PAPER

Microbiological quality of raw donkey milk from Campania Region

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Abstract

Microbiological quality of raw milk from eight healthy donkeys reared in Campania Region was investigated. A total of 152 samples were analyzed in order to evaluate the milk safety status trough monitoring mesophilic total bacterial count (TBC) at 32°C and 20°C, psychrophilic TBC at 5°C, *Enterobacteriaceae*, *Salmonella* spp., *Listeria monocytogenes, Staphylococcus aureus* and somatic cell count (SCC). The ranges for mesophilic bacteria at 32°C, 20°C and psychrophilic bacteria at 5°C were, respectively, 2.80-4.00 Log CFU/mL, 2.84-3.92 Log CFU/mL and 1.27-2.12 Log CFU/ml. *Enterobacteriaceae* showed a load ranging between 0.68-1.93 Log CFU/mL. No pathogenic bacteria were isolated. Estimated SCC values were always under 50.000 cells/mL. Additionally quantitative changes of bacterial population in raw bulk milk during eight storing days at 8°C and 3°C, were evaluated. Firstly, fresh bulk milk was contaminated by bacteria with a mean TBC at 32°C and 20°C of 2.71 Log CFU/mL and 2.64 Log CFU/mL, respectively, whereas TBC at 5°C and *Enterobacteriaceae* were not detected. After eight days of storage at 8°C, TBC at 32°C, 20°C and *Enterobacteriaceae* increased by three Log and TBC at 5°C by five Log. On the other side, after eight days of storage at 3°C no gradual Log increase was detected. Our results showed that donkey milk could be a good healthy ingredient for feeding where good hygienic procedures are applied and storage is kept at temperature lower than 3°C.

Introduction

In recent times in Italy, interest in donkey breeding for milk production has increased due to nutrient composition of its milk, which is very close to breast milk. When human breastfeeding is not possible, the use of replacer must provide best nutritional and healthy needs for infants. Possible breast-milk substitutes include: commercial infant formula, liquid animal milk (cow or goat), powdered animal milk, dehydrated milk. Donkey's milk is already used as possible milk (Polidori *et al.*, 2009) to replace cow milk in young children affected by cow milk allergy (Monti *et al.*, 2007; Vincenzetti *et al.*, 2008). Similarity consists in the lipid fraction, characterized by high level of linoleic and linolenic acid, in the Ca/P ratio and in the low total protein fraction (Salimei *et al.*, 2004), well-represented by whey proteins such as α-lactalbumin (α-LA), β-lactoglobulins (β-LG) and lysozyme (LYS) (Fantuz *et al.*, 2001). Lysozyme with other factors may reduce the incidence of gastrointestinal infections in infants (Businco *et al.*, 2000). It is known as natural antimicrobial agent acting directly on bacterial cell walls (Chiavari *et al.*, 2005) and its concentration results two times higher than human milk (Chiavari *et al.*, 2005; Vincenzetti *et al.*, 2008) and variable during the different stages of donkey lactation (Vincenzetti *et al.*, 2008). Lysozyme may be responsible for the low bacterial load reported in literature (Salimei *et al.*, 2004) and for having positive effects on raw milk storage (Zhang *et al.*, 2008).

The aims of the present study were therefore to evaluate i) the individual health status of the donkey's mammary gland during lactation stage through monitoring total bacteria count (TBC), *Enterobacteriaceae* and somatic cell count (SCC); and ii) the safety status of donkey milk by investigating the presence of pathogens such as *Salmonella* spp., *Listeria monocytogenes* and *Staphylococcus aureus*. Furthermore quantitative changes of bacterial population in raw bulk milk during storage at two different chilling temperatures, 8°C and 3°C respectively, were also evaluated.

Materials and methods

Sampling

This study was carried out within seven months (from April to November 2010) in an approved farm (Regional Centre of Equestrian Increase) in Campania Region. Samples were collected from eight clinically healthy donkeys (five Martina Franca and three Ragusana breed). Animals were about four years old, fed with hay and oat. Milk production yielded on average 775 mL per mare per milking. Sampling comprised two phases. In the first 7-months phase, a total of 152 raw milk samples were collected from animals with a different lactation stage. The foals were physically separated from the dams three hours before sampling. The mammary glands were cleaned with a mixed of water and chlorine solution and then dried. The first two ejections were discarded. A milking machine installed on a wheeled trolley type with a modified sheep cluster (set at vacuum level 40 kPa, pulse ratio 60-40%, pulse rate 120 cycle/min) was used. Samples were transported cooled to the laboratory and pH measurements were made using FiveEasy ™ pHmeter (Mettler-Toledo Inc., Columbus, OH, USA). Bacteriological analyses were carried out within three hours after sampling. In the second phase, quantitative changes of microbial population during storage were investigated. Bulk milk containing mixed-milk obtained from each donkey was aseptically collected and placed into two different sterile laboratory glass bottles (DURAN Group GmbH, Wetheim, D). Bottles containing 800 ml of milk were stored respectively at 8°C and 3°C for eight days. Bacteriological analyses and pH measurements were carried out every day for considered storage time.

Microbiological and somatic cell count analyses

In the first phase enumeration of both TBC at 32°C, 20°C, 5°C and *Enterobacteriaceae* was

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investigated by culture method. Thus, individual aliquots (10 mL) of sampled milk from each animal were aseptically placed into different sterile bags and then homogenized in 90 mL of a quarter-strength Ringer Solution (BR0052, Oxoid Ltd., Hampshire, UK). One ml was inoculated onto Plate Count Agar (PCA, CM0325, Oxoid Ltd.) incubated for five days at 32°C, 20°C and for 10 days at 5°C for TBC enumeration and onto Violet Red Bile Glucose Agar (VRBGA, CM1082, Oxoid Ltd.) incubated for 24 h at 37°C for *Enterobacteriaceae* enumeration.

Analyses for *Salmonella* spp. were done in accordance with ISO 6579:09.2006 using a two-step enrichment procedure. Briefly, 10 mL of milk from each animal were pre-enriched in 90 mL of Buffered Peptone Water (BPW, CM1049, Oxoid Ltd.) for 24 h at 37°C. Then, one ml of the pre-enriched broth was incubated for 24 h at 37°C in 10 mL of Kauffmann Tetrathionate-Novobiocin Broth (CM1048, Oxoid Ltd.) supplemented with Novobiocin Selective Supplement (SR0181, Oxoid Ltd.) in accordance with the manufacturer's instructions and 0.1 mL was incubated for 24 h at 41.5°C in 10 mL of Rappaport-Vassiliadis Soya Pepton Broth (CM0866, Oxoid Ltd.). Subsequently one loopful from each enriched broth was spread onto Mannitol Lysine Crystal Violet Brilliant Green Agar (MLCB, CM0783, Oxoid Ltd.) and onto Xylose-Lysine-Desoxycholate Agar (XLD, CM0469, Oxoid Ltd.). Plates were incubated for 24 h at 37°C. Colonies were biochemically tested by using the API 20E System in accordance with the manufacturer's instructions (bioMérieux SA, Marcy-l'Etoile, France).

Analyses for *L. monocytogenes* were done in accordance with ISO 11290-1:2004. An amount of 10 ml of donkey milk was incubated in 90 mL of Fraser Broth (CM0895, Oxoid Ltd.) with Half Fraser Supplement (SR0166, Oxoid Ltd.) for 24 h at 30°C. Subsequently, 0.1 mL was incubated in 10 ml of Fraser Broth (CM0895, Oxoid Ltd.) with Fraser Supplement (SR0156, Oxoid Ltd.) for 24 h at 37°C. One loopful was then streaked onto Palcam Agar (CM0877, Oxoid Ltd.) added with Palcam Selective Supplement (SR0150, Oxoid Ltd.) and onto Chromogenic Listeria Agar (CM1084, Oxoid Ltd.) supplemented with Listeria Selective Supplement (SR0226, Oxoid Ltd.) and Listeria Differential Supplement (SR0244, Oxoid Ltd.). Both plates were incubated for 48 h at 37°C. *Listeria monocytogenes* like-colonies were biochemically tested by using the API Listeria System in accordance with the manufacturer's instructions (bioMérieux SA). *Staphylococcus aureus* counting procedure was performed by spreading one ml of milk onto Baird-Parker Agar Base

Table 1. Total bacterial count at 32°C expressed as Log CFU/mL for each animal.

Donkey	1	2	3	4	5	6	7	8
Total samples (152)	24	23	22	21	20	18	18	6
Mean	3.44	3.88	3.75	4.00	3.78	3.19	3.37	2.8
Standard deviation	1.19	0.98	1.12	1.16	1.18	0.98	0.92	0.84
Maximum	6.78	6.14	6.04	6.16	6.03	5.22	5.40	4.19
Minimum	1.95	2.57	1.90	2.13	2.27	2.20	2.21	1.73

Table 2. Total bacterial count at 20°C expressed as Log CFU/mL for each animal.

Donkey	1	2	3	4	5	6	7	8
Total samples (152)	24	23	22	21	20	18	18	6
Mean	3.51	3.85	3.69	3.92	3.71	3.22	3.32	2.84
Standard deviation	1.13	0.89	0.98	1.22	1.21	1.00	0.96	0.68
Maximum	6.70	5.46	6.18	6.16	6.02	5.19	5.27	3.62
Minimum	2.14	2.46	2.34	1.95	2.34	2.00	2.27	1.64

Table 3. Total bacterial count at 5°C expressed as Log CFU/mL for each animal.

Donkey	1	2	3	4	5	6	7	8
Total samples (152)	24	23	22	21	20	18	18	6
Mean	1.71	2.12	1.72	2.02	1.97	1.27	1.36	1.34
Standard deviation	2.12	1.87	2.10	2.34	2.43	1.91	1.70	1.49
Maximum	6.29	6.07	6.23	6.33	5.95	5.36	4.69	3.00
Minimum	0	0	0	0	0	0	0	0

Table 4. *Enterobacteriaceae* expressed as Log CFU/mL for each animal.

Donkey	1	2	3	4	5	6	7	8
Total samples (152)	24	23	22	21	20	18	18	6
Mean	1.15	1.93	1.67	1.82	1.52	1.32	1.69	0.68
Standard deviation	1.25	1.25	1.38	1.50	1.43	1.34	1.17	1.07
Maximum	3.26	3.93	3.48	3.88	4.42	3.35	3.30	2.34
Minimum	0	0	0	0	0	0	0	0

Table 5. Total bacterial count at 32°C expressed as Log CFU/mL in relation of lactation stage (days *post-partum*).

Days	0-60	60-120	120-180	180-240
Total samples (152)	43	54	27	28
Mean°	3.70	3.9	3.35	3.39
Standard deviation	1.08	1.21	0.92	0.85
Maximum	6.03	6.79	5.23	5.28
Minimum	2.20	2.10	1.90	2.28

°No significant difference (P>0.05).

Table 6. Total bacterial count at 20°C expressed as Log CFU/mL in relation of lactation stage (days *post-partum*).

Days	0-60	60-120	120-180	180-240
Total samples (152)	43	54	27	28
Mean°	3.64	3.96	3.36	3.29
Standard deviation	1.07	1.15	0.86	0.89
Maximum	6.02	6.70	5.08	5.08
Minimum	2.00	2.30	2.34	1.95

°No significant difference (P>0.05).

(CM0275, Oxoid Ltd.) supplemented with Egg Yolk Tellurite Emulsion (SR0054, Oxoid Ltd.). Suspicious colonies were tested for biochemical properties using API Staph System in accordance with the manufacturer's instructions (bioMérieux SA). Estimation of SCC was obtained by flow cytometry (Fossomatic 5000 Foss Electric A/S).

In the second phase, aliquots (10 mL) from each stored bottle (8°C and 3°C, respectively) were analyzed for TBC at 32°C, 20°C, 5°C and for *Enterobacteriaceae* enumeration by culture method as previously described. Measurements of pH were also investigated. The analysis of variance was followed by Tukey's multiple comparison tests using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA).

Results and discussion

During the evaluation period (seven months), all tested samples showed pH values on average of 7.08±0.05. Results for microbiological analyses in relation to each animal are shown in Tables 1 to 4. Results for microbiological analyses in relation to the lactation stage (days *post-partum*) are shown in Tables 5 to 8. Limited data are reported from literature on microbiological characteristics of donkey's milk. In our findings raw milks collected at farm level were slightly contaminated by bacteria with mean values never higher than 4 Log CFU/mL (Table 1). Possible reasons for low bacterial load and constant pH values could be on one side the presence of natural concentration of antimicrobial compounds like lactoferin and moreover lysozyme which acts directly on bacteria (Chiavari *et al.*, 2005) and contributes to maintain unvarying pH values (Coppola *et al.*, 2002; Salimei *et al.*, 2004; Zhang *et al.*, 2008) and on the other side due to the strictly hygienic milking procedures applied during sampling. Our results agree with Coppola *et al.* (2002) study where bacterial load of 4.6 Log CFU/mL, 1.5 Log CFU/mL and 3.2 Log CFU/mL were proved respectively for mesophilic bacteria, psychrotrophic bacteria and *Enterobacteriaceae*. Moreover in other studies mesophilic bacteria ranges from 1 to 2.39 Log CFU/mL (Pilla *et al.*, 2010) and results of 4.46 Log CFU/mL were also obtained by Salimei *et al.* (2004). All our samples were negative for *Salmonella* spp., *Listeria monocytogenes* and *Staphylococcus aureus* even though pathogen detection in donkey milk is proved in literature: *Staphylococcus aureus* was detected by Pilla *et al.* (2010) using molecular analyses and *Enterobacter sakazakii* was isolated by Conte and Passantino (2007) using culture method. SCC must be considered as a good system to evaluate the healthy status of the mammary gland as well as the quality of its milk. In this study SCC values tested always under 50.000 cells/mL as in Pilla *et al.* (2010) study. Our findings met values under 100.000 cells/mL reported by Beghelli *et al.* (2009). No significative differences were evidenced ($P<0.05$) among the lactation stages for TBC at 32°C and 20°C and *Enterobacteriaceae.* Significative differences were evidenced for TBC at 5°C (Tables 5 to 8).

During storage period early pH value was 7.12 (Figure 1). No significative differences ($P<0.05$) were evidenced for pH values between two tested storage temperatures. Results for microbiological analyses are shown in Figures 2 to 5. Immediately after sampling bulk milk was contaminated by bacteria with a mean TBC at 32°C and 20°C of 2.71 and 2.64 Log CFU/mL respectively. TBC at 5°C and *Enterobacteriaceae* population were not detected. After eight days at 8°C, TBC at 32°C, 20°C and *Enterobacteriaceae* increased by three Log (Figures 2, 3 and 5) and TBC at 5°C by five Log (Figure 4). After eight days at 3°C no Log increase was detected for TBC at 32°C, 20°C (Figures 2 and 3) whereas peaks (days 1, 4, 6) were detected for TBC at 5°C (Figure 4) and for *Enterobacteriaceae* (days 1, 7) (Figure 5). Our findings are not in agreement with Šari *et*

Table 7. Total bacterial count at 5°C expressed as Log CFU/mL in relation of lactation stage (days *post-partum*).

Days	0-60	60-120	120-180	180-240
Total samples (152)	43	54	27	28
Mean	2.16[a]	2.21[a]	0.63[b]	0.34[c]
Standard deviation	1.07	1.15	0.86	0.89
Maximum	5.95	6.34	4.62	3.82
Minimum	0	0	0	0

[a,b,c]Values within a row without a common superscript are significantly different at $P<0.05$.

Table 8. Enterobacteriaceae expressed as Log CFU/mL in relation of lactation stage (days *post-partum*).

Days	0-60	60-120	120-180	180-240
Total samples (152)	43	54	27	28
Mean°	1.59	1.62	1.82	1.88
Standard deviation	1.07	1.15	0.86	0.89
Maximum	4.43	3.94	3.27	4.05
Minimum	0	0	0	0

°No significant difference ($P>0.05$).

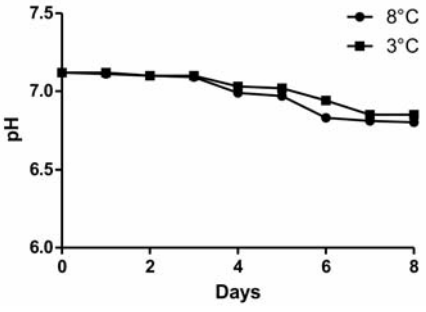

Figure 1. Values of pH *vs* days of storage at two different temperatures.

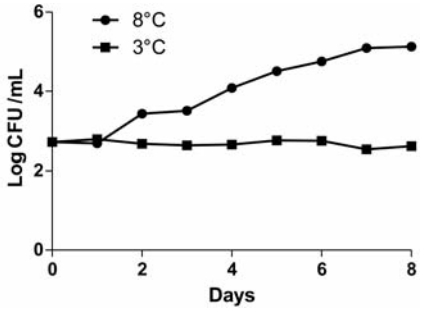

Figure 2. Total bacterial count at 32°C expressed as Log CFU/mL *vs* days of storage at two different temperatures.

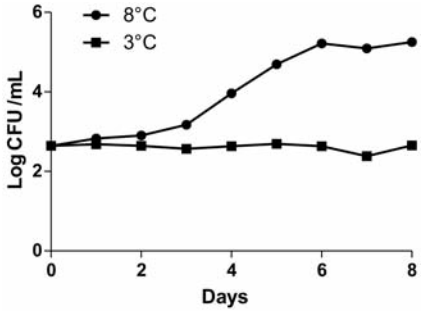

Figure 3. Total bacterial count at 20°C expressed as Log CFU/mL *vs* days of storage at two different temperatures.

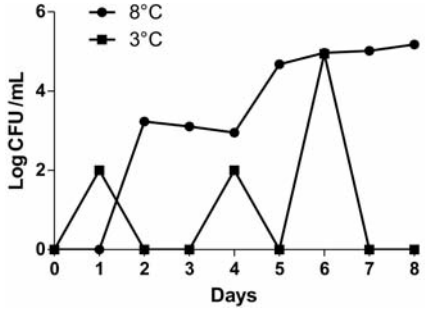

Figure 4. Total bacterial count at 5°C expressed as Log CFU/mL *vs* days of storage at two different temperatures.

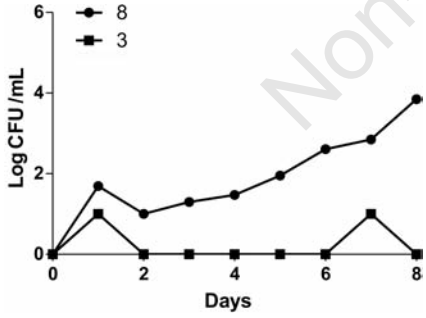

Figure 5. *Enterobacteriaceae* expressed as Log CFU/mL *vs* days of storage at two different temperatures.

al. (2012) study, where a gradual Log increase was described for TBC and *Enterobacteriaceae* in donkey milk stored at 4°C. Significative differences ($P<0.05$) between 8°C temperature and 3°C temperature were found. According to our results from the 3°C storage experiment keeping milk at refrigerated temperature immediately after milking process might be an effective measure to control bacteria growth and a possible long shelf life can be reached. However, the *Enterobacteriaceae* detection shows the importance to improve the hygienic practices on farm level.

Conclusions

Based on our results potential antimicrobial activity and good shelf life of raw donkey milk might be attributed to its natural compounds acting in synergism each other and to the hygienic milking procedures applied at farm level. Thus it might be considered as healthy base ingredient for feeding in case of low immune defense system, elderly, convalescent, children with cow milk allergy and when breast-feeding is not possible. As limitation of our study the experimental farming conditions should be considered and further studies are required to investigate milk quality in intensive farming conditions in order to facilitate its production and distribution on a large scale.

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