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, Vincenetti *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 an 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 assase 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 (Sabar ouds 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 x 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., 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 10^2 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. Nazzaro 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

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>FAT</th>
<th>SNF</th>
<th>CLR (Cholesterol)</th>
<th>H2O</th>
<th>Temperature</th>
<th>PTN (Protein)</th>
<th>LCT (Lactose)</th>
<th>SLT (Salt)</th>
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<tbody>
<tr>
<td>1</td>
<td>0.66</td>
<td>8.63</td>
<td>32.4</td>
<td>00</td>
<td>30</td>
<td>3.16</td>
<td>4.74</td>
<td>0.71</td>
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<tr>
<td>2</td>
<td>0.70</td>
<td>7.06</td>
<td>26.8</td>
<td>00</td>
<td>31</td>
<td>2.59</td>
<td>3.88</td>
<td>0.58</td>
</tr>
<tr>
<td>3</td>
<td>0.69</td>
<td>7.15</td>
<td>27.2</td>
<td>00</td>
<td>31</td>
<td>2.62</td>
<td>3.93</td>
<td>0.59</td>
</tr>
<tr>
<td>4</td>
<td>0.59</td>
<td>8.49</td>
<td>31.9</td>
<td>00</td>
<td>31</td>
<td>3.11</td>
<td>4.67</td>
<td>0.70</td>
</tr>
<tr>
<td>5</td>
<td>0.65</td>
<td>8.53</td>
<td>32.0</td>
<td>00</td>
<td>29</td>
<td>3.13</td>
<td>4.69</td>
<td>0.70</td>
</tr>
<tr>
<td>6</td>
<td>0.74</td>
<td>8.49</td>
<td>32.5</td>
<td>00</td>
<td>33</td>
<td>3.11</td>
<td>4.67</td>
<td>0.70</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>0 hr (cfus)</th>
<th>1hr (cfus)</th>
<th>5hr (cfus)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>263</td>
<td>76</td>
<td>16</td>
</tr>
<tr>
<td>2</td>
<td>&gt;300</td>
<td>97</td>
<td>46</td>
</tr>
<tr>
<td>3</td>
<td>232</td>
<td>54</td>
<td>05</td>
</tr>
<tr>
<td>4</td>
<td>253</td>
<td>59</td>
<td>02</td>
</tr>
<tr>
<td>5</td>
<td>248</td>
<td>64</td>
<td>13</td>
</tr>
<tr>
<td>6</td>
<td>223</td>
<td>39</td>
<td>08</td>
</tr>
</tbody>
</table>
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-acetylamino-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).

Inspite 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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