated with spontaneous fermentation [56,57].

In conclusion, in this short communication, we report a preliminary description of the microbial diversity of donkey milk by using a metagenomic approach. The effect of three-day refrigeration storage was also investigated. Our results revealed a high abundance of spoilage in Gram-negative communities. Cold storage reduced the microbial biodiversity of the milk, encouraging the dominance of *Pseudomonas* spp. The microbial communities detected in this work might negatively affect the quality of the raw product and its technological transformation. Moreover, although non-conventional foodborne pathogens have been found, the occurrence of microbial species belonging to risk group 2 in fresh milk poses a moderate safety concern, especially considering the main target group of consumers (i.e., infants, immunocompromised, and elderly people).

Therefore, more details on the microbial composition of donkey milk are required to elucidate the impact on the technological, safety, and functional potential of this high-value niche product and to encourage its exploitation.

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Conflicts of Interest: The authors declare no conflict of interest.

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