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EFFECT OF DIFFERENT TEMPERATURES ON THE MICROBIOLOGICAL STATUS OF DONKEY MILK

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SUMMARY

Donkey milk is usually sold for human consumption directly at farms and agricultural households and it is used in the raw state because of its well-known medicinal properties. Sometimes, however, such milk has poor microbiological status due to inappropriate conditions during hand milking and storage. The aim of this paper was to examine the microbiological status of donkey milk exposed to different temperature treatments (refrigerated at 4 °C for 3 h, frozen at -18 °C for 1 day, and pasteurized at 80 °C for 10 minutes). The number of molds and yeasts, total viable count, Enterobacteriaceae, Escherichia coli, coagulase-positive Staphylococcus, and the presence of foodborne pathogens, including Salmonella spp., Listeria monocytogenes, and Campylobacter spp. were evaluated to estimate the microbiological status of donkey milk. As expected, freezing and pasteurization reduced the number of tested microorganisms, and no examined foodborne pathogens were detected. The total viable count was reduced by 0.64 log CFU/mL by freezing and by 2.23 log CFU/mL by pasteurization. The total molds and yeasts and total Enterobacteriaceae count were significantly reduced by pasteurization. In conclusion, frozen and pasteurized donkey milk is safer than raw milk. In order to be able to recommend the best temperature treatment before consumption, the effects of freezing and pasteurization on the quality and antibacterial properties of donkey milk have to be further investigated.

Key words:
donkey milk, hand milking, microorganisms

Abbreviations:
DNA - deoxyribonucleic acid; DM - donkey milk; CFU - colony-forming unit; TVC - total viable count

INTRODUCTION

The time and location of donkey domestication is still unknown. Archeologists have identified remains of early domestic donkeys dating to 8.500 years ago in northeastern Africa. Analysis of ancient deoxyribonucleic acid (DNA) shows that the Nubian wild ass was the ancestor of modern donkey (Marshall & Weissbrod, 2011; Wang et al., 2020). Over the centuries, donkeys have spread from Africa across Asia, Europe, and further (Messias et al., 2021). Donkeys were mainly used for transport of people and goods, for hard agricultural work, and for their meat, milk, and skin (Kugler et al., 2008). In ancient times, donkey milk (DM) was used for its healing and cosmetic properties. Hippocrates treated many diseases with DM, while Egyptian queen Cleopatra took baths in DM to preserve her beauty (Mansueto et al., 2013).

Over time donkeys have lost their value due to mechanization in the developed countries, and the population of donkey have been reduced (Camillo et al., 2018; Messias et al., 2021). Today the interest in DM in human consumption is receiving renewed attention. Due to its nutritional, medical, and functional properties, DM is suitable for feeding infants, for the sick, and the elderly (Martini et al. 2021). Donkey milk is considered as a good alternative to breast-feeding, since it is the most similar to mother's milk in terms of its quality (lactose, lipid, and protein status). It has low levels of casein, which is convenient for feeding infants with food intolerance and cow's milk allergies (Monti et al., 2007). Besides, DM is rich in oligosaccharides, which have a valuable role in prevention of cardiovascular diseases. Recent reports suggested that DM also has strong antioxidant activity enhancing the immune system. It can also inhibit the growth of metastasis, which is important for anti-cancer treatment. Donkey milk has large amount of lysozyme, which has ability to regulate intestinal microflora (Bertino et al., 2022). All the above mentioned has resulted in increase in DM production and setting up of new farms, especially in Italy, Greece, China, and Brazil (Malissiova et al., 2016; Mottola et al., 2018; Seyiti & Kelimu, 2021; Messias et al., 2021). The interest in establishing donkey farms has also risen in Serbia lately. However, donkey farms are still family-based farms, with small production and with the lack of modern management, which primarily refers to poor hygienic conditions.

Donkey milk is still traditionally consumed in the raw state, as it is well-established opinion that heat treatments and pasteurization destroy its quality and composition characteristics (Cosentino et al., 2016). Fresh raw and frozen milk is often available through new routes of sale, such as vending machines and internet sales, which provide no data on the temperature, duration of storage, and microbiological controls (EFSA, 2015). Consuming raw DM can be potentially dangerous as it can contain pathogenic microorganisms transmissible to humans (Mottola et al., 2018). Microbiological hazards associated with raw milk are *Campylobacter* spp., *Salmonella* spp., *Escherichia coli*, *Listeria monocytogenes*, and *Staphylococcus aureus*, among others (EFSA, 2015).

According to the European Union and Serbian legislation, "raw milk" is defined as milk which is produced by secretion of the mammary gland of farmed animals and which has not been heated to more than 40 °C or undergone any treatment that has an equivalent effect. The microbial criteria for such milk gained from donkeys is ≤ 1 500 000 CFU/mL for plate count at 30 °C (Regulation, 2004; Regulation, 2017). Raw milk can be contaminated directly through organisms of animals, or indirectly from fecal contamination, and post-milking environmental contamination (EFSA, 2015).

The aim of this paper was to present the microbiological status of Domestic Balkans DM exposed to different temperature treatments (refrigerated, frozen, and pasteurized). In order to estimate the microbiological status of DM the study evaluated the number of yeasts and molds, total viable count (TVC), *Enterobacteriaceae*, *Escherichia coli*, coagulase-positive *Staphylococcus*, and *Campylobacter* spp., as well as the presence of foodborne pathogens, including *Salmonella* spp., and *Listeria monocytogenes*.

MATERIAL AND METHODS

Sampling and Milk Sample Preparation

During the period from May 2023 to June 2023 three bulk samples of raw DM were collected from "Zasavica" Special Nature Reserve in Serbia. The milk originated from clinically healthy Domestic Balkan donkeys. The animals were milked manually in the morning, and the milk samples were collected aseptically in 500 mL sterile glass bottles. After postmilking refrigeration, milk was transported to the laboratory of the Scientific Veterinary Institute in Novi Sad in cooling transport boxes at ≤ 4 °C. Each batch of milk was then divided into 3 portions: (1) refrigerated at 4 ℃ for 3 h; (2) frozen at -18 ℃ for 1 day; (3) pasteurized at 80 ℃ for 10 minutes.

Microbiological analysis

Ten mL of DM were aseptically taken into a Stomacher blender bag containing 90 mL sterile saline (Biokar Diagnostic, France). It was homogenized for 2 min using a homogenous stomacher (Mayo International SRL, Italy). Aliquot of 1 mL of homogenate was transferred to a test tube containing 9 mL sterile saline to make 10^{-2} dilution and shaken well with vortex mixer. Further serial dilutions were prepared. Microbiological analysis were performed following standard ISO methods for enumeration of yeasts and molds (SRPS ISO 21527-1:2011), total viable count (SRPS EN ISO 4833-1:2014), *Enterobacteriaceae* (SRPS EN ISO 21528-2:2017), *Escherichia coli* (SRPS ISO 16649-2:2008), coagulase-positive *Staphylococcus* (SRPS EN ISO 6888-1:2021), and *Campylobacter* spp. (SRPS EN ISO 10272-2:2017). For the detection of *Salmonella* spp. (SRPS EN ISO 6579-1:2017/A1:2020), and *Listeria monocytogenes* (SRPS EN ISO 11290-1:2017) 25 mL of DM was used. All experiments were performed in triplicate.

Statistical analysis

Data were analyzed using statistical software R version 3.2.2 (R Foundation for Statistical Computing, Vienna, Austria). One-way analysis of variance (ANOVA) followed by the Duncan test was used to statistically assess the differences between the microbial population, at the significance level of $P < 0.05$.

RESULTS AND DISCUSSION

Contamination of milk with yeasts, molds, and different bacteria may originate from air, soil, bedding, feed, grass, feces, udder, water, humans, and the storage equipment (Hassan & Frank, 2011). As pointed out in the review by Conte & Panebianco (2019), bacterial microflora of DM may consist of *Bacillus cereus*, *Campylobacter* spp., coliforms, *Cronobacter sakazakii*, *Enterobacter cloacae*, *Enterobacter agglomerans*, *Escherichia coli*, *Escherichia hermannii*, *Listeria* spp., *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Staphylococcus chromogenes*, *Staphylococcus intermedius*, *Staphylococcus sciuri*, *Staphylococcus warneri*, *Staphylococcus xylosus*, *Streptococcus hycus*, *Streptococcus epidermidis*, *Streptococcus equi*, *Streptococcus equisimilis*, *Streptococcus intermedius*, *Streptococcus zooepidemicus*, *Streptococcus dysgalactiae*, and others.

The microbiological status of refrigerated, frozen, and pasteurized DM is presented in Table 1. The average numbers of yeasts and molds in raw and frozen DM were similar, while the pasteurization significantly ($P < 0.05$) reduced the number of yeasts and molds. The study conducted at a farm in Cyprus showed the presence of yeasts and molds in only 2 out of 11 samples of DM, with the average of 3.86 log CFU/mL (Aspri et al., 2017). Šarić et al. (2023) reported the number of yeasts and molds below the limit of quantification (<1 log CFU/mL) in raw DM from Serbia.

TVC is the most widely used measure of microbial quality of raw milk, and it is primarily used as an indicator of the overall hygienic production practices at a farm (Martin et al., 2023). In this study, the mean values of TVC in DM were not higher than 5.34 log CFU/mL, defining it as high-quality milk according to the Regulation on the quality of raw milk (e.g. 1.500.000 CFU/ml, that is 6.18 log CFU/mL) (Regulation, 2017). Similar finding was reported earlier (5.23 ± 0.55 log CFU/mL) (Giacometti et al., 2016). However, other studies reported lower bacterial count of raw DM. DM from 11 different dairy donkey farms located in Italy had TVC ranging from 4.85 to 4.95 log CFU/mL (Mottola et al., 2018), while DM from Greece and Cyprus had TVC values ranging from 3.04 to 4.80 log CFU/mL (Malissiova et al., 2016). Studies conducted earlier at "Zasavica" Special Nature Reserve in Serbia showed slightly better results, with TVC up to 4.6 ± 0.02 log CFU/mL (Gubić et al., 2014), and 4.79 ± 0.55 log CFU/mL (Šarić et al., 2023) in raw DM.

The *Enterobacteriaceae* count in raw DM in our study was similar to those reported by Giacometti et al. (2016) (2.20 ± 1.69 log CFU/mL), but much higher than those obtained by Martini et al. (2018). In this study, the number of *Escherichia coli*, coagulase-positive *Staphylococcus*, and *Campylobacter* spp. were below the limit of quantification (<1 log CFU/mL) in all of the DM samples. Studies show a wide range of coagulase-positive *Staphylococcus* in raw DM (from <1.00 to 2.96 ± 1.36 log CFU/mL) (Aspri et al., 2017; Martini et al., 2018; Mottola et al., 2018). The foodborne pathogens *Salmonella* spp. and *Listeria monocytogenes* were not detected in any of the DM samples, as reported in other studies (Sarno et al., 2012; Aspri et al., 2017; Mottola et al., 2018; Šarić et al., 2023).

Table 1. Microbiological status of donkey milk

Parameters	Temperature treatments		
	Refrigerated (4 °C for 3h)	**Frozen (-18 °C for 1 day)**	**Pasteurized (80 °C for 10 min)**
Molds and yeasts (log CFU/mL)	1.35 ± 0.05^{a}	1.18 ± 0.42^{a}	$<1^{b}$
Total viable count (log CFU/mL)	5.34 ± 0.26^{a}	4.70 ± 0.14^{b}	3.11 ± 0.11^{c}
Enterobacteriaceae (log CFU/mL)	2.18 ± 0.02^{a}	2.17 ± 0.01^{a}	$<1^{b}$
Escherichia coli (log CFU/mL)	<1	<1	<1
Coagulase-positive *Staphylococcus* (log CFU/mL)	<1	<1	<1
Campylobacter spp. (log CFU/mL)	<1	<1	<1
Salmonella spp. (absence in 25 mL)	ND	ND	ND
Listeria monocytogenes (absence in 25 mL)	ND	ND	ND

Legend: ^{a,b,c} = average values in the same row marked with different letters significantly differ ($P < 0.05$)

In this study, freezing resulted in reduction of TVC by 0.64 log CFU/mL. Reduction of approximately 1-2 log CFU/mL was achieved by freezing DM at -20 °C for 4 days (Ozturkoglu-Budak, 2016). On the other hand, freezing did not reduce the number of yeasts and molds, and the number of *Enterobacteriaceae* in our study. Freezing of DM is a common practice to extend its shelf life (Papademas et al., 2022), and make it always or easily available on the market (Martini et al., 2018).

Although pasteurization of milk is a more common thermal treatment in dairy industry because it enhances the product safety (Papademas et al., 2022), it is not common among DM producers. They claim that heat treatments destroy all the antimicrobial compounds of DM and its medicinal value. They strongly recommend consumption of raw DM (Keipopele et al., 2018). Pasteurization resulted in significant ($P < 0.05$) reduction of TVC by 2.23 log CFU/mL. However, pasteurization at 65 °C for 30 minutes resulted in much higher reduction (for 4.45 log CFU/mL) in earlier study (Martini et al., 2018).

Previous studies also investigated other thermal treatments of DM, including ultra-high temperature and freeze-drying, also known as lyophilization (Aspri et al., 2016). Thermal treatments have different impact on microbiological status of DM. However, to be able to recommend the best temperature treatment before consumption of DM, the effects of freezing and pasteurization on the quality and antibacterial properties have to be further investigated. According to Yvon et al. (2019), thermal treatments at 72 °C for 8 min and at 80 °C for 10 min significantly decrease the activity of lysozyme in DM. On the other hand, low-temperature long-time pasteurization treatment of DM has no impact on the lysozyme activity. Lysozyme is a powerful enzyme present in DM with antimicrobial activity against bacteria, viruses, fungi, and parasites (Martini et al., 2019).

CONCLUSION

In conclusion, the quality of the tested raw DM is categorized as first class milk according to the Regulation on the quality of raw milk. Low levels of *Escherichia coli*, coagulase-positive *Staphylococcus*, and *Campylobacter* spp., and the absence of foodborne pathogens *Salmonella* spp. and *Listeria monocytogenes* indicate good hygienic practice used at "Zasavica" Special Nature Reserve during hand-milking of donkeys. The hygienic quality of DM was higher after pasteurization compared to freezing. The microbiological results suggest that DM should be pasteurized before consumption. However, having in mind that DM is primarily intended for consumption by infants, the sick and the elderly, further investigations are needed to guarantee the nutritional quality and antibacterial properties of DM.

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