

Physico-Chemical Composition and Antimicrobial Protein Content of Early Lactation Donkey Milk

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Abstract

The influence of early lactation on chemical composition and the concentration of antimicrobial proteins of donkey's milk produced in Cyprus were investigated. Milk samples from 10 female donkeys in their first season of lactation were collected at 7, 15 and 30d postpartum. The average contents of donkey milk gross composition were 1.40% protein, 0.16% fat and 8.74% total solids. Results showed that lactation had a significant negative effect on protein concentration, while total solid concentration showed an increased followed by a decrease. Composition of antimicrobial proteins also showed a significant decreased during lactation period except from lactoferrin which showed an increase. On the other hand, throughout the lactation, pH and fat were constant.

Keywords: Donkey milk; Antimicrobial proteins; Immunoglobulin-A; Lactation

1 Introduction

Milks from non traditional animal species (i.e., donkey, camel, and buffalo) are gaining interest due to the fact that they are considered suitable to supplement the needs of special population groups such as infants and elderly. Donkey milk has been studied less compared to that of ruminant milks, but in recent years interest in donkey's milk production and commercialization have significantly increased. This increase in interest is due to its unique nutritional and physicochemical characteristics as well as its functional properties such as antimicrobial, immunomodulatory, anti-inflammatory, and anti-hypertensive properties (Aspri, Economou, & Papademas, 2017; Brumini et al., 2013; Jirillo & Magrone, 2014; Mao et al., 2009; Zhang, Zhao, Jiang, Dong, & Ren, 2008).

The nutritional composition of donkey milk is very similar to that of human milk and it has been reported to be an adequate replacement for children with cow milk protein allergy, mainly due to its tolerability, nutritional contents and good taste (Aspri et al., 2017; Monti et al., 2012). Donkey milk is characterized by low fat and protein content and high lactose content. It is also characterized by low casein content and a particularly high whey protein concentration, rich in lysozyme which is around 6000 times more than lysozyme content of bovine milk (Guo et al., 2007; Salimei et al., 2004).

Previous studies have shown that the strong antimicrobial activity of donkey milk arise from its high concentrations of antimicrobial agents such as lysozyme and lactoferrin (Tidona et al., 2011; Vincenzetti et al., 2008). Lysozyme is the main protein in donkey's milk that plays an impor-

tant role of fighting infections in breastfeeding infants, while lactoferrin is the second antimicrobial protein in donkey milk (Uniacke-Lowe, Huppertz, & Fox, 2010). In addition, these two antimicrobial proteins work synergistically with other proteins such as lactoperoxidase and immunoglobulins (Polidori & Vincenzetti, 2010). The high content of these antimicrobial proteins are considered to be the reason for the low microbial counts in donkey milk (Chiavari, Coloretti, Nanni, Sorrentino, & Grazia, 2005; Salimei et al., 2004; Sarić et al., 2012; Vincenzetti et al., 2008). As some donkey milk producers are marketing "early lactation milk" the aim of this research was to study the influence of early lactation stage on some physicochemical parameters of donkey milk (TS, protein, fat and pH) and also on the concentration of antimicrobial proteins of donkey milk.

2 Materials and Methods

2.1 Animals and sampling procedure

This study was carried out at "Golden donkey's farm" located in Skarinou, Cyprus. The farm had three breeds of donkeys (Asia, Cyprus and Israel) and their crosses. Raw milk samples were obtained by hand milked of 10 female donkeys, after parturition on 1st, 15th and 30th day of lactation, always at the same time of the day. The animals were maintained within the same conditions, and receiving the same feeding level and composition. Their diet was composed of hay, wheat and silage. The milk samples of an average volume of 100mL were collected in standard containers, and transported same day to the laboratory, and kept at 4°C for further analysis.

2.2 Physicochemical analysis

Determination of pH

The pH of the donkey milk samples was determined by potentiometry. The pH meter glass probe was first calibrated using standard buffers at pH 4 and 7 before being used to measure the

sample pH. Sample pH was measured by submerging the tip of the probe into the sample for ~1-2 min until a stable reading was registered on the pH meter scale. Measurements were done in triplicates and average values were reported.

Protein content

The protein content of milk samples was determined using the Bradford Protein assay (Bradford, 1976). Bovine serum albumin (BSA) was prepared for standard curve using the following concentrations, 50 µg/ml to 300 µg/ml. After incubated for 5 minutes at room temperature, absorbance measurements were determined spectrophotometrically (Infinite PRO 200, Tecan, Switzerland), at 595 nm. The Bradford reagent was used as a blank. Measurements were done in triplicates and average values were reported.

Fat content

Milk fat content was determined by a butyrometric method according to Gerber (IDF ISO 488, 2008). Milk samples (11 ml) were mixed with sulfuric acid (10 ml) and 1 ml of isoamyl alcohol in butyrometer and closed with rubber cork. The mixture was mixed and placed in a water bath at 65°C. The sample was centrifuged in a Gerber centrifuge for 5 min at 1000 rpm and the butyrometer was placed back into the water bath for another 5 min. The fat content is expressed as a percentage on the butyrometer. Measurements were done in triplicates and average values were reported.

Total Solids Content

Total solids were determined by the oven method in accordance with ISO 6731 (IDF standard 21, 2010a). 2g of fresh donkey milk samples were transferred to a pre-weighed round flat bottom aluminum dish. Then, the dishes were transferred to a hot air oven at 102 °C for 2 hours. Then the dish was transferred to a desiccator for 30 min to cool down and weighted. Total solids content was calculated by the following formula:

$$\text{Total solids (\%)} = \frac{m_2 - m_0}{m_1 - m_0} \times 100 \quad (1)$$

where m_0 is the mass (g) of the dish, m_1 is the mass (g) of the dish and the test portion and m_2 is the mass (g) of the dish and the dried test portion. Measurements were done in triplicates and average values were reported.

2.3 Antimicrobial proteins and IgA

Lysozyme content of donkey milk

Lysozyme was quantified according to a sensitive fluorescence-based method using EnzChek® kit (Life Technologies, Carlsbad, CA, USA). The assay is based on the assessment of the lytic activity of lysozyme in the cell walls of *Micrococcus lysodeikticus* which have been labeled with the fluorescent dye fluorescein. The test was performed according to the manufacturer's instructions and the absorbance was determined spectrophotometrically (Infinite PRO 200, Tecan, Switzerland), at 497nm and 518 nm. All samples were analyzed in triplicate.

Lactoferrin content of donkey milk

Quantitative determination of LF in the skim milk samples was performed using a commercial ELISA kit, the Bovine Lactoferrin ELISA Quantification Kit (Bethyl Laboratories, Montgomery, TX). The procedures were performed according to manufacturer's instructions. A standard curve was prepared using the supplied lactoferrin standard diluted to concentrations of 31.2 $\mu\text{g/ml}$, 62.5 $\mu\text{g/ml}$, 125 $\mu\text{g/ml}$, 250 $\mu\text{g/ml}$, 500 $\mu\text{g/ml}$ and 1000 $\mu\text{g/ml}$ using the sample diluent buffer (50 mM Tris, 0.14 M NaCl, 1% bovine serum albumin, 0.05% Tween 20, pH 8.0). The final absorbance of the samples and the standards was measured at 450 nm, using an ELISA plate-reader (Infinite F200; Tecan, Mannedorf, Switzerland), and the concentration of lactoferrin calculated by extrapolating from a standard curve. All samples were analysed in triplicate.

Immunoglobulin A content of donkey milk

Total IgA in milk was determined by using Horse IgA ELISA Quantitation Kit (Bethyl Labora-

tories Inc., Montgomery, TX, USA), according to the manufacturers' instructions. A standard curve was prepared using the supplied lactoferrin standard diluted to concentrations of 1000 nm/ml, 500 nm/ml, 250 nm/ml, 125 nm/ml, 62,5 nm/ml, 31.25 nm/ml, 15.6 nm/ml, 0 nm/ml using the sample diluent buffer. The final absorbance of the samples and the standards was measured at 450 nm, using an ELISA plate-reader (Infinite F200; Tecan, Mannedorf, Switzerland), and the concentration of IgA calculated by extrapolating from a standard curve. All samples were analyzed in triplicate.

2.4 Statistical Analysis

All experiments were carried out in triplicates. The data are expressed as the mean \pm standard deviation (SD). Statistical analysis was performed using SPSS version 17.0 (SPSS Inc., Chicago, IL, USA). The data were subjected to one-way analysis of variance (ANOVA) to determine the differences of samples. Significant differences were compared by Tuckey test on the level of $P < 0.05$.

3 Results and Discussions

3.1 Physicochemical analysis

The changes in the physico-chemical parameters and composition (protein, fat, pH, TS) of donkey milk during the early lactation period (30 days) are presented in Table 1.

The pH value of donkey milk, ranging from 7.03 to 7.06, did not vary significantly throughout the lactation period (30 days), which was consistent with the findings of Salimei et al. (2004). According to the literature the pH of donkey milk after milking is 7.19 ± 0.10 (Curadi, Giampietro, Lucenti, & M., 2001). This suggests that the pH value was not influenced by breed or stage of lactation (Chiavari et al., 2005; Guo et al., 2007; Malacarne, Martuzzi, Summer, & Mariani, 2002; Polidori, Beghelli, Mariani, & Vincenzetti, 2009). The average pH value (7.03 ± 0.02) of donkey milk was higher than that of cow milk which is in the range of 6.60 to 6.80. According to Salimei et al. (2004), this may be due to the lower casein (CN)

Table 1: Chemical composition of donkey milk at different stages during the lactation period

	Day 7			Day 15			Day 30		
	Mean	Min	Max	Mean	Min	Max	Mean	Min	Max
Protein Content (g/100ml)	1.55 ± 0.41 ^b	1.02	2.19	1.75 ± 0.38 ^c	1.30	2.64	0.90 ± 0.21 ^a	1.02	1.94
Fat (%)	0.15 ± 0.08 ^b	0.05	0.30	0.18 ± 0.22 ^c	0.05	0.80	0.14 ± 0.07 ^a	0.05	0.30
TS (%)	8.73 ± 0.57 ^a	8.13	10.02	9.10 ± 0.40 ^b	8.42	9.69	8.40 ± 0.40 ^a	8.29	9.22
pH	7.03 ± 0.13 ^a	6.83	7.27	7.01 ± 0.11 ^a	6.81	7.19	7.06 ± 0.07 ^a	6.98	7.21

Values are means ± standard deviation; values within the same row followed by different superscript letters significantly differ ($p < 0.05$).

TS = Total Solids

and phosphate contents in donkey milk than in cow milk (Salimei et al., 2004). The phosphate content of cow milk is 1000 mg/L, while the phosphate content of donkey milk is very low, 638, 42 mg/L (Fantuz et al., 2012).

The total protein content of donkey milk (approximately $1.4 \pm 0.29\%$) was similar to that of human milk and much lower than that of cows', goats' or sheep's milk (Guo et al., 2007). The observed average milk protein content (1.4%) was lower (1.72 %, 1.89 %) than reported by Salimei et al. (2004) and Giosue, Alabiso, Russo, Alicata, and Torrisi (2008). The protein content of donkey milk at 15 days after lactation is statistically significant higher compared to the first day and the 30th day of lactation (1.75 ± 0.38 , 1.55 ± 0.41 and 0.90 ± 0.21 respectively, $p < 0.05$). The results are consistent with the findings of Salimei et al. (2004), which have reported a falling trend in the protein level of donkey milk during lactation. This may be due to the differential expression of milk protein synthesis genes during the lactation period. The levels of early lactation protein mRNA increased after parturition, they reach the maximum after a few weeks and then they start decreasing (Demmer, Ross, Ginger, Piotte, & Grigor, 1998).

In contrast to the protein content, the fat content of donkey milk does not show a statistically significant difference between the different sampling days. However, other studies showed a significant variation of donkey milk fat content throughout the lactation period (Cosentino, Paolino, Freschi, & Calluso, 2012; Martemucci & D'Alessandro, 2012; Salimei et al., 2004). These differences indicate that fat content could be affected by breed,

breeding area and forage, milking technique, and interval between milkings, as also reported by Fox (2003). The average fat content of donkey milk was $0.16 \pm 0.02\%$, which is consistent with that reported in the literature for donkey's milk (Ivankovic et al., 2009; Salimei et al., 2004).

The total solids content of donkey milk was significantly higher on the 15th day of lactation compared to the 1st and 30th of lactation (9.14 ± 0.38 , 8.73 ± 0.56 and 8.35 ± 0.39 respectively, $p < 0.05$). The observed average dry matter content ($8.74 \pm 0.25\%$) was consistent with the data for dry matter content (8.84 %) reported by Salimei et al. (2004) and are not affected by breed, lactation stage or milking conditions. The mean dry matter observed in current donkey milk study was of $8.73 \pm 0.11\%$ which is consistent with the values reported in the literature for donkey milk (Chiavari et al., 2005; Guo et al., 2007; Salimei et al., 2004). The total solids content in the donkey milk according to Polidori et al. (2009) can be up to 8.80 g / 100g, which is lower than the solid residue in cow's milk (12.5 - 13.00 g / 100 g), sheep (17.5 - 19.5 g / 100 g) and human milk (11.70 - 12.90 g / 100 g).

3.2 Antimicrobial Proteins

The amount of lysozyme, lactoferrin and IgA concentrations (mg/ml) at different stages of donkey lactation are presented in Table 2.

Table 2: Lysozyme, lactoferrin and IgA concentrations in donkey milk at different stages of lactation

	Day 7	Day 15	Day 30
Lysozyme (U/ml)	225.08 ± 7.05 ^b	224.11 ± 6.00 ^a	201.94 ± 10.96 ^a
Lactoferrin (μg/ml)	111.03 ± 41.04 ^b	Nd	135.08 ± 27.68 ^c
IgA (ng/ml)	2674.40 ± 133.72 ^c	2576.47 ± 143.04 ^a	2267.32 ± 145.12 ^a

Values are means ± standard deviation; values within the same row followed by different superscript letters significantly differ ($p < 0.05$).

Nd=Not determined

Lysozyme

The EnzChek fluorescence-based assay measures lysozyme activity on *Micrococcus lysodeikticus* cells, which are labeled to such a degree that the fluorescence is quenched. Lysozyme action can relieve this quenching and the increase in fluorescence that is proportional to lysozyme activity. The mean lysozyme activity value was 217.04 ± 13.09 U/ml. Our results were in accordance with other studies carried out by Gubić et al. (2014); Guo et al. (2007); Pilla, Dapra, Zecconi, and Piccinini (2010); Sarić et al. (2012); Sarić et al. (2014); Vincenzetti et al. (2011). The lysozyme content at 30th day was significantly lower compared to the lysozyme content at 1st and 15th day of lactation (201.94 ± 10.96 , 225.08 ± 7.05 and 224.11 ± 5.99 respectively, $p < 0.05$).

The high lysozyme content of donkey milk plays an important role in the prevention or reduction of intestine infections in infants and is also responsible for the low bacteria count of donkey milk reported in literature (Salimei et al., 2004). Moreover, the high lysozyme content does not affect the growth or the acidification activity of probiotic strains, making donkey milk a good base for the production of probiotic fermented milk beverages (Chiavari et al., 2005; Coppola et al., 2002). Finally, the high lysozyme activity could explain the low incidences of mastitis in donkeys (Conte et al., 2006). Donkey milk lysozyme belongs to C-type calcium-binding lysozyme and is able to bind calcium ions; this binding leads to more stable complex with an enhanced antimicrobial activity (Wilhelm et al., 2009).

Lactoferrin

Results of lactoferrin content in donkey milk showed that lactoferrin content at 30th day of lactation was significantly higher compared to the 1st day of lactation (135.08 ± 27.68 and 111.03 ± 41.04 respectively, $p < 0.05$), which is in agreement with literature. A study carried out by Gubić et al. (2015) showed that the concentration of lactoferrin is increased over the lactation period. Moreover, according to Adlerova, Bartoskova, and Faldyna (2008), there is an important correlation between lactation and lactoferrin levels. In general, the content of lactoferrin in donkey milk is higher than in ruminant milk, but much lower than in mare and in human milk (Kanyshkova, Buneva, & Nevinsky, 2001).

Lactoferrin is an iron-binding protein that displays many biological functions such as antioxidant, antiviral, anti-inflammatory, immunomodulatory and anti-carcinogenic activity (Kuwata et al., 1998; Ward, Paz, & Conneely, 2005). Furthermore, it controls the proper composition of the intestinal microflora by suppressing the growth of pathogenic bacteria while promoting the growth of beneficial bacteria such as *Lactobacillus* and *Bifidobacterium* (Madhusudan, Ramachandra, Udaykumar, Nagraj, & Jagjivan, 2017). Different studies suggest that lactoferrin and lysozyme work synergistically to inhibit the growth of pathogenic bacteria and contribute to donkey milk's strong overall antibacterial activity against both Gram-positive and Gram-negative bacteria (Sarić et al., 2012; Tidona et al., 2011). In fact, Lf can bind different components in the outer membrane, thereby opening "pores" to enhance susceptibility of Gram-negative bacteria to the lysozyme by increas-

ing in membrane permeability (Ellison & Giehl, 1991; Leitch & Willcox, 1999).

Immunoglobulin-A (IgA)

The concentration of IgA of donkey milk was significantly lower at the 30th day of lactation compared to the 1st and 15th day of lactation. (2267.32 ± 159.91 , 2674.47 ± 178.14 and 2576.47 ± 167.34 respectively, $p < 0.05$). The average value of IgA was 2.50 g/L.

Immunoglobulins (Igs) are a defence family of globular proteins with antimicrobial and other protective bioactivities. They play an important role in transferring immunity to the newborn by establishing an optimal microfloral population in the gut of the newborn inactivate bacteria by binding to specific sites on the bacterial surface, while its own immune system is developing (Gapper, Copestake, Otter, & Indyk, 2007).

4 Conclusion

In conclusion, lactation stage affected the gross composition of donkey milk but had no significant effect on pH and fat content. It can be concluded that the chemical composition of milk in terms of fat, protein, pH, total solid residue, lysozyme, lactoferrin and IgA showed significant differences throughout the different stages of lactation except for fat and pH. However, further research is needed to establish how donkey's nutrition affects the quality of milk; as it is a type of milk of particular interest with unique composition.

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