

Original Research Article

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Microbial Profile and Antimicrobial Effect of Donkey Milk against *Staphylococcus aureus*

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ABSTRACT

In India, there is a growing interest in donkey milk mainly because of its antimicrobial properties. The aim of this study was to know the microbial profile and to investigate antibacterial effect of raw donkey milk against *Staphylococcus aureus* (*S. aureus*). Average values of fat, SNF, cholesterol, proteins, lactose, salt, temperature and pH were 0.67, 8.05, 30.4, 2.95, 4.43, 0.66, 30.83 °C and 7.08 respectively. The total viable count in the neat donkey milk samples varied from 220 to 2080 cfu/ml (918 or 2.96 log cfu/ml on average). All samples were negative for moulds/yeast and also for pathogenic bacteria like *S. aureus*, *Salmonella* spp., *E. coli*, *Bacillus cereus* and *Streptococcus* spp. In two samples, the bacterial count reduced below the detection limit of <1 log cfu/ml after 5hr of incubation which indicates the antimicrobial effects of donkey milk. In disc diffusion test a clear zone of inhibition was noticed.

Keywords

Donkey milk,
Microbial profile,
Antimicrobial
effect, Lysozyme

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Introduction

In India, donkey milk (DM) is gaining lot of attention for human (children) consumption in view of its medicinal and therapeutic values with special reference to its antimicrobial property. Since ages, donkey milk has been known for its significant medicinal and therapeutic properties (Zhang *et al.*, 2008, Vincenzetti *et al.*, 2008). Some of these properties of DM are in prevention of

atherosclerosis (Tafaro *et al.*, 2007), antibacterial (Saric *et al.*, 2012; Tidona *et al.*, 2011; Zhang *et al.*, 2008) antiviral (Brumini *et al.*, 2013), antiproliferative and anti-tumor effects (Mao *et al.*, 2009) but majority of its application is by considering it as a alternative to cow/mother milk for children affected with milk allergy. The DM is having higher tolerability, palatability, nutritional adequacy (Iacono *et al.*, 1992; Mansueto *et al.*, 2013; Monti *et al.*, 2007, 2012) Low fat and casein

content, high percentage of lactose, lysozymes and essential amino acids (Vincenzetti *et al.*, 2008). The objective of this study was to know the microbial profile and to investigate antibacterial effect of raw DM against *Staphylococcus aureus* (*S. aureus*).

Materials and Methods

Sample collection

Fresh donkey milk samples were collected from eight apparently healthy donkeys in their third month of lactation. Initially, the udder was cleaned with water and then dried with cloth & then the animals were milked. Immediately after collection, the milk was transported to laboratory and pH of the milk was recorded.

Milk chemical and biochemical analyses

Ten milliliter of DM from each sample was subjected into sensor based milk analyzer (Ksheeraa – by Dairy equipments limited, India) to know the chemical and biochemical analyses like FAT, SNF (Solid not fat), CLR (Cholesterol), Water, Temperature, PTN (Protein), LCT (Lactose) and SLT (Salt).

Microbial profile

To assess total bacterial or viable count, serial log dilution of milk samples was conducted in sterile saline (1ml milk + 9ml saline) and 100µl of Neat (undiluted), 10^{-1} , 10^{-2} dilutions were spread on PCA (Plate count agar) in duplicate by L spreaders (Himedia) to assess total bacterial or viable count and plates are incubated at 37 °C for overnight. The results were then expressed as cfu/mL.

Neat milk samples (100µl) in duplicate were spread on SDA (Sabarouds dextrose agar), MSA (Mannitol salt agar), MCA (McConkey agar), XLD (Xylose lysine deoxycholate),

BCA (*Bacillus cereus* agar), Deoxy Lactose agar and Edwards medium to identify the pathogenic moulds/yeast, *S. aureus*, Lactose fermenters/Lactose non-fermenters, *Salmonella*, *Bacillus cereus*, *Coliforms* and *Streptococcus*, respectively and incubated overnight at 37°C.

Antibacterial assay

The antibacterial assay was performed on milk samples using *S. aureus* reference strain (MTCC No. 96) (Accession No. JN247783). After overnight incubation on BHI at 37 °C, well-isolated colony of *S. aureus* was selected and transferred with an inoculating loop to a tube of sterile saline and vortexed thoroughly. The density of the bacterial suspension was adjusted to 0.5 McFarland (1.5×10^8 cfu / ml) using McFarland standards (Himedia, India). Further decimal dilutions (1ml of bacterial suspension + 9ml of saline) to get 10^7 cfus, 10^6 cfus, 10^5 cfus, 10^4 and 10^3 cfus in sterile saline were prepared from initial suspensions.

The DM samples (9ml) were spiked to mimic the contamination with the bacterial suspension (1ml) at the level of contamination of 10^3 cfu/mL.

10 mL of each artificially contaminated sample were placed into a sterile beaker and kept in water bath at 38 ± 0.5 °C for 5 hours duration. Changes in the number of tested bacteria were monitored at every one & five hour by spreading 200 µl on MSA or on BHI agar in duplicate. Non inoculated DM was used as negative control, while artificially contaminated nutrient broth (Himedia, India) was used as positive control. Further circular Whatman filter paper were suspended in neat, 10^{-1} and 10^{-2} diluted milk samples and these dried discs were placed on the MHA plate streaked with 0.5 Mcfarland *S. aureus* bacterial suspension, incubated overnight (Disc diffusion test).

Results and Discussion

All samples on an average showed a pH of 7.08 & temperature of 30.83 °C. The average values of fat, SNF, cholesterol, proteins, lactose & salt were 0.67, 8.05, 30.4, 2.95, 4.43 and 0.66, respectively. The total viable count in the neat donkey milk samples varied from 220 – 2080 cfu/ml (918 or 2.96 log cfu/ml on average). The mesophilic total viable bacterial count in our study was in agreement with Pilla *et al.*, 2010 (1 to 2.39 log cfu/ml) and in contrast with other studies like Malissiova *et al.*, 2015 (6.7 X 10³ log cfu/ml); Sarno *et al.*, 2012 (less than 4 log cfu/ml); Count ranging from 4 to 4.7 log cfu/ml (Chiavarin *et al.*, 2005, Zhang *et al.*, 2008, Ivankovi *et al.*, 2009, Addo and Ferragut 2015, Salimei *et al.*, 2004, Coppola *et al.*, 2002, Sorrentino *et al.*, 2010) and Cavallarin *et al.*, 2015 (5 log cfu/ml) (Table 1).

All samples were negative for moulds/yeast and also for pathogens like *S. aureus*, *Salmonella* spp., *E. coli*, *Bacillus cereus*, lactose fermenters / non fermenters and

Streptococcus spp. Our study revealed the absence of pathogens in raw DM, similar studies conducted earlier also revealed the same results with the exception of some studies which showed the presence of *S. aureus* (Pilla *et al.*, 2010) and *B. cereus* (Cavallarin *et al.*, 2015 - 1.3 X 10² cfu/mL, Bartoszewicz, *et al.*, 2008, Scatassa *et al.*, 2011) in raw DM samples.

The number of viable cells of the tested *S. aureus* decreased significantly in artificially contaminated DM (Table 2) in comparison with positive controls after one hr and five hours of incubation. In samples 3 & 4 the bacterial count reduced below the detection limit of <1 log cfu/ml after 5 hr of incubation which indicates the antimicrobial effects of DM. Similar kind of study was carried by Saric *et al.*, (2014) in which they showed the antimicrobial effect of DM against *S. aureus*, *Listeria monocytogenes* and *E. coli*. Nazzero *et al.*, (2010) and Tidona *et al.*, (2011) reported the antibacterial activity of DM toward *S. aureus* and *L. monocytogenes*, respectively.

Table.1 Chemical and biochemical analyses of donkey milk samples

| Sample No. | FAT | SNF | CLR (Cholesterol) | H ₂ O | Temperature | PTN (Protein) | LCT (Lactose) | SLT (Salt) |
|------------|------|------|-------------------|------------------|-------------|---------------|---------------|------------|
| 1 | 0.66 | 8.63 | 32.4 | 00 | 30 | 3.16 | 4.74 | 0.71 |
| 2 | 0.70 | 7.06 | 26.8 | 00 | 31 | 2.59 | 3.88 | 0.58 |
| 3 | 0.69 | 7.15 | 27.2 | 00 | 31 | 2.62 | 3.93 | 0.59 |
| 4 | 0.59 | 8.49 | 31.9 | 00 | 31 | 3.11 | 4.67 | 0.70 |
| 5 | 0.65 | 8.53 | 32.0 | 00 | 29 | 3.13 | 4.69 | 0.70 |
| 6 | 0.74 | 8.49 | 32.5 | 00 | 33 | 3.11 | 4.67 | 0.70 |

Table.2 Enumeration of *S. aureus* in spiked DM samples at different duration of incubations

| Sample No. | 0 hr (cfus) | 1hr (cfus) | 5hr (cfus) |
|------------|-------------|------------|------------|
| 1 | 263 | 76 | 16 |
| 2 | >300 | 97 | 46 |
| 3 | 232 | 54 | 05 |
| 4 | 253 | 59 | 02 |
| 5 | 248 | 64 | 13 |
| 6 | 223 | 39 | 08 |

This reduction in bacterial count in artificially contaminated DM was mainly attributed to lysozyme which is present in high concentration and therefore it is responsible for antibacterial effect of DM (Coppola *et al.*, 2002; Vincenzetti *et al.*, 2008; Zhang *et al.*, 2008; Tidona *et al.*, 2011; Sarno *et al.*, 2012 and Saric *et al.*, 2014). Lysozyme exhibits antibacterial effect particularly towards gram positive bacteria by cleaving the linkages between N-acetylmuramic acid and 2-acetyl-amino-2-deoxy-D-glucose residues in cell wall (Floris *et al.*, 2003; Van Herreweghe and Michiel, 2012). The lysozyme activity against the gram negative bacteria is less sensitive compared to gram positive bacteria may be due to strong barrier of LPS for the penetration of lysozyme to the action sites of gram negative cell wall (Floris *et al.*, 2003). In spite of this speculation, there are studies which reported the antimicrobial effect of DM against gram negative bacteria like *E. coli*, *Salmonella* and other *Enterobacteriaceae* (Zhang *et al.*, 2008; Tidona *et al.*, 2011; Saric, *et al.*, 2012; Saric, *et al.*, 2014). This indicates the presence of some other factors in DM that also exhibit antimicrobial effect along with lysozyme. Lactoferrin is designated as secondary antimicrobial factor in DM, since it is present in significantly lower concentration in DM in comparison to lysozyme (Coppola *et al.*, 2002; Tidona *et al.*, 2011; Vincenzetti *et al.*, 2008; Zhang *et al.*, 2008).

Negative controls did not reveal any growth of *S. aureus* at different periods of incubation. In disc diffusion test there was a clear zone of inhibition surrounding the Whatman filter paper discs which further indicates the antimicrobial effect of DM. A significant antimicrobial effect was exhibited by DM against *S. aureus* in artificially contaminated milk as well as in disc diffusion test. Based on the results obtained in this study we conclude that DM is healthy and exhibits antibacterial effect against *S. aureus*.

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