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Short communication
Σύντομη ανακοίνωση

Natural antimicrobial effect of donkey milk evaluated with screening microbiological tests

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ABSTRACT: Donkey milk has been used since ancient times and is well known for its many beneficial properties. The aim of this study was to confirm the antimicrobial activity of donkey milk using a commercial screening assay and standard microbiological methods. The antimicrobial activity of donkey milk, along with cow, sheep, and goat milks, was initially tested by Delvotest® SP NT, a test based on the rapid growth and acid production of *Bacillus stearothermophilus*. This test showed false positive results to antibiotics in donkey milk, while all other milk samples were negative. This indicates the presence of natural antimicrobial agents in donkey milk. Further, the antimicrobial activity of all milk samples was evaluated using agar diffusion assay, against *Staphylococcus aureus* (ATCC 25923), *Bacillus subtilis* subsp. *spizizenii* (ATCC 6633), *Listeria monocytogenes* 4e (ATCC 19118), *Kocuria rhizophila* (ATCC 9341), *Escherichia coli* (ATCC 25922) and *Salmonella* Typhimurium (ATCC 14028). Only donkey milk showed small inhibition zones of *Bacillus subtilis* subsp. *spizizenii* and *Salmonella* Typhimurium at pH 6 and 8, respectively. To check the ability of foodborne pathogens to survive and grow in milk, all milk samples (donkey, cow, sheep, and goat milks) were contaminated at two levels (≈ 10 CFU/mL and ≈ 400 CFU/mL) with *Salmonella* Typhimurium and stored under different conditions (at 37 °C for 4 hours and at 4 °C for 24 hours). The highest number of *Salmonella* Typhimurium cells was recorded in cow milk, regardless of storage conditions. In this study, donkey milk demonstrated significant antibacterial activity against *Salmonella* Typhimurium in all storage conditions, which may be attributed to its naturally high content of lysozyme and lactoferrin.

Keyword: Donkey milk; Delvotest, *Bacillus, Salmonella*

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INTRODUCTION

Donkey milk (DM) shows a natural inhibitory effect against certain Gram-positive and Gram-negative bacterial strains (Martini et al., 2021). Several *in vitro* studies have confirmed the efficacy of DM in inhibiting the growth of specific foodborne pathogens, such as *Listeria monocytogenes* (Tidona et al., 2011; Šarić et al., 2014c; Fratini et al., 2016; Massouras et al., 2020), *Staphylococcus aureus* (Šarić et al., 2014c; Chandrashekar et al., 2018; Ebrahimi et al., 2021), *Enterococcus faecalis* (Nazaro et al., 2010), *Staphylococcus haemolyticus* (Massouras et al., 2020), *Bacillus cereus* (Tidona et al., 2011), *Shigella dysenteriae* (Zhang et al., 2008), *Salmonella* spp. (Zhang et al., 2008; Šarić et al., 2014b) and *Escherichia coli* (Šarić et al., 2014a). *Salmonella* serovars, *Escherichia coli* O157 and *Mycobacterium* spp. have not yet been reported in raw DM (Massouras et al., 2020; Papademas et al., 2022).

The main antimicrobial agent in milk is lysozyme, a highly conserved enzyme that appears to be of leukocyte origin (D'Alessandro et al., 2011) and has been identified in essentially all vertebrates (Ellison and Giehl, 1991). Lysozyme, also called muramidase, hydrolyzes β-1,4-glycosidic bonds between N-acetylmuramic acid and N-acetylglucosamine residues of peptidoglycan, leading to lysis of the bacterial cell (Benkerroum, 2008; Van Herreweghe and Michiel, 2012). It is very effective against Gram-positive bacteria, while Gram-negative bacteria are partially protected by an outer membrane, the lipopolysaccharide of the cell wall, which makes it difficult for lysozyme to reach their target peptidoglycan sites. However, the exact mechanism of its action is still not fully understood, as the hydrolytic degradation of peptidoglycan does not explain the resistance of certain Gram-positive bacteria, nor the effect of lysozyme on viruses and eukaryotic microorganisms. In addition, the occurrence of Gram-negative bacteria naturally sensitive to lysozyme may be explained by the interaction of lysozyme with the lipid layer of the inner membrane (Benkerroum, 2008). Based on the lysozyme content, mammalian milk is classified into two groups: the first with high lysozyme levels ranges from 200 to 1,330 mg/L (human, equine and canine milks) and the second with 3,000 to 6,000 times lower lysozyme than those in the first group (bovine, ovine and caprine) (Benkerroum, 2008). In DM, the lysozyme content has been reported to range from 1,000 to 2,000 mg/L (Fratini et al., 2016), which can reach levels of 4,000 mg/L (Guo et al., 2007; Zhang et al., 2008; Brumini et al., 2016). Variations in lysozyme levels are related to breed, lactation period and season (D'Alessandro et al., 2011; Naidenova, 2022). High lysozyme concentrations in DM, ranging from 670 to 3,740 mg/L, have been recorded in Italian and domestic Balkan donkeys (Šarić et al., 2012; Brumini et al., 2016).

Lactoferrin plays a secondary antimicrobial role in DM (Ozturkoglu-Budak, 2018). Lactoferrin was first identified as a high-affinity iron chelator in human milk but was later discovered to have additional mechanisms of action against bacteria beyond those related to iron deficiency (Ellison and Giehl, 1991). Lactoferrin exhibits antibacterial, antiviral, antifungal, and antiprotozoal activities (Jenssen and Hancock, 2009; Martini et al., 2021). Lactoferrin can damage the outer membrane of Gram-negative organisms and may increase their susceptibility to lysozyme. Each protein, lactoferrin and lysozyme, is bacteriostatic by itself; together they may exert a bactericidal effect for Gram-negative bacteria and almost always coexist in vivo (Ellison and Giehl, 1991). Lactoferrin is a minor component of DM and its concentration ranges from 80 to 133 mg/L (Tidona et al., 2011; Vincenzetti et al., 2008).

In raw milk delivered to the market, maximum residue limits (MRLs) of pharmacologically active substances are established by Commission Regulation (EU) No 37/2010. Raw milk refers to milk from cows, less commonly from sheep or goats. For a drug residue-free milk supply, several methods, such as microbial inhibition methods, immunological methods, enzymatic assays, gas chromatography, and liquid chromatography, are used to detect drug residues in milk (Kand and Kondo, 2001). Microbiological methods for detecting antibiotic residues in milk are most often used as screening tests, because they allow the examination of many samples, are simple to perform, and do not require special equipment. Many studies have confirmed that natural antimicrobial substances whose concentrations are increased in colostrum and milk of cows with mastitis, can give false-positive results in screening microbiological tests for the detection of antibiotic residues (Carlsson et al., 1989; Tyler et al., 1992; Van Eenennaam et al., 1993).

The starting point of this preliminary study is the assumption that the antimicrobial effect of DM, which is the result of naturally high concentrations of lysozyme, can be detected by simple microbiological methods, as well as by a commercial screening test for the detection of antibiotic residues in milk. The natural antimicrobial effect of DM, considering the rise of antibacterial resistance, may have health benefits fighting infections and supporting the immune system of consumers.

MATERIALS AND METHODS

Donkey milk samples

DM samples were collected from 25 Domestic Balkan donkeys in the Special Nature Reserve "Zasavica". DM samples were collected from clinically healthy individuals in the middle stage of lactation (120-150 days post partum). Sampling was performed by hand milking in the morning, where milk from 5 donkeys was collected in one sterile glass bottle. To compare the natural antimicrobial properties, in addition to DM, we also used samples of raw cow milk (n=5), sheep milk (n=5), and goat milk (n=5). Milk samples were collected from healthy animals (jennies, cows, sheep and goats), originating from local farms. All the selected farms followed similar hygienic procedures before, during and after hand milking. Based on anamnestic data, the animals from which the milk was collected were not treated with antibiotics. LC-MS/MS analysis confirmed that the milk samples did not contain antibiotic residues, including sulfonamide, amoxicillin, ampicillin, cephalexin, cefquinome, ceftiofur, dicloxacillin, cloxacillin, oxacillin, penicillin G, doxycycline, chlortetracycline, oxytetracycline, and tetracycline (data not shown). All milk samples were frozen and stored at -20 °C until analysis.

Delvotest® SP NT

All milk samples were tested by Delvotest® SP NT (DSM Food Specialties, Delft, the Netherlands) according to the manufacturer's instructions, immediately upon delivery to the laboratory and re-examined after heat treatment for 10 min at 80 °C.

Agar plate test

The natural antimicrobial activity of milk samples was scanned using agar diffusion assay, against six reference strains: *Staphylococcus aureus* (ATCC 25923), *Bacillus subtilis* subsp. *spizizenii* (ATCC 6633), *Listeria monocytogenes* 4e (ATCC 19118), *Kocuria rhizophila* (ATCC 9341), *Escherichia coli* (ATCC 25922), and *Salmonella* Typhimurium (ATCC 14028). Suspensions with a density of 0.5 McFarland were prepared from reference strains. The suspensions were diluted 1:20 in a sterile saline solution and inoculated with a sterile swab onto the surface of test agar at pH 6 (Merck, Darmstadt, Germany; dehydrated medium 10663), pH 7.2 (Merck, Darmstadt, Germany; dehydrated medium 15787) and pH 8 (Merck, Darmstadt, Germany; dehydrated medium 10664) in Petri dishes (Ø 90 mm). After 10 min, sterile paper filter discs (6 mm) soaked with cow, sheep, goat, and donkey milk, were placed on the surface. Discs soaked into sterile saline were used as negative controls, while ampicillin (10 µg) and sulfadimidine (200 µg) discs were used as positive controls. All agar plates were incubated for 24 h at 37 °C, after which they were examined for the zones of growth inhibition around the discs (diameter of the zone of inhibition).

Artificial contamination of sample

Artificial contamination of milk samples was carried out by suspension of reference culture *S.* Typhimurium, in two levels of contamination: ≈ 10 CFU/mL and ≈ 400 CFU/mL, in duplicate. Non-inoculated milk samples were used as negative control, while tryptone soya broth (TSB, Biokar Diagnostics, Beauvais, France) inoculated with *S.* Typhimurium was used as a positive control. The number of viable *S.* Typhimurium cells was detemined after 4 h of incubation at 37 °C, after which the samples were stored at 4 °C for an additional 24h, and viable counts were determined again. The contents of the test tubes were homogenized on a vortex, and two decimal dilutions were prepared. The contents of each tube and decimal dilutions were spread in an amount of 100 µL onto the surface of xylose lysine deoxycholate agar (XLD, Biokar Diagnostics, Beauvais, France) in duplicate. After incubation of XLD agar for 24 h at 37 °C, *S.* Typhimurium colonies were counted. The results were then expressed as CFU/mL of milk. Statistical analyses were performed by statistical software R version 3.2.2 (R Foundation for Statistical Computing, Vienna, Austria). To determine the statistical differences the analysis of variance (ANOVA), followed by a Duncan's test, was performed. The comparison was evaluated at $p < 0.05$.

RESULTS AND DISCUSSION

The initial assumption of the study was that screening with microbiological methods could confirm the natural antimicrobial effect of antibiotic-free DM. According to Delvotest® SP NT, all examined milk samples (cow, goat, and sheep milk) were negative, free from inhibitors or containing antibiotics below the detection limit of the test, except DM samples which were positive, indicating the presence of antibiotic residues.

The Delvotest® SP NT test is based on the rapid growth and acid production of *Bacillus stearothermophilus* and the change in the color of the indicator from purple to yellow, if the milk does not contain inhibitory substances. The method was recognized by the Association of Official Analytical Chemists in 1982 (Katz, 1982; Kelley, 1982). The Delvotest® SP NT is most suitable for testing individual cows, and there is less data on its use for testing sheep and goat milk (Stead et al., 2008; Sierra et al., 2009), as well as DM (Tskali et al., 2018).

The main disadvantage of screening microbiological tests for the detection of antibiotic residues in milk is that they are not specific for antimicrobial substances, i.e. they do not reveal their identity and origin (Carlsson et al., 1989). Positive results may occur when testing milk samples containing inhibitory substances that are natural components of milk, such as lactoferrin and lysozyme, and whose concentrations is usually higher in colostrum (Korhonen, 1977) and bovine mastitis milk samples (Carlsson et al., 1989; Kang and Kondo, 2001) or in cows challenged with endotoxin (Tyler et al., 1992; Van Eenennaam et al., 1993; Andrew et al., 1997). Lysozyme and lactoferrin were shown to inhibit the growth of *Bacillus stearothermophilus* var. *calidolactis* spores, used as test organism in Delvotest P (Carlsson and Björck, 1987; Kaneen and Hurd, 1990). Compared to cow's milk, DM is characterized by naturally high content of components with antimicrobial effects, such as lysozyme, immunoglobulins, lactoferrin, and lactoperoxidase (Guo et al., 2007; Šarić et al., 2012; Fratini et al., 2016; Naydenova, 2022). Concentrations of lysozyme and lactoferrin in bovine milk are low, on average only 0.13 mg/L of lysozyme and lactoferrin between 20 and 350 mg/L (International Dairy Federation, 1985; Carlsson and Björck, 1987). The DM samples collected at the Special Nature Reserve "Zasavica" contained lysozyme in range from 980 to 3,740 mg/L, while lactoferrin ranged from 2.2 to 54.3 mg/L (Šarić et al., 2014a).

Since a positive reaction in Delvotest correlates well with increases in lactoferrin and lysozyme concentrations (Carlsson et al., 1989), Delvotest® SP NT may not be suitable for antibiotic residue testing of DM, nor is DM listed in the manufacturer's instruction or in the FAQ section. Contrary to our observation, Tskali et al. (2018) reported a negative result using Delvotest P (Gist-Brocades, NY) when testing DM samples from the Arcadian breed in Greece for antibiotic residues.

A positive result, using Delvotest® SP NT, was also obtained by retesting the same samples of DM previously heat-treated at 80 °C for 10 minutes, indicating insufficient inhibition of natural antimicrobial substances. Contrary, Yvon et al. (2019) reported significant decrease in lysozyme activity at 80 °C for 10 min with a reduction of 60.79 ± 11.65%. Additionally, a decrease in lysozyme activity was reported at 72 °C for 8 min with a reduction of 27.15 ± 5.32%. During heat treatment of DM at 75 °C, a small loss in enzyme stability was observed, with a decrease of 8.25% compared to raw DM, whereas heat treatment at 85 °C destroyed approximately 60% of lysozyme (Ozturkoglu-Budak, 2018). Partial denaturation of lysozyme at 80 °C for 20 min resulted in a strongly reduced antimicrobial activity compared with native lysozyme (Ibrahim et al., 1997). Denaturation of lysozyme begins at 85 °C, while lactoferrin was more sensitive to heat treatment and begans to lose stability at 65 °C (Ozturkoglu-Budak, 2018).

As shown in a previous study (Šarić et al., 2014a), the high level of lysozyme in DM (980- 3,740 mg/L) from the same farm as our DM samples meant that reported reduction in lysozyme (>60%) was not sufficient to obtain a negative result with the Delvotest® SP NT. In the agar diffusion screening test, the antimicrobial effect of cow, sheep, goat, and donkey milk was tested using suspensions of six reference bacterial strains. Small zones of growth inhibition, up to 2 mm, were observed only around the discs soaked with DM for two indicator strains: *B. spizizenii* (pH 6) and *S.* Typhimurium (pH 8). However, growth of individual colonies of *B. spizizenii* was observed in the zone of initial growth inhibition. At pH 6, the inhibition zones were 39 mm for ampicillin and 32 mm for sulfadimidine, whereas at pH 8, they were 32 mm for ampicillin and 18 mm for sulfadimidine.

The number of viable *S.* Typhimurium cells in artificially contaminated milk samples after 4 hours of incubation at 37 °C and after 24 hours at 4 °C is shown in Table 1.

The highest count (CFU/mL) of *S.* Typhimurium was recorded in cow's milk samples at both levels of contamination. Therefore, we excluded cow's milk in the next statistical analysis. Combined cultivation of certain bacteria and cooperation between organisms through the exchange of metabolic products and nutrients in raw milk can enhance bacterial growth

Table 1. The number of viable *Salmonella* Typhimurium (CFU/mL) cells in artificially contaminated milk.

Group	Contamination level ≈ 10 CFU/mL		Contamination level ≈ 400 CFU/mL	
	4 h on 37 °C	24 h on 4 °C	4 h on 37 °C	24 h on 4 °C
DM	80 ± 15.81^{dU}	58.2 ± 13.83^{cZ}	2,942 ± 175.98^{eU}	2,328 ± 442.85^{dU}
Cow milk	1,014 ± 116.53^{a}	670 ± 86.60^{a}	41,100 ± 3,489.27^{a}	25,700 ± 2,109.50^{a}
Sheep milk	178 ± 40.25^{dZ}	100 ± 29.15^{bcY}	12,920 ± 402.49^{dZ}	7,740 ± 920.87^{bX}
Goat milk	804 ± 103.83^{bX}	124 ± 20.74^{bXY}	29,540 ± 2,161.71^{cY}	5,720 ± 903.88^{cY}
TSB	484 ± 74.36^{cY}	148 ± 41.47b^{bX}	36,280 ± 5,233.74^{bX}	4,660 ± 550.45^{cZ}

Different small letters (a, b, c, d, e) in the column indicate statistically significant differences between all groups ($p < 0.05$). Different big letters (X, Y, Z, U) in the column indicate statistically significant differences between all groups excluding cow milk ($p < 0.05$).

(Pacheco and Segrè, 2019; Hahne and Lipski, 2021). Comparing DM with cow, sheep, and goat milk, it showed significant ($p < 0.05$) antimicrobial effect on *S.* Typhimurium. At the low level of contamination, the number of viable cells of *S.* Typhimurium were 80±15.81 CFU/mL (37 °C for 4 h) and 58.2±13.83 CFU/mL (4 °C for 24 h), while the number of viable cells of *S.* Typhimurium, at the high level of contamination, were 2,942±175.98 CFU/mL (37 °C for 4 h) and 2,328±442.85 CFU/mL (4 °C for 24 h).

The inhibitory effect of DM on Gram-negative bacteria is explained by the synergistic action of lysozyme and lactoferrin (Zang et al., 2008; Tidona et al., 2011). Although both lysozyme and lactoferrin individually have a bacteriostatic effect, together they can have a bactericidal effect on *E. coli*, *Salmonella*, and *Vibrio cholerae* (Elison and Giehl., 1991). Previous studies have confirmed the antimicrobial activity of DM obtained from an indigenous Chinese breed of donkey against *Salmonella choleraesuis* and *Shigella dysenteriae* (Zhang et al., 2008), as well as Domestic Balkan DM against *S.* Typhimurium, *S.* Enteritidis and *S.* Livingstone (Šarić et al., 2014b). Šarić et al. (2014b) observed that the antibacterial potential of DM was influenced by the initial bacterial count: at the lowest level of contamination (10^2 CFU/mL), raw DM showed bactericidal activity resulting in the destruction of 99.9% of bacteria after five days of storage at 4 °C. The antimicrobial activity of DM can be described as bacteriostatic in milk samples with higher levels of contamination (10^3 CFU/mL and 10^4 CFU/mL).

CONCLUSION

The antimicrobial properties of DM can be detected using simple screening microbiological tests. The naturally high content of lysozyme and lactoferrin makes this type of milk unsuitable for screening with rapid tests for antibiotic residues. Although decades of empirical experience from the previous century have shown the benefits of DM consumption for human health, particularly in preventing intestinal infections in children compared to those fed with cow's milk, this practice has, unfortunately, completely disappeared. The increasing prevalence of adverse reactions (intolerance and allergies) associated with the consumption of intensively produced cow's milk highlights the need for greater research efforts and promotion of DM for the preservation of human health. As shown in this study, the antimicrobial effect of raw DM against *S.* Typhimurium, which can cause severe foodborne disease, may be considered as a valuable natural antimicrobial agent.

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