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

Pasquale Russo 1,*, Daniela Fiocco 2, Marzia Albenzio 1, Giuseppe Spano 1, and Vittorio Capozzi 3

1 Department of Sciences of Agriculture, Food, and Environment, University of Foggia, via Napoli 25, 71122 Foggia, Italy; marzia.albenzio@unifg.it (M.A.); giuseppe.spano@unifg.it (G.S.)
2 Department of Clinical and Experimental Medicine, University of Foggia, Viale Pinto 1, 71122 Foggia, Italy; daniela.fiocco@unifg.it
3 Institute of Sciences of Food Production, National Research Council (CNR), c/o CS-DAT, Via Michele Protano, 71121 Foggia, Italy; vittorio.capozzi@ispa.cnr.it

* Correspondence: pasquale.russo@unifg.it

Received: 12 March 2020; Accepted: 25 March 2020; Published: 28 March 2020

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 shaking 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.

| Sample | Sequences | Av. Length | Total Mb | Av. Quality | OTUs | Shannon Value |
|--------|-----------|------------|----------|-------------|------|---------------|
| Mt0–16S | 22,483 | 443.12 | 9.96 | 35.69 | 268 | 2.580 |
| Mt3–16S | 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).

![Figure 1](A) ![Figure 1](B)
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].
Figure 2. Cont.
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, *Streptococcaceae*, 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 reinkei, 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 $3.1 \times 10^8$ cfu mL$^{-1}$ to $3.9 \times 10^8$ 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 microbiota [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 of the cheese.
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.

**Author Contributions:** Investigation, P.R., D.F., M.A., G.S., and V.C.; conceptualization, P.R., D.F., M.A., G.S., and V.C.; literature search, P.R., and V.C.; writing—original draft preparation, P.R., and V.C.; writing—review and editing, P.R., D.F., M.A., G.S., and V.C. All authors have read and agreed to the published version of the manuscript.

**Funding:** Pasquale Russo is the beneficiary of a grant by MIUR in the framework of ‘AIM: Attraction and International Mobility’ (PON R&I2014-2020) (practice code D74I18000190001). The authors acknowledge Massimo Franchi and Francesco De Marzo of the Institute of Sciences of Food Production—CNR for the skilled technical support provided during the realization of this work.

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

**References**

1. Aspri, M.; Economou, N.; Papademas, P. Donkey milk: An overview on functionality, technology and future prospects. *Food Rev. Int.* 2016, 33, 316–333. [CrossRef]

2. Camillo, F.; Rota, A.; Biagini, L.; Tesi, M.; Fanelli, D.; Panzani, D. The current situation and trend of donkey industry in Europe. *J. Equine Vet. Sci.* 2018, 65, 44–49. [CrossRef]

3. Miraglia, N.; Salimei, E.; Fantuz, F. Equine milk production and valorization of marginal areas—A review. *Animals* 2020, 10, 353. [CrossRef] [PubMed]

4. Giacometti, F.; Bardasi, L.; Merialdi, G.; Morbarigazzi, M.; Federici, S.; Piva, S.; Serraino, A. Shelf life of donkey milk subjected to different treatment and storage conditions. *J. Dairy Sci.* 2016, 99, 4291–4299. [CrossRef] [PubMed]

5. Turchi, B.; Pedonese, F.; Torraca, B.; Fratini, F.; Mancini, S.; Galiero, A.; Montalbano, B.; Cerri, D.; Nuvoloni, R. *Lactobacillus plantarum* and *Streptococcus thermophilus* as starter cultures for a donkey milk fermented beverage. *Int. J. Food Microbiol.* 2017, 256, 54–61. [CrossRef] [PubMed]

6. Faccia, M.; Gambacorta, G.; Martemucci, G.; Natrella, G.; D’Alessandro, A.G. Technological attempts at producing cheese from donkey milk. *J. Dairy Res.* 2018, 85, 327–330. [CrossRef] [PubMed]

7. Altomonte, I.; Salaria, F.; Licitra, R.; Martini, M. Donkey and human milk: Insights into their compositional similarities. *Int. Dairy J.* 2019, 89, 111–118. [CrossRef]

8. Souroullas, K.; Aspri, M.; Papademas, P. Donkey milk as a supplement in infant formula: Benefits and technological challenges. *Food Res. Int.* 2018, 109, 416–425. [CrossRef] [PubMed]

9. Conte, F.; Panebianco, A. Potential hazards associated with raw donkey milk consumption: A review. *Int. J. Food Sci.* 2019, 2019, 5782974. [CrossRef]

10. Quigley, L.; O’Sullivan, O.; Stanton, C.; Beresford, T.P.; Ross, R.P.; Fitzgerald, G.F.; Cotter, P.D. The complex microbiota of raw milk. *FEMS Microbiol. Rev.* 2013, 37, 664–698. [CrossRef]

11. Quigley, L.; O’Sullivan, O.; Beresford, T.P.; Ross, R.P.; Fitzgerald, G.F.; Cotter, P.D. Molecular approaches to analysing the microbial composition of raw milk and raw milk cheese. *Int. J. Food Microbiol.* 2011, 150, 81–94. [CrossRef] [PubMed]

12. Zhang, F.; Wang, Z.; Lei, F.; Wang, B.; Jiang, S.; Peng, Q.; Zhang, J.; Shao, Y. Bacterial diversity in goat milk from the Guanzhong area of China. *J. Dairy Sci.* 2017, 100, 7812–7824. [CrossRef] [PubMed]
13. Alexandraki, V.; Kazou, M.; Angelopoulou, A.; Arena, M.P.; Capozzi, V.; Russo, P.; Fiocco, D.; Spano, G.; Papadimitriou, K.; Tsakalidou, E. The Microbiota of Non-cow Milk and Products. In *Non-Bovine Milk and Milk Products*; Tsakalidou, E., Papadimitriou, K., Eds.; Academic Press: London, UK, 2016; pp. 117–159.

14. Cavallarin, L.; Giribaldi, M.; Soto-del Rio, M.; de los Valles, D.E.; Barbarino, G.; Gennero, M.S.; Civera, T. A survey on the milk chemical and microbiological quality in dairy donkey farms located in NorthWestern Italy. *Food Control* **2015**, *50*, 230–235. [CrossRef]

15. Malissiova, E.; Arsenos, G.; Papademas, P.; Fietouris, D.; Manouras, A.; Aspri, M.; Nikolopoulou, A.; Giannopoulou, A.; Arvanitoyannis, J.S. Assessment of donkey milk chemical, microbiological and sensory attributes in Greece and Cyprus. *Int. J. Dairy Technol.* **2016**, *69*, 143–146. [CrossRef]

16. Soto del Rio, M.D.L.D.; Dalmasso, A.; Civera, T.; Bottero, M.T. Characterization of bacterial communities of donkey milk by high-throughput sequencing. *Int. J. Food Microbiol.* **2017**, *251*, 67–72. [CrossRef] [PubMed]

17. Machado, S.G.; Baglinière, F.; Marchand, S.; Van Coillie, E.; Vanetti, M.C.; De Block, J.; Heyndrickx, M. The Biodiversity of the Microbiota Producing Heat-Resistant Enzymes Responsible for Spoilage in Processed Bovine Milk and Dairy Products. *Front. Microbiol.* **2017**, *8*, 302. [CrossRef]

18. Yuan, L.; Sadiq, F.A.; Burmølle, M.; Wang, N.I.; He, G. Insights into psychrotrophic bacteria in raw milk: A review. *J. Food Prot.* **2019**, *82*, 1148–1159. [CrossRef]

19. Raats, D.; Offëk, M.; Minz, D.; Halpern, M. Molecular analysis of bacterial communities in raw cow milk and the impact of refrigeration on its structure and dynamics. *Food Microbiol.* **2011**, *28*, 465–471. [CrossRef]

20. Porcellato, D.; Aspholm, M.; Skeie, S.B.; Monshaugen, M.; Brendehaug, J.; Mellegård, H. Microbial diversity of consumption milk during processing and storage. *Int. J. Food Microbiol.* **2018**, *266*, 21–30. [CrossRef]

21. Fernández de Palencia, P.; López, P.; Corbi, A.L.; Pelaez, C.; Requena, T. Probiotic strains: Survival under simulated gastrointestinal conditions, in vitro adhesion to Caco-2 cells and effect on cytokine secretion. *Eur. Food Res. Technol.* **2008**, *227*, 1475–1484. [CrossRef]

22. Klindworth, A.; Pruesse, E.; Schweer, T.; Peplies, J.; Quast, C.; Horn, M.; Glöckner, F.O. Evaluation of a general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Res.* **2012**, *41*, e1. [CrossRef] [PubMed]

23. Zhang, J.; Kobert, K.; Flouri, T.; Stamatakis, A. PEAR: A fast and accurate Illumina Paired-End reAd mergeR. *Bioinformatics* **2014**, *30*, 614–620. [PubMed]

24. Marcel, M. Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet J.* **2011**, *17*, 10–12.

25. Edgar, R.C.; Haas, B.J.; Clemente, J.C.; Quince, C.; Knight, R. UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics* **2011**, *27*, 2194–2200. [CrossRef]

26. Huang, Y.; Niou, B.; Gao, Y.; Fu, L.; Li, W. CD-HIT Suite: A web server for clustering and comparing biological sequences. *Bioinformatics* **2010**, *26*, 680–682. [CrossRef]

27. Hantsis-Zacharov, E.; Shakéd, T.; Senderovich, Y.; Halpern, M. *Chryseobacterium oranimense* sp. nov., a psychrotolerant, proteolytic and lipolytic bacterium isolated from raw cow’s milk. *Int. J. Syst. Evol. Microbiol.* **2008**, *58*, 2635–2639. [CrossRef]

28. Yuan, L.; Sadiq, F.A.; Liu, T.J.; Li, Y.; Gu, J.S.; Yang, H.Y.; He, G.Q. Spoilage potential of psychrotrophic bacteria isolated from raw milk and the thermo-stability of their enzymes. *J. Zhejiang Univ. Sci. B* **2018**, *19*, 630–642. [CrossRef]

29. Masiello, S.N.; Martin, N.H.; Trmčić, A.; Wiedmann, M.; Boor, K.J. Identification and characterization of psychrotolerant coliform bacteria isolated from pasteurized fluid milk. *J. Dairy Sci.* **2016**, *99*, 130–140. [CrossRef]

30. Schmidt, V.S.; Kaufmann, V.; Kulozik, U.; Scherer, S.; Wenning, M. Microbial biodiversity, quality and shelf life of microfiltered and pasteurized extended shelf life (ESL) milk from Germany, Austria and Switzerland. *Int. J. Food Microbiol.* **2012**, *154*, 1–9. [CrossRef]

31. Odeyemi, O.A.; Alegbeleye, O.O.; Strateva, M.; Stratev, D. Understanding spoilage microbial community and spoilage mechanisms in foods of animal origin. *Compr. Rev. Food Sci. Food Saf.* **2019**. [CrossRef]

32. Zhang, R.; Hua, W.; Zhu, W.; Mao, S. Characterization of bacterial community of raw milk from dairy cows during subacute ruminal acidosis challenge by high-throughput sequencing. *J. Sci. Food Agric.* **2015**, *95*, 1072–1079. [CrossRef] [PubMed]
33. Schukken, Y.; Chuff, M.; Moroni, P.; Gurjar, A.; Santisteban, C.; Welcome, F.; Zadoks, R. The “other” Gram-negative bacteria in mastitis *Klebsiella, Serriata*, and more. *Vet. Clin. N. Am. Food A* 2012, 28, 239–256. [CrossRef] [PubMed]

34. Fernández, M.; Porcel, M.; de la Torre, J.; Molina-Henares, M.A.; Daddaoua, A.; Llamas, M.A.; Roca, A.; Carriel, V.; Garzón, I.; Ramos, J.L.; et al. Analysis of the pathogenic potential of nosocomial *Pseudomonas putida* strains. *Front. Microbiol.* 2015, 6, 871. [CrossRef] [PubMed]

35. Brooke, J.S. *Stenotrophomonas maltophilia*: An emerging global opportunistic pathogen. *Clin. Microbiol. Rev.* 2012, 25, 2–41. [CrossRef] [PubMed]

36. Hirai, J.; Uechi, K.; Hagihara, M.; Sanakashi, D.; Kinjo, T.; Haranaga, S.; Fujita, J. Bacteremia due to *Citrobacter braakii*: A case report and literature review. *J. Infect. Chemother.* 2016, 22, 819–821. [CrossRef] [PubMed]

37. Schmidt, V.S.; Wenning, M.; Scherer, S. *Sphingobacterium lactis* sp. nov. and *Sphingobacterium alimentarium* sp. nov., isolated from raw milk and a dairy environment. *Int. J. Syst. Evol. Microbiol.* 2012, 62, 1506–1511. [CrossRef] [PubMed]

38. Yuan, L.; Sadiq, F.A.; Liu, T.; Flint, S.; Chen, J.; Yang, H.; Gu, J.; Zhang, G.; He, G. Psychrotrophic bacterial populations in Chinese raw dairy milk. *LWT Food Sci. Technol.* 2017, 84, 409–418. [CrossRef]

39. Kaur, M.; Singh, H.; Sharma, S.; Mishra, S.; Tanuku, N.R.S.; Pinnaka, A.K. *Sphingobacterium bevisgrammientis* sp. nov., isolated from yak milk. *Int. J. Syst. Evol. Microbiol.* 2018, 68, 636–642. [CrossRef]

40. Liu, W.; Xi, X.; Sudu, Q.; Kwok, L.; Guo, Z.; Hou, Q.; Menhe, B.; Sun, T.; Zhang, H. High-throughput sequencing reveals microbial community diversity of Tibetan naturally fermented yak milk. *Ann. Microbiol.* 2015, 65, 1741–1751. [CrossRef]

41. Anandham, R.; Heo, J.; Krishnamoorthy, R.; SenthilKumar, M.; Gopal, N.O.; Kim, S.J.; Kwon, S.W. *Azospirillum ramasamyi* sp. nov., a novel diazotrophic bacterium isolated from fermented bovine products. *Int. J. Syst. Evol. Microbiol.* 2019, 69, 1369–1375. [CrossRef]

42. McInnis, E.A.; Kalanetra, K.M.; Mills, D.A.; Maga, E.A. Analysis of raw goat milk microbiota: Impact of stage of lactation and lysozyme on microbial diversity. *Food Microbiol.* 2015, 46, 121–131. [CrossRef] [PubMed]

43. Quigley, L.; McCarthy, R.; O’Sullivan, O.; Beresford, T.P.; Fitzgerald, G.F.; Ross, R.P.; Stanton, C.; Cotter, P.D. The microbial content of raw and pasteurized cow milk as determined by molecular approaches. *J. Dairy Sci.* 2013, 96, 4928–4937. [CrossRef] [PubMed]

44. Zhang, X.Y.; Zhao, L.; Jiang, L.; Dong, M.L.; Ren, F.Z. The antimicrobial activity of donkey milk and its microflora changes during storage. *Food Control* 2008, 19, 1191–1195. [CrossRef]

45. von Neubeck, M.; Baur, C.; Krewinkel, M.; Stoessel, M.; Kranz, B.; Stressler, T.; Fischer, L.; Hinrichs, J.; Scherer, S.; Wenning, M. Biodiversity of refrigerated raw milk microbiota and their enzymatic spoilage potential. *Int. J. Food Microbiol.* 2015, 211, 57–65. [CrossRef] [PubMed]

46. Xin, L.; Meng, Z.; Zhang, L.; Cui, Y.; Han, X.; Yi, H. The diversity and proteolytic properties of psychrotrophic bacteria in raw cows’ milk from North China. *Int. Dairy J.* 2017, 66, 34–41. [CrossRef]

47. Zhang, D.; Palmer, J.; Teh, K.H.; Biggs, P.; Flint, S. 16S rDNA high-throughput sequencing and MALDI-TOF MS are complementary when studying psychrotrophic bacterial diversity of raw cows’ milk. *Int. Dairy J.* 2019, 97, 86–91. [CrossRef]

48. Rasolofo, E.A.; St-Gelais, D.; LaPointe, G.; Roy, D. Molecular analysis of bacterial population structure and dynamics during cold storage of untreated and treated milk. *Int. J. Food Microbiol.* 2010, 138, 108–118. [CrossRef]

49. Alothman, K.A.; Silcock, P.; Bremer, P.J. Comparing PTR-MS profile of milk inoculated with pure or mixed cultures of spoilage bacteria. *Food Microbiol.* 2017, 64, 155–163. [CrossRef]

50. Unicke-Lowe, T.; Fox, P.F. Equid milk. In *Encyclopedia of Dairy Sciences*, 2nd ed.; Fuquay, J.W., Fox, P.F., McSweeney, P.H., Eds.; Academic Press: San Diego, CA, USA, 2011; pp. 518–529.

51. Iannella, G. Donkey cheese made through pure camel chymosin. *Afr. J. Food Sci.* 2015, 9, 421–425.

52. Saric, L.C.; Saric, B.M.; Mandic, A.I.; Hadnadev, M.S.; Gubic, J.M.; Milovanovic, I.L.; Tomic, J.M. Characterization of extra-hard cheese produced from donkeys’ and caprine milk mixture. *Dairy Sci. Technol.* 2016, 96, 227–241. [CrossRef]

53. EFSA. Panel on Biological Hazards. Scientific Opinion on the public health risks related to the consumption of raw drinking milk. *EFSA J.* 2015, 13, 3940. [CrossRef]

54. Giribaldi, M.; Antoniazzi, S.; Gariglio, G.M.; Coscia, A.; Bertino, E.; Cavallarin, L. A preliminary assessment of HTST processing on donkey milk. *Vet. Sci.* 2017, 4, 50. [CrossRef] [PubMed]
55. D’Alessandro, A.G.; Martemucci, G.; Loizzo, P.; Faccia, M. Production of cheese from donkey milk as influenced by addition of transglutaminase. *J. Dairy Sci.* 2019, 102, 10867–10876. [CrossRef] [PubMed]

56. Capozzi, V.; Fragasso, M.; Romaniello, R.; Berbegal, C.; Russo, P.; Spano, G. Spontaneous food fermentations and potential risks for human health. *Fermentation* 2017, 3, 49. [CrossRef]

57. Capozzi, V.; Fragasso, M.; Russo, P. Microbiological safety and the management of microbial resources in artisanal foods and beverages: The need for a transdisciplinary assessment to conciliate actual trends and risks avoidance. *Microorganisms* 2020, 88, 306. [CrossRef] [PubMed]

© 2020 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).