ANALYSIS OF BACTERIAL DIVERSITY IN MILK FROM FOUR VARIOUS ANIMAL SPECIES USING METAGENOMIC APPROACHES

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Abstract. To fully understand the foodborne diseases attributed to the intake of raw milk samples as conveyors of antimicrobial resistance genes (ARGs), metagenomic sequencing methods are used worldwide to increase food safety because they allow the molecular characterization of pathogens without conducting primary isolation stages. The diversity of the bacterial communities within raw and pasteurized milk samples from diverse assets (i.e., horse (3), camel (3), goat (3), cow (3), and three pooling samples as controls) gathered from Saudi Arabia were assessed using 16S rRNA sequencing as a standard DNA barcoding approach, showing significant numbers of bacteria. The dissimilar bacterial communities among the tested samples were classified under different classes (including Bacteroidia, Alphaproteobacterial, Clostridia, etc.), families (including *Lachnospiraceae, Pseudomonadaceae, Staphylococcaceae*, etc.), and species (including *Arthrobacter-citreus, Escherichia, Staphylococcus-aureus,* etc.). Unexpectedly, the pooled pasteurized milk samples that were used as the controls were contaminated, and the top percentage was from *Bacilli.* Furthermore, 477 novel species were detected, and the largest amount was from raw horse milk (37.3%), followed by raw camel milk (21.6%), while countless species were discovered to be descended from Proteobacteria. Generally, the samples of raw horse milk had the highest levels of species richness and diversity, as shown by the alpha diversity analysis. The beta diversity analysis showed no exact similar community structures among species within the examined samples. To better comprehend the microbiological diversity, the use of high-throughput sequencing with a hypothetical basis is highly recommended to obtain useful microbial reserves from raw and pasteurized milk samples.
Keywords: *raw and pasteurized milk, metagenomic analysis, 16S rRNA, microbiology, microbiota, Saudi Arabia*

Abbreviations. ARGs, antimicrobial resistance genes; OUT, operational taxonomic unit; PCA, principal component analysis; MRSA, methicillin-resistant *Staphylococcus aureus;* IMIs, intramammary infections

Introduction

Dairy products, especially milk products (either raw or pasteurized), are essential foods for adults and kids globally. Because of the increasing consumption of dairy products, which harbor complex microbial communities, over the last decade the milk microbiota is evaluated constantly using culture-independent techniques such as real-time PCR, quantitative PCR, high-throughput DNA sequencing, and flow cytometry methods (Ryu et al., 2021). Moreover, metagenomic sequencing technology, which is known as one of the high-throughput technologies that is conducted in laboratories, is able to facilitate the environmental study of food products. It can also identify the identities of

microbiota such as yeast and bacteria by targeting metagenomics using gene fragment DNA sequencing without the need to culture the organisms. Commonly, the 16S rDNA gene is used as a conserved fragment ([20]et al., 2009; Garmendia et al., 2012; Quigley et al., 2013; Salazar et al., 2018; Martin et al., 2018[1]et al., 2020).

Nevertheless, the field of metagenomics has been covered in several studies recently to gain obvious insights into the microbial compositions in various kinds of foods, such as meat, kimchi, raw milk, spinach, wine, kefir, cheese, and bean sprouts ([25]). As a result, certain characteristics of the microbial composition that contribute to microorganisms, such as the color, texture, smell, damage, shelf-life, and flavor, have been specified (Garrido-Cardenas and Manzano-Agugliaro, 2017; Salazar et al., 2018; Porcellato et al., 2021). For instance, various species of bacteria were detected (i.e., *Actinobacteria, Firmicutes, Bacteroidetes,* and *Proteobacteria*) among samples from raw milk, assembled from forty milking farms in four various districts of Korea (Ryu et al., 2021). At the phylum level, the basic microbiome within the milk samples was from Actinobacteria, and at the genus level *Pseudomonas* formed the greatest number (Ryu et al., 2021).

Raw milk samples, furthermore, contain various species of bacteria in many countries around the world, such as Ireland, United States, and Saudi Arabia, as assessed using different techniques such as 16S rRNA gene, culture-based, and metagenomic sequencing methods (Quigley et al., 2013; Liu et al., 2020; Alghizzi and Shami, 2021). It was also found that such bacteria harbor antimicrobial resistance genes (ARGs) that threaten human health and raise the need to understand the importance of the proper storage and transportation of raw milk (Kuehn, 2018; Liu et al., 2020; Alghizzi and Shami, 2021). Surprisingly, the metagenomic sequencing of raw milk samples collected from United States showed more ARGs than for pasteurized milk, and the ARGs differ depending on the geographic location, possibly related to the regionally unassociated milk microbiota (Langer et al., 2012; Liu et al., 2020).

The existing study was planned to examine the microbial diversity by applying the metagenomic method, consisting of various stages including DNA preparation, DNA quality control (DNA QC), product purification, library quality control, and sequencing. As a reason for quantifying and comparing the bacterial richness on every level in all tested samples (from pasteurized and raw milk samples from diverse origins), a bioinformatics analysis was performed via alpha diversity analysis, beta diversity analysis, and principal component analysis (PCA). A species phylogenetic analysis was also implemented to understand the relationship within the identified bacterial species and to determine their ancestors.

Materials and Methods

Samples Collection

From four different locations throughout Riyadh city in Saudi Arabia, fifteen raw and pasteurized milk samples were collected: 3 raw horse milk samples, 3 raw camel milk samples, 3 raw goat milk samples, and 3 raw cow milk samples. Moreover, 3/15 samples; gathered from local shops and they were already pasteurized, were used as controls. For further processing, approximately 50 milliliters of each sample was transported on dry ice to the laboratory within 24 h in a disinfected and labelled tube.

DNA Extraction

The producer's guidelines for the DNeasy® PowerFood® Microbial Kit (100) (QIAGEN, Germany) with 2 ml bead tubes, which are better for DNA isolation from uncultured food, were tracked on the tested sample to extract their DNA and undergo subsequent metagenomic techniques.

Experiment Workflow and Sequence Quality Control

The 16S rRNA fusion primers and 30 ng qualified DNA samples were mixed up together to perform polymerase chain reaction (PCR) tests, which were then purified by Agencourt AMPure XP beads, disbanded in elution buffer, and marked to finish building the library. Then, an Agilent 2100 Bioanalyzer detected the library size and concentration. Depending on the insert sizes of the qualified libraries, they were sequenced on a HiSeq platform. To obtain high-quality clean reads, the raw data were purified and the reads with average Phred quality scores of less than 20 over a 25 bp sliding window were truncated and the reads that kept 75% of their original lengths were removed. Adapter sequences were used in the next step in order to eliminate all of these issues, including the contaminated reads (i.e., 15 bases overlapped by reads and adapters with no maximal similarities between 3 bases), the reads with vague bases (N base), and the low-complexity reads (i.e., ten consecutive identical base reads).

Bioinformatics Analysis

Clean reads that can overlap together were embedded for marking and further clustered into operational taxonomic units (OTUs). Moreover, it is necessary to cluster the sequences into OTUs with 97% similarity to quantify the bacterial richness on each level in all samples. The tags were clustered into OTUs (operational taxonomic units) with USEARCH (v7.0.1090), as follows: (1) by using UPARSE, the tags were clustered into OTUs with a 97% threshold, where the singular OTU representative sequences could be gained; (2 by using UCHIME (v4.2.40), the chimeras were filtered (for 16S rDNA and ITS sequences, chimeras in OTUs were screened and filtered by mapping them to the gold database (v20110519) and UNITE (v20140703), respectively, whereas de novo chimera screening was carried on OTUs for 18S rDNA sequences); (3) to calculate OTU abundance tables, all tags were mapped to representative OTU sequences by using USEARCH GLOBAL (USEARCH v7 .0.1090, UCHIME v4.2.40). The Ribosomal Database Project database, moreover, was used to specify the taxonomic classifications to representative OTU sequences.

Nevertheless, for the alpha diversity analysis (including Chao1, observed species, ACE, Shannon's diversity, Simpson's diversity, and Good's coverage values), mothur software (v.1.31.2) (Schloss et al., 2009) was used. The beta diversity analysis was also used to assess the variations in bacterial species complexity within samples by using Quantitative Insights into Microbial Ecology (QIIME) (v1.80) software (Lozupone and Knight, 2005; Lozupone et al., 2007, 2011). A normalization process was devised because of the differences in sequencing depths among the different samples; thus, sequences were mined randomly depending on the smallest sequence number for all samples, and such sequences formed a new 'OTU table biom' file to cluster the beta diversity distance. Plus, various values, such as the Bray–Curtis (to reverse the controversies among two communities, which amounts to between zero (exact similar community structure) and one) and UniFrac (which applies the system evolution information to compare the

compositions of species communities within samples) values. Generally, the results can be used as a measure of the beta diversity by conducting a principal component analysis (PCA) (using R software (v3.1.1) package ade4). GraPhlAn can also be used to image the taxonomy compositions and abundances. Therefore, the distances of evolution among the species can be identified, and the greater differences between samples form the bigger index values. The weighted UniFrac approach considers the richness of the sequences, while unweighted UniFrac does not.

Species Phylogenetic Analysis

To represent evolutionary relationships amongst creatures, a phylogenetic tree was built to reflect how species or other groups developed from a series of common ancestors using FastTree version 2.1.3. The utmost likelihood algorithm was used to generate phylogenetic trees, and the visualization of the phylogeny tree was carried on OTUs using R software (v3.1.1).

Results

Bacterial Composition and Abundance Among Raw and Pasteurized Milk Samples

After filtering, the whole examined samples were of high quality and equaled more than 20 bp, depending on the Phred score, and their p-values were significant (<0.05). Additionally, large numbers of clean sequence reads (i.e., 513.83) were identified and the top value (i.e., 37.50) was identified from all tested samples except the horse set, while the lowest value (i.e., 22.89) was from the goat set (*Table 1*). However, the bacterial classes within such tested samples were variable (e.g., Bacteroidia, Alphaproteobacterial, Clostridia, Betaproteobacteria, Actinobacteria, Gammaproteobacteria, Bacilli, etc.). Relying on *Figure 1a*, the highest existence was from the Bacilli class within cow raw milk samples, while the lowest was from the Clostridia class among goat raw milk samples. Under these classes, in addition, many bacterial families were spotted, such as *Lachnospiraceae, Rhodocyclaceae, Propionibacteriaceae, Pseudomonadaceae, Mycobacteriaceae, Staphylococcaceae, Streptococcaceae, Moraxellaceae, Bacillaceae, Micrococcaceae, Enterobacteriaceae, Enterococcaceae,* etc. *Mycobacteriaceae, Enterobacteriaceae,* and *Bacillaceae;* nonetheless, the greater fractions formed between the detected bacterial families were from raw camel milk, raw goat milk, and control samples, respectively (as shown in *Figure 1b*). Pasteurized control milk samples were also contaminated by different classes and families of bacteria.

Around 1038 abundant species were identified among the samples, including *Anoxybacillus flavithermus, Acinetobacter kookii, Escherichia, Bacillus cereus, Klebsiella pneumoniae,Lactococcus lactis, Lactococcus raffinolactis, Macrococcus caseolyticus, Mycobacterium wolinskyi, Propionibacterium acnes, Pantoea vagans, Staphylococcus aureus, Streptococcus agalactiae,* and *Streptococcus mitis*. The greatest presence levels between the observed species were from *Arthrobacter citreus, Enterococcus saccharolyticus, Escherichia,* and *Anoxybacillus flavithermus,* and they were from camel, horse, cow, goat, and control samples, respectively (*Figure 1c*).

Uncovered Novel Species Throughout Metagenomic Assembly

The quantities of unique OTUs in multi samples are displayed via Venn diagrams and the samples are filled with different colors. The numbers in each non-overlapping area

indicate the numbers of sample-specific OTUs, while numbers in the overlapping areas show the OTUs that are common between samples (*Figure 2*). In other words, 44 species were detected in all examined samples, whilst 103 species (in raw camel milk samples), 26 species (in raw cow milk samples), 77 species (in raw goat milk samples), 178 species (in unpasteurized horse milk samples), and 93 species (in pasteurized milk control samples) were recorded as novel species, according to *Figure 2*.

***Table 1.** Data filtering for unpasteurized and pasteurized milk samples gathered from Saudi Arabia (A=control sample, C=cow sample, G=goat sample, H=horse sample, and K=camel sample)*

Sample name	Read length (bp)	Raw data (Mbp)	N base (%)	Poly base (%)	Clean data (Mbp)	Data utilization ratio (%)
A1	300:298	41.530	2.952	0.001	37.50	90.30
A2	299:298	42.318	3.976	0.001	37.50	88.61
A3	299:297	34.074	2.664	0.001	31.14	91.40
C1	299:297	41.629	3.418	0.000	37.50	90.08
C2	299:296	42.914	4.380	0.000	37.50	87.39
C3	298:296	42.038	3.326	0.001	37.50	89.21
G1	297:296	25.612	2.751	0.000	22.89	89.36
G2	296:296	42.567	3.542	0.001	37.50	88.10
G3	294:296	42.366	3.558	0.000	37.50	88.52
H1	294:297	33.597	2.612	0.001	30.71	91.40
H2	293:297	36.775	2.654	0.001	33.53	91.17
H3	300:297	37.012	2.639	0.000	33.74	91.15
K1	298:297	42.212	3.789	0.001	37.50	88.84
K2	297:297	30.648	2.796	0.002	27.93	91.13
K3	296:297	37.304	2.657	0.001	33.89	90.85

Furthermore, *Figure 3* illustrates the phylogenetic tree of common ancestors of determined species from the tested samples, which were from *Acidobacteria, Actinobacteria, Aminicenantes, Armatimonadetes, Bacteroidetes, Candidatus−Saccharibacteria, Chloroflexi, Cyanobacteria, Deinococcus−Thermus, Firmicutes, Fusobacteria, Gemmatimonadetes, Lentisphaerae, Nitrospirae, Parcubacteria, Planctomycetes, Proteobacteria, Spirochaetes, Tenericutes,* and *Verrucomicrobia.* Generally, many species were found to be descended from Proteobacteria, followed by Firmicutes and Actinobacteria.

Species Richness and Diversity Assessed Through Alpha and Beta Diversity Analyses

For the alpha diversity analysis, *Figure 4* shows that the samples of raw horse milk had the highest observed species, Chao1, ACE, and Shannon diversity values, as the bacteria within the raw horse samples had the maximum level of diversity. The raw goat milk samples had the uppermost species richness and diversity as assessed via the Simpson diversity and Good's coverage values (reordered as significant p values of 0.08). Further, the higher Good's coverage index value indicated less unexplored species in the tested samples from raw goat milk.
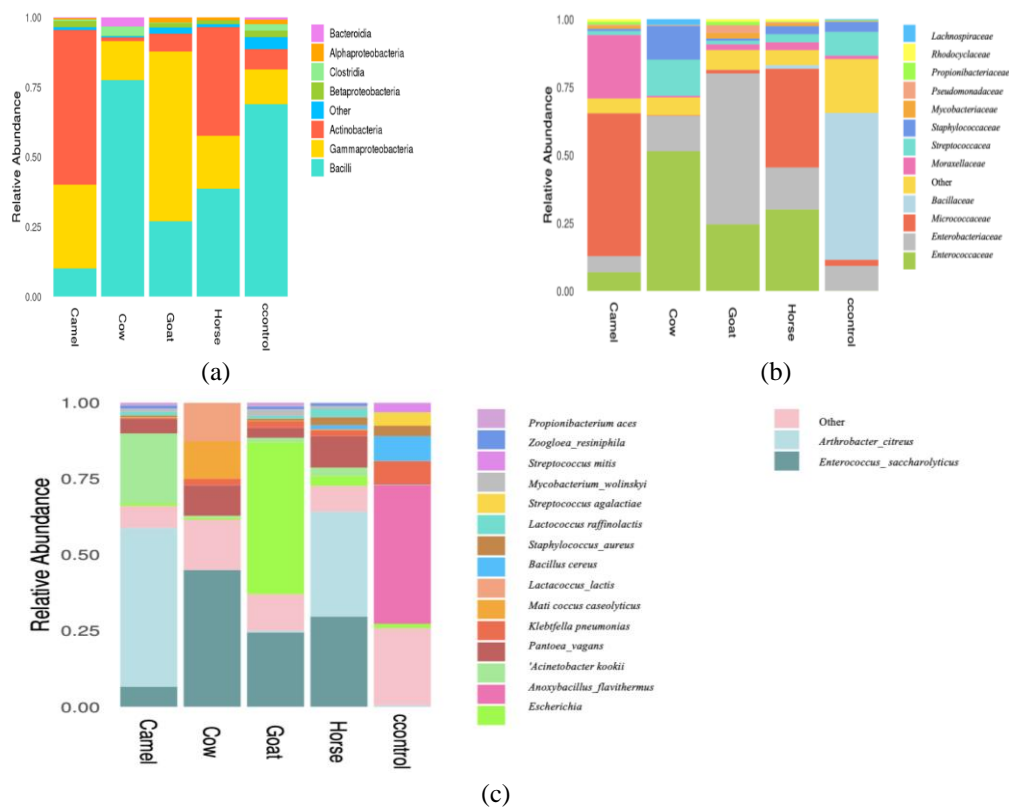
Figure 1. *Bacterial abundance levels within raw and pasteurized milk samples gathered from Saudi Arabia: (a) illustration of bacterial classes' abundance levels within tested samples; (b) illustration of bacterial families' abundance levels within tested samples; (c) species richness among tested samples*
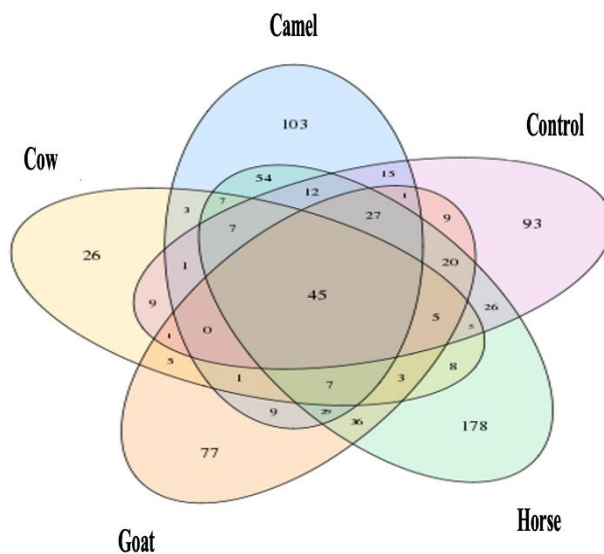

Figure 2. *OTU Venn map illustrating the numbers of sample-specific OTUs in each non-overlapping area (blue represents OTU numbers of raw camel milk samples, pink represents OTU numbers of pasteurized control milk samples, green represents OTU numbers of horse raw milk samples, orange represents OTU numbers of goat raw milk samples, and yellow represents OTU numbers of cow raw milk samples, while the integers in the overlapping areas show the number of OTUs that were shared by the samples)*
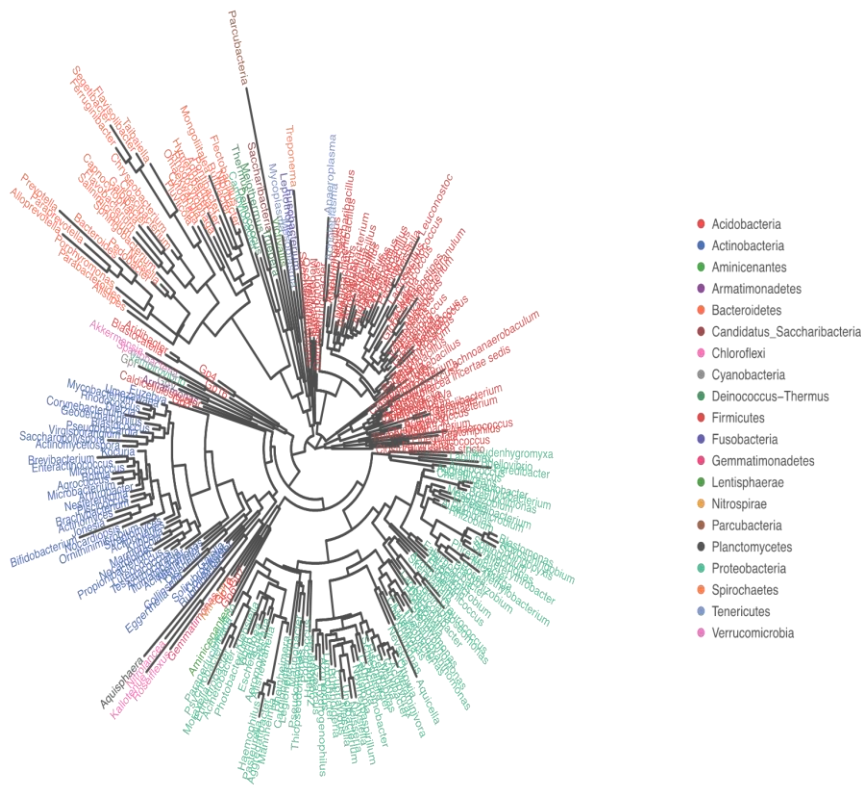
Figure 3. The phylogenetic tree analysis for common ancestors of the determined bacterial species in unpasteurized and pasteurized milk samples gathered from Saudi Arabia
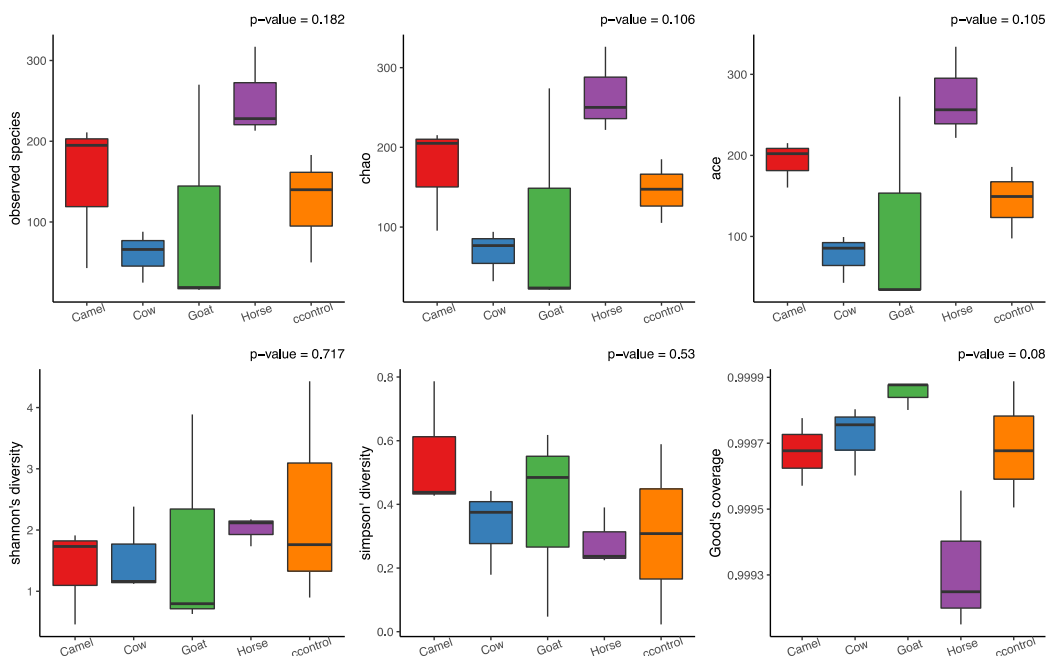

Figure 4. The alpha diversity analysis of the phylogenetic diversity, showing the witnessed species, Chao1, ACE, Shannon, Simpson, and Good's coverage index values of each raw (horse, camel, cow, and goat) and pasteurized (control) milk sample collected from Saudi Arabia

Additionally, the beta diversity analysis showed no exact similar community structure among the species within the examined samples because none of them had a zero Bray–Curtis value. The raw goat milk samples had the most species complexity, while the lowest species complexity was found between raw horse milk samples (*Figure 5A*). Likewise, the control sample had more species than the other tested samples based on the weighted UniFrac index values, the raw goat milk samples were above the level of species based on the unweighted UniFrac index, and the raw horse milk samples gave the lowest values for these indexes (*Figure 5B,C*). Additionally, *Figure 6* shows the result of the PCA analysis, showing that the shorter the distance between samples, the greater the similarity of the bacterial composition among them and the less variation. The high complexity and diversity of the discovered bacterial species among the raw milk samples from the various sets, especially in the horse raw milk samples, were examined for the first time through metagenomics in this study.
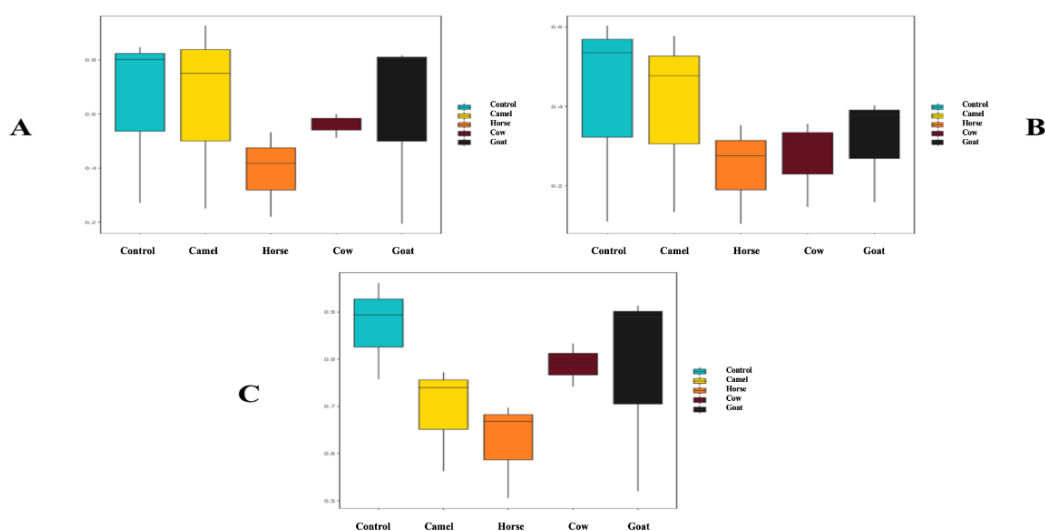

Figure 5. The beta diversity analysis, showing the Bray–Curtis (A), weighted UniFrac (B), and unweighted UniFrac (C) values of each raw (horse, camel, cow, and goat) and pasteurized (control) milk sample collected from Saudi Arabia
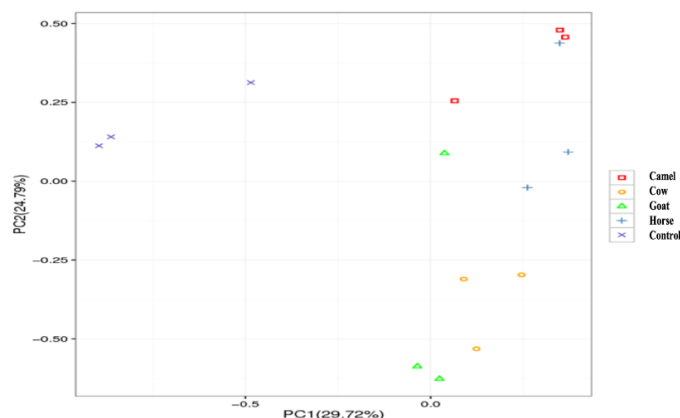

Figure 6. The PCA analysis for different raw and pasteurized milk samples (camel, cow, goat, horse) collected from Saudi Arabia, showing that the shorter distance between samples, the greater the similarity of the bacterial compositions among them and the less variation

Discussion

The existence of multiple microbial classes and families within raw milk samples in this study supported the findings of Gao et al. (2021) study, and some of these detected raw milk core microbiota are classified depending on their living conditions and locations, included teat skin bacteria (e.g., *Staphylococcaceae, Streptococcaceae*), rumen or gut bacteria (e.g., *Clostridiaceae*), and psychrotrophs (e.g., *Pseudomonadaceae*). However, teat skin bacteria in raw milk can be prevented by wiping the animal's teat before the milking process with a disinfectant containing lactic acid with 0.6% w/w chlorhexidine or iodine (the best option for decreasing staphylococcal isolates), lactic acid with salicylic acid, lactic acid with 1.6% w/w hydrogen peroxide (the best option for decreasing streptococcal isolates), diamine, chlorhexidine, chlorine dioxide, iodine, and lactic acid (Fitzpatrick et al., 2021). Surprisingly, the pasteurized milk control samples were also contaminated by bacteria because of the milk packing tankers, which were polluted with such bacteria (Vahedi et al., 2013; Porcellato et al., 2021).

Most of these discovered species within the raw and pasteurized milk samples are known as pathogenic bacteria, e.g., *Staphylococcus aureus,* which causes methicillin-resistant *Staphylococcus aureus* (MRSA), the presence of which was attributed to the lack of hygiene during the collection, preparation, and pasteurization of the samples (Alghizzi and Shami, 2021). The presence of *Escherichia,* which grows in the large intestine, was attributed to fecal contamination (Vahedi et al., 2013; Bellassi et al., 2021). Nevertheless, *Streptococcus agalactiae, Staphylococcus aureus,* and *Escherichia* within raw milk are essential bacterial strains that have been related to intramammary infections (IMIs) and mastitis (Fitzpatrick et al., 2021). Thus, the teat disinfection process has become the first step of the milking routine for modern cattle in many countries, including Ireland and the UK (Fitzpatrick et al., 2021; Breen, 2019).

Generally, many species were found to be descended from *Proteobacteria,* followed by *Firmicutes* and *Actinobacteria*, which supported the study results found by Zhang et al. (2015), who blamed the mastitis, which can be caused by pathogenic bacteria. Additionally, *Firmicutes* and *Bacteroidetes* are the dominant phyla among the human gut microbiota, followed by *Proteobacteria* and *Actinobacteria*; therefore, the consumption of any contaminated food might increase the composition level of these gut microbiota, causing irritable bowel syndrome, diarrhea, multiple sclerosis, Parkinson's disease, Alzheimer's disease, lung disease, asthma, or autism (Rizzatti et al., 2017; Magne et al., 2020). *Bacteroidetes* can also affect the drug action, e.g., patients with metastatic melanoma and high rates of *Bacteroidetes* were found to be less influenced by colitis following treatment with ipilimumab (Martin et al., 2018).

The high complexity and diversity of the discovered bacterial species among the raw milk samples from the various sets, especially for the horse raw milk samples examined in this study for the first time, could be related to environmental contamination, feed efficiency, resilience to mastitis, and teat skin microbiota (Alghizzi and Shami, 2021). Resilience to mastitis was suggested by Derakhshani et al. (2020) to protect the composition of the milk microbiota, which was affected negatively by mastitis. Tarrah et al. (2022) also concluded that the diversity of the tested cow milk microbiota in Italy was affected by the weather, so that when they applied the Chao1 index they found that the microbiota was more diverse in winter than summer. On the other hand, the lack of standard operating procedures to compare raw data from different studies makes the detection of specific factors of the microbial compositions within raw and pasteurized milk samples difficult (Parente et al., 2020; Porcellato et al., 2021).

In addition, the quality and safety of milk are completely associated with the milk microbiota, which are essential factors used to characterize the differences in the microbial formation of raw milk. Hence, the enhancement of the quality of the water used by milk handlers to wash their hands and milking equipment and the education level of the milk handlers must be enhanced to avoid milk-borne diseases through improving the milk quality (Berhanu et al., 2021; Yuan et al., 2022). However, the use of chlorine to clean and sterilize the milking equipment has negative side effects on the milk microbiota and milk quality (Cremonesi et al., 2020).

Conclusions

Recently, the interest in the studies on the characterization of the microbial communities in unpasteurized and pasteurized milk samples via applying high-throughput sequencing methods has been raised significantly in order to enhance public health, while a great fraction of such studies have been concentrated on raw cow milk rather than other types, such as raw horse milk, which is utilized in Saudi society to recover from asthma. Hence, this study focused on raw horse milk for the first time as one of the examined milk sources, and it ended up that 37.3% of the 477 discovered novel species of bacteria were from such milk sources. Additionally, the highest richness and diversity levels of bacterial species were found in the raw horse milk samples.

Furthermore, plenty of major sources of bacteria can be found in raw milk, including normal udder flora and intramammary contagions, as well as bacteria attributed to mastitis, the milking conditions, fecal contamination, and the environment. Precisely, unclean udders and teats, the feed efficiency, the inappropriate disinfection of the milking equipment, and poor cooling are factors commonly associated with high bacterial numbers, which consequently affect the milk quality. Because of the diverse bacterial compositions among the tested pasteurized milk samples, boiling and pasteurizing raw milk before consumption and using teat sanitization products as a pre-milking stage to decrease the microflora of the teat skin are highly recommended steps.

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Authors' Declaration of Interests. The authors declare that there are no competing interests that could be perceived as prejudicing the impartiality of the research reported.

Ethical Statement. This study was carried out in accordance with the recommendations of the PNU Institutional Review Board (IRB) number: 19-0169.

Data Availability Statement. The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

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