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Molecular diagnosis for camel raw milk microbiota

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Abstract

The existence of diverse microbes in unprocessed camel milk poses a significant threat to the well-being of a large population, especially infants and toddlers. The objective of this study was to ascertain the existence of microorganisms in unprocessed raw camel milk by employing a molecular-based technique in combination with a histological examination of bacteria. The identification of microbial species was achieved by employing PCR amplification and sequencing of 16s rRNA gene fragments. Various micorganisms found includes the probiotic *Lactobacillus species, Staphylococcus succinic, Macrococcus casealyticus, Bacillus cohnii,* and *Salinicoccus kunmingensis.* To prevent microbial contamination in raw milk, it is necessary to adequately heat or pasteurise the milk and to wash and sterilise the udder before milking the camel. This is because raw milk contains microbes that cause multiple diseases. Moreover, in the current era of the COVID-19 pandemics, ensuring proper sanitary conditions in milk and its derivatives might potentially mitigate the transmission of various diseases among consumers shortly.

Keywords: Camel, Microbiota, 16s rRNA gene, PCR.

1. Introduction

Camel milk has recently entered both the local and international milk markets due to its multiple advantages. Seeing the rapid growth of the milk market due to increasing demand for a greater variety of processed products the threat of microbial contamination is the biggest challenge [1]. To satisfy the standards set by other nations, it is necessary to employ state-of-the-art research and development in the appropriate sectors [2]. Raw camel milk is widely consumed in many regions due to its known presence of beneficial lactic acid bacteria and its long-established reputation as a safe source of nourishment for humans [3]. Lactic acid is generated as a secondary product of fermentation by rod-shaped bacilli or spherical cocci lactic acid bacteria. The predominant lactic acid bacteria in camel milk are derived from the genera *Lactobacillus, Lactococcus, Leuconostoc, Streptococcus, Pediococcus, Aerococcus*, and *Oenococcus.* Various research studies confirm this claim [4-7], and the bacteria *Streptococcus salivarius subsp* [8] has been found in abundance in raw camel milk. *Streptococci* spp. such as *Streptococcus thermophilus, S. agalactiae,* and *S.moroccensisrifensis* have been discovered and described because of recent genetic research in human and animal sources [9-10]. In 2020, the value of milk-based dairy products in Saudi Arabia was 4,807,70 million US dollars, and the market is expected to grow at a compound annual growth rate of 4.71 percent from 2021 to 2026.

The Dromedary Camel, scientifically known as *Camelus dromedarius*, is highly valued in Saudi Arabia because of its high food and fiber quality. Somalia and Kenya produce fresh camel milk at a rate equivalent to 64% of the global production of 2.85 million metric tons. Camel milk is the sole source of nutrition for the people of many Middle Eastern countries, and it is especially true for nations located in the grazing zone (Sausi Arabia, Joreden, Egypt, Yemen) or along migration routes. In 2010, the Kingdom of Saudi Arabia had approximately 850,000 camels of various breeds [11], derived from four native camel breeds such as Maghatier, Shul, Majahiem, and Soffer [12]. The high milk production made these species very important breeds that are geographically constrained and unique.

Globally, mastitis is a serious problem for the health of dairy cattle and other animals [13]. The symptoms are comprised of an inflammation of the mammary gland caused by microbial infection, which has a significant impact on animal welfare and severe performance consequences, thereof resulting in massive economic losses in Saudi Arabia and other Middle Eastern countries [14]. Drinking

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tainted milk puts a person at risk for a variety of illnesses, and there is even a chance that the COVID-19 virus will spread if people continue to do so despite the risks [14-18]. Mastitis harms milk quality and has a high mortality rate in dairy cattle [18]*; Streptococcus agalactiae* was found responsible for fatal acute gangrenous mastitis in dromedary camels [19]. As a result of the advancement of DNA markers and molecular biology tools, new ways have been discovered that can lead to the discovery of novel genetic markers, modification of genetic material, and the selection of high-quality traits and organisms (references). Marker-assisted selection in Saudi Arabian camels [20], is especially useful for improving the qualities that cannot be easily improved through phenotypic selection [21].

The polymerase chain reaction (PCR) was found to be highly important in microbiological research in camel and the use of many DNA markers had a significant impact on the performance of species' genomic analysis. Over the last two decades, DNA markers have emerged as a critical tool in the study of camel genetic evolution and microbial analysis (21) and according to some scientists [22], the observed heterozygosity in Bactrian camels ranged from 0.359 to 0.978, while the expected heterozygosity ranged from 0.449 to 0.879. In recent years, the discovery of genetic variability at the DNA sequence level has resulted in the development of a diverse set of applications for several markers that were previously only useful in the context of genomic research. The emerging genetic applications have resulted in the expansion of the field of genomics and microbiology and according to Manee *et al.* [23] the genome-wide characterization and analysis of microsatellite sequences in native camel species from Saudi Arabia [23] employed the MAS techniques such as marker-assisted genetic selection, genetic augmentation, and species selection. Because of DNA markers, have enabled significant advances in farm animal genetics and microbiology over the last two decades, and the diverse aspects of these animals have improved over a long period of research [12]. The sequencing of 16s rRNA gene and other molecular DNA markers has enabled the application of functional genomics to animal species improvement. A large number of DNA sequence genetic polymorphisms have been identified and validated as markers for determining the genetic basis of observed phenotypic variation as a result of recent advances in DNA technology; especially the molecular markers are indicators of changes that occur at the DNA level, and the polymerase chain reaction has emerged as a critical tool for molecular DNA testing, recognition of DNA polymorphisms (also known as fingerprints), genotyping analysis, and genome mapping in animals. In farm animals both the sequencing of the 16S rRNA gene and RAPD techniques are valuable additions to microbiological tools used for molecular diagnosis. The detection of polymorphism is useful for DNA analysis which is made possible by using a random array of single RAPD primers [24]. The goal of this study was to recognize and classify the bacteria found in camel raw milk using microbiological and molecular techniques, specifically 16s rRNA gene sequencing.

2. Methods and materials

2.1. Isolation of bacteria

Camel farms in Jeddah, Saudi Arabia, served as collection points for milk samples; and collected samples were taken from 44 different camel farms. Raw camel milk samples (up to 104 in total) were serially diluted in sterile distilled water. Following that, 100 ml aliquots of each dilution were placed on nutrient agar (NA) plates (50g) and incubated for 72 hours at 30 degrees Celsius. A variety of bacterial colonies were obtained, and their isolates were frozen in a 20% glycerol solution and stored at -80 degrees Celsius. The raw camel milk, the udder, and four different nipples were all tested, and the presence of M1, M2, and M3 bacterial strains was discovered which were later for gram-staining purposes.

2.2. Biochemical characterization

2.2.1. Gram-staining

Gram staining was carried out following the procedure given by Kumar, et al. [25].

2.2.2. Oxidase evaluation

We used a 1% aqueous solution of tetramethyl phenylenediamine as a testing material (26). A Whatman No-1 filter paper strip was placed in a glass petri dish, followed by two drops of a freshly prepared 1% tetramethyl-phenylenediamine solution. Sterile toothpicks were used to apply a bacteria loop to the impregnated area of the strip. The bacteria were isolated from a culture that had grown for 24 hours on an NA medium. On appearance of purple coloration after 10 to 60 seconds, the samples are considered to have passed the test.

2.2.3. Catalase Evaluation

The catalase activity of the bacteria was measured within 24 hours of culture in the NA medium. A loop of catalase was combined with a drop of hydrogen peroxide on a clean glass slide, and the resulting reaction was observed. This procedure was carried out for each bacterial culture [27].

2. 3. DNA extraction from bacterial genomes and purification

Following bacterial cell collection with anticoagulant EDTA (0.5M, pH8), DNA was purified using a QIAamp Genomic DNA Purification Kit. To obtain genomic DNA, a QIAamp DNA extraction kit was used, and the samples were kept at -20 degrees celsius under storage.

2.4. Polymerase Chain Reaction and Amplification of 16S ribosomal RNA.

The 16S rRNA gene was amplified using primers that are specific to each conserved region. The forward primer base sequence was 5'- CAGCGGTACCAGTTGCTGCTCAG-3', and the reverse primer base sequence was 5'- CTCTCTGCAGGCTACC TTGTACGACTTT-3'. We performed 30 cycles of amplification, each, lasting for 1 minute at 94 °C, 30 seconds at 58 °C, and 1.5 minutes at 72 °C. The final cycle was followed by an additional 10 minutes of incubation at 4 °C. The denaturing process took four minutes at a temperature of 94 degrees Fahrenheit (34.4°C). After being resolved by electrophoresis on agarose gel and observed under UV light, the amplified gene fragments were sequenced.

2.5. Species evaluation

Basic Local Alignment Search Tool (BLAST) was used to determine whether any of the 16S rDNA sequences

were present in the NCBI GenBank. MEGA 7.0.26 used the neighbor-joining method to construct the phylogenetic tree. The Kimura model was employed to compute the evolutionary distances using the dendrogram as a starting point [27-30].

3. Results

After collecting 54 samples of raw camel milk from five different camel breeds, each sample was placed on a nutrient agar plate and incubated for 24 hours at 30 degrees Celsius before being examined for the presence of bacterial growth. The pure colonies were infected with blood, chocolate, and Mac-Conkey media before being transferred to the Eppendorf tube. This was done before the relocation of pure colonies. These pure colonies were then used to determine phenotypic characteristics, physical characteristics, and molecular recognition of microbes found in raw camel milk. Raw camel milk was tested for the presence of pathogenic and nonpathogenic bacteria using the gram staining technique, as well as oxidase and catalase assays (Figures 1 and 2, respectively). *Staphylococcus succinus, Macrococcus casealyticus, Bacillus cohnii,* and *Salinicoccus kunmingensis* were all found during analysis; however, *Lactobacillus* was not included due to its favorable nature as a probiotic bacteria.

3.1. 16S rRNA gene Sequence and bacterial species differentiation

High-quality genomic DNA was extracted from the selected bacterial strains to be used in the process of determining the molecular characteristics of individual bacterial isolates. The levels of genomic DNA produced ranged from 60 to 140 ng/l. Ribosomal DNA was amplified using PCR with universal forward and reverse primers targeting a conserved region of 16S rRNA. After the PCR sample was amplified, it was run on a 1% agarose gel, and the results were examined with a UV trans-illuminator. After sequencing amplified DNA fragments, we used the BLAST program to search our database for a match (found at the link: www.ncbi.nlm.com). We found that certain pathogenic bacteria, such as *Staphylococcus succinus, Macrococcus casealyticus, Bacillus cohnii*, and *Salinicoccus kunmingensisbut* were present in raw camel milk, as depicted in Figure 3, Figure 4, and Table-1.

The high consumption of camel milk necessitates an immediate consideration of the product's microbial properties, as demonstrated in the current study of raw camel milk that has not been subjected to any thermal treatment (e.g., decontamination) containing a higher quantity of bacteria. *Staphylococcus succinus, Macrococcus casealyticus, Bacillus cohnii,* and *Salinicoccus kunmingensis* are among the microbes that can contaminate raw camel milk and cause it to spoil under room temperature. Significant logistical challenges arose in this line of work in the form of isolating and identifying microbiological contaminants in raw camel milk. Further, the bacterial counts found in raw camel milk produced in pastoral areas may overestimate the actual number of bacteria present in the milk.

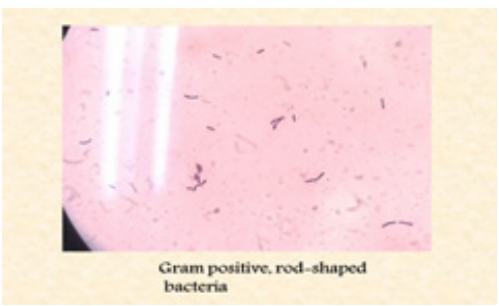

Fig. 1. Using Gram staining: Small, purple, rod-shaped, gram-positive bacteria were observed under a light microscope of 10,000 magnification range.

Fig. 2. H200803-012-E16-4C-3-1492R (*Staphylococcus Staphylococcus succinus* strain 16 (Gram-positive, catalase- and oxidase-positive).

Fig. 3. H200729-002-C03-2D-1-1-1492R (*Macrococcus caseolyticus* strain, gram positive/ oxidase positive/ catalase-positive.

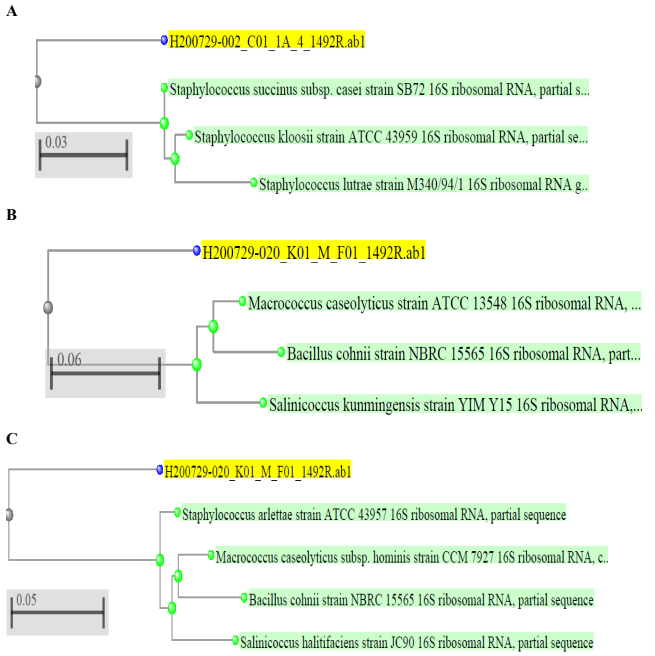

Fig. 4. Phylogenetic analysis of bacterial isolates, A, B, and C as *Staphylococcus* spp, *Macrococcus* spp, *Bacillus* spp, and *Salinicoccus* spp, from raw camel milk samples based on partial nucleotide sequences of 16S rRNA gene. The tree was constructed using the NCBI Blast.

Table 1. Shows the gram staining of bacterial species results and name of the bacterial isolates species.

Sample No.	Positive staining	Negatives staining	Sample name
1	+	0	*Staphylococcus succinus*
2	+	0	*Staphylococcus kloosii*
3	+	0	*Micrococcus scohnii*
4	+	0	*Micrococcus casealyticus*
5	+	0	*Staphylococcus lutrae*
6	+	0	*Staphylococcus arlettae*
7	+	0	*Micrococcus scohnii*
8	+	0	*Micrococcus casealyticus*
9	+	0	*Staphylococcus succinus*
10	+	0	*Staphylococcus scohnii*
11	+	0	*Bacillus cohnii*
12	+	0	*Salinicoccus kunmingensis*
13	+	0	*Salinicoccus halitifaciens*

However, on the farm, insufficient time was spent inspecting the camel udders, which resulted in the emergence of these problems.

The microbial changes of raw camel milk are quite high, and according to the results found, microbial quality varies throughout the value chain. There were large variations in the amount of bacterial contamination found in camel milk samples, and value chain analysis revealed that retail outlets on the ground level had the highest levels of contamination. The total number of *Coliform* bacteria discovered in the present study was significantly higher than expected. In addition to demonstrating the unsanitary conditions in which milk is handled and sold, the presence of these *Coliform* bacteria can result in serious human health problems. According to milk samples taken at various points along the supply chain, most of the contamination occurred at retail outlets rather than on the farms where the milk was produced. The level of microbial contamination was discovered to vary significantly. According to the current investigation, there were significantly more *Coliform* bacteria than is considered safe. These *Coliform* bacteria have the potential to cause serious infections in humans and work as a sign that milk processing and distribution were not done properly.

Phylogenetic analysis of bacterial isolates from raw camel milk samples based on partial nucleotide sequences of the 16S rRNA gene is shown in Figure 4. Figure 5 shows an alignment of 16S rDNA sequences present in NCBI GenBank.

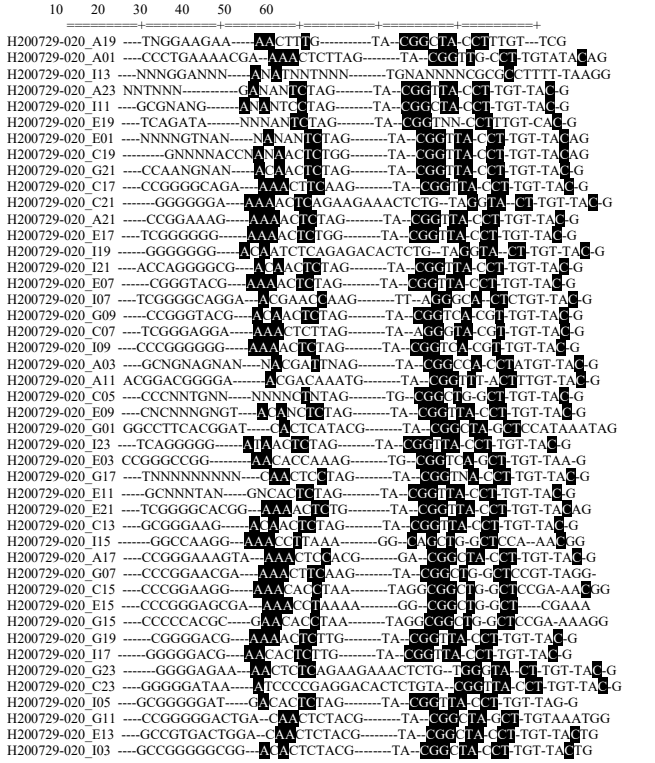

Fig. 5. Gblocks 0.91b Results, Processed file: input: fasta, Alignment assumed to be: DNA, A new number of positions: is 209 (selected positions are underlined).

4. Discussion

The majority of the raw camel milk samples tested positive for bacterial contamination, with various types of bacteria present to varying degrees in each sample. There are currently no sophisticated guidelines in place for determining the microbiological quality of camel milk. As a result, to assess the quality of camel milk, the current study used the standard microbiological parameters suitable for microbial estimation of camel milk, which is between 1 to 105 CFU/ml, with 102 CFU/ml being the starting point. TBC is a useful standard to follow when it comes to ensuring a clean environment for raw camel milk handling and processing based on the standard guidelines of TBC [31]. According to a current study, the average TBC found in raw camel milk was consistent with previous reports [31]. Variations in the microbial load of the milk product were due to contamination at the udder base, water content used to clean milking utensils, and time that passed between production and sale. Raw milk which has not been pasteurized and is collected directly from the camel, has been shown to contain more bacteria than milk collected using industrial methods [32]. The current study found that the mean coliform counts (CC) in milk samples in central and southern regions of Saudi Arabia was 83 in Log CFU/ml, lower than previous values found [31], possibly by using a larger sample size [32]. Further, 6.85 log coliform CFU/ml found in Morocco [33] was less than the 6.75 log coliform CFU/ml found in southwestern Algeria [34]. We observed that current findings contrast with the 6.75 log coliform CFU/ml found in southwestern Algeria; and the current investigation revealed that the average coliform

count was significantly higher compared to previous findings [35, 36]. According to some recent research, the most common bacteria found in raw camel milk are *Staphylococcus succinus, Macrococcus casealyticus, Bacillus cohnii,* and *Salinicoccus kunmingensis. Staphylococcus aureus* infected more than 70% (n=23) of camel milk samples due to poor hygiene and subclinical mastitis. In 31% of the cases, raw milk samples tested positive for *E. coli.* This is consistent with the findings of an earlier study, which discovered *E. coli* contamination in 39.13% of camel milk samples collected in Sudan's Bahrain region. Consuming camel milk that has not been pasturised significantly increases the likelihood of becoming ill due to the presence of disease-causing bacteria [37]. Some Africans consume camel milk directly from the camel, putting them at risk for a variety of diseases transmitted from animals to humans since raw animal milk contains more harmful bacteria than sheep or cow milk [38]. Taking this into consideration the outcomes from a current study, and also according to the latest research, the Middle East Respiratory Syndrome (MERS) virus is common in camel milk and can be easily transmitted to humans. Camel milk has a higher nutritional value comparable to cow milk; however, it contains less total fat and saturated fat, as well as provides fewer calories of energy. Camel milk has significantly higher iron and vitamin C levels than cow's milk [38]. Current research suggests that camel milk must be boiled and then pasteurized to eliminate dangerous and potentially pathogenic microbes. Because of the potential presence of a new coronavirus known as COVID-19, all animal-derived products must be thoroughly cleaned and cooked before consumption. Our study found that camel milk purchased from a store was significantly more likely to contain bacteria than camel milk obtained directly from a farm. Incorrect post-harvest milk processing procedures, as well as udder infections, are both potential sources of microbes in the market areas.

There have been reports of seasonal variations in the amounts of fat, protein, lactose, and chloride found in raw camel milk [39]. Microbiological investigation revealed that the quality of raw camel milk is poor [39-40] due to the unsanitary conditions that exist during milking, storage, transportation, and processing. Because of a lack of refrigeration during storage and transport to processing plants, as well as higher ambient temperatures, highly contaminated raw camel milk samples were collected during the summer months. Because of the presence of bacteria, drinking camel milk raw poses a potential health risk and should instead be properly cooked before consumption [40]. These findings provide compelling evidence that appropriate sanitary measures must be implemented all along the value chain of camel milk. To avoid milk contamination, farm space, employees, milking equipment and water must be neat and clean. According to the value chain model, most MRL farmers sell their milk to processors rather than directly to consumers [41, 42-44].

The quality of the milk was compromised at multiple points along value chains. These flaws included the failure to wear personal protective equipment, the use of non-food grade materials for milking equipment, storage equipment, and utensils, and the failure to cool milk while it was bulked and shipped [45]. Although home pasteurization can reduce the risk of contracting *Brucellosis* and other milk-borne zoonoses, the practice is not widely used. Re-contamination is still possible after pasteurization if the product is handled improperly [46]. Asbesto-toxins and heat-stable toxins such as *Staphylococcus aureus* entero-toxins may also survive boiling or pasteurization, as many heat-resistant spores are produced by *Clostridium perfringens* and *Bacillus* species [47]. According to some recent research [48], milk is frequently adulterated in Kenya to increase its volume (for example, by adding water to it) or improve its shelf life (i.e., the addition of inhibitory substances), increasing the chances of microbial contamination. Consuming milk tainted with pollutants and pathogens because of the addition of untreated water made the production and processing process hazardous to public health. Even though, the cooperatives and processor nodes along the formal value chain used approved milk handling practices and produced milk that exceeded KeBS's minimum requirements. However, due to consistency, it was unsuitable for use in the production of luxury food items such as cheese, which requires exceptionally high-quality raw milk. Further, it has been discovered that improper milk-handling practices in the unformalized value chain are to be blamed for the poor quality of milk produced [49]. The findings were also extremely reliable throughout the entire process and according to a recent study, poor food handling processes in agri-food value chains are the root cause of the food safety issues that exist in Sub-Saharan Africa [50], It is also critical to provide farmers and other supply chain members with the tools they need to comply with applicable regulations and food standards [51]. Lack of quality assurance programs or quality-based remuneration mechanisms may hinder milk quality and safety improvements. Milk has a short shelf life and the proper pasteurisation eliminates most bacteria, however, the result only lasts 10–15 days in the fridge. The wax paper used to package processed milk left traces on the milk, some of which are evident and may surprise consumers.

The concept of "food safety is expected" increasingly getting attention in the realm of food microbiology, specifically in a current study on untreated camel milk that has not undergone any form of thermal treatment [52]. Contaminated food containing bacteria, viruses, and other pathogenic microorganisms, can readily transmit from one individual to another. Certain pathogens present in food may serve as instances of microbial contamination. The microorganisms and their metabolic byproducts can be utilised to counteract harmful pathogens. Several probiotic microorganisms, particularly those abundant in bacteriocins, possess the ability to eradicate or impede the proliferation of harmful bacteria and viruses [53, 54, 55].

5. Conclusion

High-quality genomic DNA was isolated from bacterial strains to determine their molecular features. The genomic DNA molecule achieved was in a range from 60 to 140 ng/l. Polymerase Chain Reaction employing universal forward and reverse primers targeting a conserved 16S rRNA region amplified ribosomal DNA. After PCR amplification, the material was run on a 1% agarose gel and inspected with a UV trans-illuminator. Except for *Lactobacillus*, all probiotics are found in camel raw milk and the findings also revealed the presence of Gram-positive bacteria such as *Staphylococcus succinus, Macrococcus casealyticus, Bacillus cohnii,* and *Salinicoccus kunmingensis.* This study emphasizes the importance of food safety, which

should be based on improved hygienic practices such as contamination prevention and the use of appropriate types of processing types of equipment. Before milking the camel, the udder must be washed and checked for mastitis to reduce the possibility of bacterial contamination. Camel milk that has been boiled or pasteurized must be further processed before it can be consumed by humans, improving the milk's suitability for human consumption. To improve the consistency of camel milk, strict hygienic control measures should be put in place from the time it is processed until it is consumed. Because of the increased risk of the recent coronavirus (COVID-19), it is now more important than ever before to ensure that all animal products, including milk, eggs, and meat, are properly sterilized and cooked.

Conflict of interest

None

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