TITLE: Characterization of several milk proteins in Domestic Balkan donkey breed during lactation, using Lab-On-A-Chip capillary electrophoresis

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SCIENTIFIC PAPER

CHARACTERIZATION OF SEVERAL MILK PROTEINS IN DOMESTIC BALKAN DONKEY BREED DURING LACTATION, USING LAB-ON-A-CHIP CAPILLARY ELECTROPHORESIS

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ABSTRACT

Domestic Balkan donkey (*Equus asinus asinus*) is a native donkey breed, primarily found in the northern and eastern regions of Serbia. The objective of the study was to analyze proteins of Domestic Balkan donkey milk during the lactation period (from the 45th to the 280th day) by applying Lab-on-a-Chip electrophoresis. The chip-based separations were performed on the Agilent 2100 Bioanalyzer in combination with the Protein 80 Plus Lab Chip kit. The protein content of Domestic Balkan donkey milk during the lactation period of 280 days ranged from 1.40 % to 1.92 % and the content of αs1-casein, αs2-casein, β-casein, α-, β-lactoglobulin, lysozyme, lactoferrin and serum albumin was relatively quantified. Lysozyme (1040-2970 mg/L), α-lactalbumin 12 kDa (1990-2730 mg/L) and α-lactalbumin 17.7 kDa (2240-3090 mg/L) were found to be the proteins with the highest relative concentrations.

Keywords: donkey milk, protein, lab-on-a-chip electrophoresis

Highlights

- Protein profile of Domestic Balkan donkey milk during lactation period to 280. day were determined
- Donkey's milk protein profile were determined by applying Lab-on-a-Chip electrophoresis
- Domestic Balkan donkey milks is low in casein content
- Lysozyme, lactoferrin and immunoglobulins were identified
- Balkan donkey milk represents a rich source of high nutritive components

INTRODUCTION

Over the past decades donkey milk has been less studied compared to ruminant milk, but in the last few years' interest in donkey milk has considerably increased among the scientific community of Europe. Donkey milk has been successfully used in clinical studies, with children who suffer from cow's milk protein allergy (CMPA), and has good palatability [1, 2]. Its composition is more similar to human milk than ruminant milk, however. It has a relatively low lipid content and adequate lipid integration is needed for toddlers' diet [3, 4]. Other types of milk, such as mare's [5], have been proposed as a substitute for human milk, but scarce information is available regarding the use of donkey milk for this purpose.

Domestic Balkan donkey is a native breed, primarily found in the northern and eastern regions of Serbia, with about 1000 subjects reared [6, 7]. The population of this breed is nowadays reduced to a very low number. Therefore, it is very important to preserve the breed and to increase the number of animals, in order to achieve milk production in significant amounts. FAO-the organization of food and agriculture, has initiated and recommended activities for the mentioned breed protection. Specific milk characteristics and parameters are the effect of the keeping conditions and pasture feeding, climate, as well as the race [8]. Donkey milk has been traditionally used in Serbia as a natural remedy for the treatment of asthma and bronchitis. Considering this fact, there has recently been a growing demand for donkey milk in the Serbian market [9].

Donkey milk has a lower protein content than other ruminant milk ranging from 13 to 28g/L, while proteomic profile is quite similar to human milk [10, 11]. Protein content varies considerably among species and is influenced by breed, stage of lactation, feeding, climate, parity, season, and udder health status [12]. The content of casein in donkey milk ranges from 6.4 to 10.3 g/kg of total protein content. Generally, casein present in different types of milk consists of four genetic fractions: αs1-, αs2-, β- and k-casein [13]. Guo *et al*. [14] reported that the content of whey proteins in donkey milk is within the range from 4.9 to 8.0 g/kg of total protein. According to the research by Cunsolo *et al*. [15], considerable differences can exist between the primary structure of donkey and bovine αs1-casein, which could be related to the previously demonstrated low allergenic properties of donkey milk and could contribute to its better human tolerance.

The basic whey proteins in donkey milk are β-lactoglobulin, α-lactalbumin, immunoglobulins, blood serum albumins, lactoferrin and lysozyme [16, 17]. The β-lactoglobulin is present in donkey milk as a monomer whereas this protein is a dimer in

ruminant milk [11] and has better digestibility in newborns due to higher digestibility and absorption of soluble monomer proteins [18,19]. Furthermore, there is a possibility of utilization of low-cost protein in formulations for infant feeding [20].

Donkey milk contains several antimicrobial components, including lactoferrin, lactoperoxidase and lysozyme [14, 21]. Šarić *et al*. [9] investigated the antibacterial properties and the protein profile of raw milk from the native Serbian donkey breed with an emphasis on the lysozymes and lactoferrin. The average lysozyme content of 1.0 mg/mL determined is considerably higher compared to the milk of other species [13, 16].

SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel) electrophoresis analysis is a commonly used method for protein separation, which is also widely applied to donkey milk analysis. Salimei *et al*. [13] and Guo *et al*. [14] have, using this method, concluded that whey proteins such as β-lactoglobulin, lysozyme and α-lactalbumin are the most abundant in donkey milk originating from Italian breeds, and lactoferrin, serum albumin and immunoglobulins were found to be minor protein components.

Criscione *et al.* [22] used mass spectrometry and high performance liquid chromatography to characterize IEF patterns. The authors reported the absence of αs1-casein in some individual cases and the presence of αs2-casein in all donkey milk samples. Polidori *et al.* [11] analyzed donkey milk proteins using two-dimensional electrophoresis (2-DE) followed by N-terminal analysis and found and determined β-caseins with molecular weights ranging from 33.10 to 33.74 kDa and from 31.15 to 32.15 kDa and lower.

Since literature data on use of donkey milk in human nutrition and its changes during lactation is very limited, the main objective of this study was to characterize several of the proteins of Domestic Balkan donkey milk during the lactation period from the 45th to the 280th day. Moreover, the aim of this study was to evaluate the nutritional value of Domestic Balkan donkey milk from the protein point of view, by applying Lab-on-a-Chip electrophoresis. Deep knowledge of the protein composition and variability could be useful for a more appropriate use in infant feeding.

MATERIALS AND METHODS

Sample Collection

The research on Domestic Balkan donkeys, a native breed, was conducted in the Special Nature Reserve Zasavica [23]. Zasavica is located in the north-west region of Serbia and is currently home for a herd of more than 150 female donkeys. Donkey milk samples were individually collected from 10 female Domestic Balkan donkeys, after parturition from spring

(April) to winter (January) season, on the 45th, 60th, 80th, 100th, 125th, 150th, 170th, 200th, 230th and 280th day of lactation. From June to early October the animals in the grassland of Zasavica reservation were reared outdoors on pasture, where they had the possibility to consume meadow plants. During other months of the year, donkeys were reared indoors, in a covered area, and they were fed with corn and corn stalks, while hay was available *ad libitum*. From April to June the way of feeding changed substantially. Corn and fresh water clover were given to the animals before milking. Donkeys had access to water *ad libitum*.

The animals were manually milked twice a day, at 7:00 am and 3:00 pm., 120 min after separating foals from their mothers. During milking, foals remained in visual and tactile contact with their mothers. Milk was completely removed from both udders. Each individual raw milk sample was collected into glass flasks and stored in an ice box at 4 °C. For each day of sampling, which is 10 days in total, 10 individual samples were collected twice (morning and evening) for a total of 200 samples.

Protein determination

Total protein concentration was measured through nitrogen determination. Total nitrogen was determined by the application of ISO standard method [24]. A nitrogen conversion factor of 6.38 was used for the calculation of the protein content of milk samples.

Electrophoretic analysis

The proteins of donkey milk were separated and quantitated using Lab-on-a-Chip electrophoresis technique based on their molecular mass in comparison with the marker protein ladder [25]. Sample preparation was carried out according to Tidona et al. [26] with minor modifications. Milk samples were diluted in 1:1.5 (v/v) ratio, sample: buffer (0.125 m Tris-HCL, 4% SDS, 2% glycerol, 2% β-mercaptoethanol, pH 6.8) and heated at 100 °C for 5 min.

The chip-based separations were performed using Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) in combination with the Protein 80 Plus Lab Chip kit and the dedicated Protein 80 software assay on 2100 expert software. Chips were prepared according to the protocol provided by the Protein 80 Lab Chip kit. The Protein 80 ladder (1.6, 3.5, 6.5, 15, 28, 46, 63 and 95 kDa) and the internal markers were used as reference for sizing and relative quantification. According to Živančev *et al.* [27] values of LOD and LOQ for the proteins in the analyzed solutions were 5.4 and 8.4 ng/μL respectively.

Statistical analysis

The one way ANOVA analysis and Duncan post hoc test were performed to assess data differences between various samples using Statistical software version 12 (STAT SOFT inc. 2013; USA). The data means were considered to be significantly different at $P<0.05$.

RESULTS AND DISCUSSION

Protein content of Domestic Balkan donkey milk during the lactation period of 280 days is shown in Table 1.

Table 1

The protein content reached the highest value of 1.92% on the 60th day of the lactation stage. Afterwards, the concentration decreased until the end of the lactation period when it reached the value of 1.40%. The protein content in Domestic Balkan donkey milk is in agreement with others studies on Italian donkey breeds- Martina Franca, Ragusana and Amiata [4, 13, 28, 29]. The Figure 1(a, b) shows molecular weight (in kDa) of the bands present at the beginning and at the end of the lactation period. The proteins were determined based on the literature data [11, 21, 22] by comparison of molecular weights and relative concentrations.

The bands of basic casein proteins that have been discovered have molecular weights ~30.3 kDa (αs1-casein) and ~26.7-27.0 kDa (αs2-casein). The findings showed two bands with 16-16.7 and 34.5- 35.0 kDa for β-casein in donkey milk. The results of electrophoresis showed a pattern similar to that reported in the literature [11, 13, 16]. The chip-based separation profiles of soluble proteins of donkey milk quantified α-lactalbumin with approximate molecular weight of 12 kD and 17.7 kDa. β-lactoglobulin and serum albumin molecular weights in whey protein fraction were around 19.6 kDa and 66.0 kDa respectively.

Major antimicrobial proteins determined in donkey milk were immunoglobulin (Mr 37-38 kDa), lactoferrin (Mr 74-78 kDa) and lysozyme (Mr 14.7-15.0 kDa). The results obtained in this research are similar to those obtained by other authors [11, 21, 22].

Figure 1a) (Gel-like image)

Figure 1b) (Electropherograms)

The concentrations of several protein fractions in Balkan donkey milk during the lactation period are shown in Figure 2. The trend for αs1-casein content showed a high variability and ranged from 1160 to 730 mg/L, whereas αs2-casein content ranged from 110 to 74 mg/L. The content of αs1-casein began to decline significantly from the 60th day until the 150th day, compared to αs2-casein whose content started decreasing after the 150th day and continued until the end of the lactation period. Trend variations for αs1-casein content throughout the lactation period were related to the change in total protein content. During the lactation period values of αs2-casein did not change significantly until the 150th day, and followed the same pattern from the 170th to the 230th day ($P<0.05$). However, β-casein content ($P<0.05$) decreased significantly from the beginning to the end of the lactation period (from 84 to 13 mg/L).

Figure 2

The content of α-lactalbumin was 2730 mg/L in the early and 2240 mg/L in the late lactation stage, which is very similar to the content found in human milk (2200 mg/L) [16]. The α-lactalbumin content showed a significant increase four months after parturition and reached values of 2450 to 3090 mg/L, after which the content decreased and remained quite stable until the end of the lactation period. The concentration of β-lactoglobulin varied from 139 to 263 mg/L. β-Lactoglobulin concentration decreased significantly ($P<0.05$) after the 60th day and also after the 150th day of lactation. Though β-lactoglobulin is generally resistant to gastro-intestinal enzymes, in a simulated in vitro digestion of donkey milk 70% of the β-lactoglobulin was digested, which is the amount twice as high compared to the bovine counterpart [30, 31]. Also, equine β-lactoglobulin was digested significantly faster compared to bovine and caprine β-lactoglobulin [31]. Serum albumin content (113-238 mg/L) showed a tendency towards stabilization during the mid-lactation period.

High concentrations of lysozyme were quantified in Balkan donkey milk, in which it ranged from 1040 mg/L to 2970 mg/L. The lysozyme content was stable during different stages of lactation and significantly decreased ($P<0.05$) after the 150th day. Donkey milk is known to be a rich source of lysozyme (1000 mg/L) [16] and has a significantly higher content of lysozyme than human milk (400 mg/L) and bovine milk (130 mg/L), while being quite similar to that in equine milk (400-1000 mg/L) [29, 32]. Lysozyme inhibits the growth of a large number of gram positive bacteria. Šarić *et al.* [9] investigated the antibacterial properties and

the protein profile of raw milk from the native donkey Serbian breed with an emphasis on the lysozyme and lactoferrin contents. Lysozyme and α-lactalbumin showed high resistance to human gastric and duodenal juices as already reported for raw equine, cow and human milk [30, 31]. In our study, α-lactalbumin was the dominant protein fraction of donkey milk, while immunoglobulin and lactoferrin were minor components. The lactoferrin content (87-13 mg/L) decreased significantly from the beginning to the end of the lactation period, and showed similar trend as β-casein. The immunoglobulin had an increasing trend, reaching the climax on the 100^{th} day (88.3 mg/L) and decreasing thereafter. The high content of protective antimicrobial compounds in donkey milk taken from the early and middle lactation period, suggested its beneficial impact on gut health and immune defense system.

CONCLUSIONS

Lab-on-a-Chip capillary electrophoresis could be applied to the relative quantification of several milk proteins. αs1-casein, αs2-casein, β-casein, α-lactalbumin, β-lactoglobulin, lysozyme, lactoferrin and serum albumin were relatively quantified, with the highest relative concentration of lysozyme and α-lactalbumin. The concentration of all determined proteins decreased during the lactation period. The minimum significant changes were observed for αs2-casein, lysozyme and β-lactoglobulin.

It could be concluded that Balkan donkey milk represents a source of antibacterical proteins such as lysozyme and highly digestible proteins such as whey protein, α-lactalbumin and lactoferrin.

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Figure captions

Fig.1a) Gel-like image of samples taken on the 45th and the 280th day

Fig. 1b) Electropherogram of samples taken from the 45th until the280th day of the lactation stage

Fig. 2 Trend of the concentration (mg/L) of several proteins fractions in Balkan donkey milk during the lactation period

Table 1. Protein content of Domestic Balkan donkey milk (total n=200) during the lactation period of 280 days

Time of milk sampling (day)	Protein (Nitrogen x 6.38) (%)	Significance level
45	1.83 ± 0.12	*
60	1.92 ± 0.20	*
80	1.73 ± 0.23	*
100	1.70 ± 0.28	*
125	1.64 ± 0.30	*
150	1.62 ± 0.19	*
170	1.49 ± 0.28	ns
200	1.50 ± 0.19	ns
230	1.45 ± 0.19	*
280	1.40 ± 0.20	*

Results are given as mean ± standard deviation;

**p<0.05; ns-not significant*

Proteins	Mw (kDa)	45d Conc (mg/L)	45d LoaC gel images	280d LoaC gel images	280d Conc (mg/L)
lactoferrin	74.0-78.0	41.45			5.15
serum albumin	66.0	113.15			140.70
immunoglobulins	37.0-38.0	47.60			9.30
β-caseins	34.5-35.0	84.20			13.00
αS1-casein	30.3	1160.00			730.00
αS2-casein	26.7-27.0	110.80			74.35
β-lactoglobuline	19.6	260.20			163.00
α-lactalbumin	17.7	3090.00			2240.00
β-caseins	16.0-16.7	55.53			38.87
lysozyme	14.7-15.0	2970.00			1040.00
α-lactalbumin	12.0	2730.00			1990.00

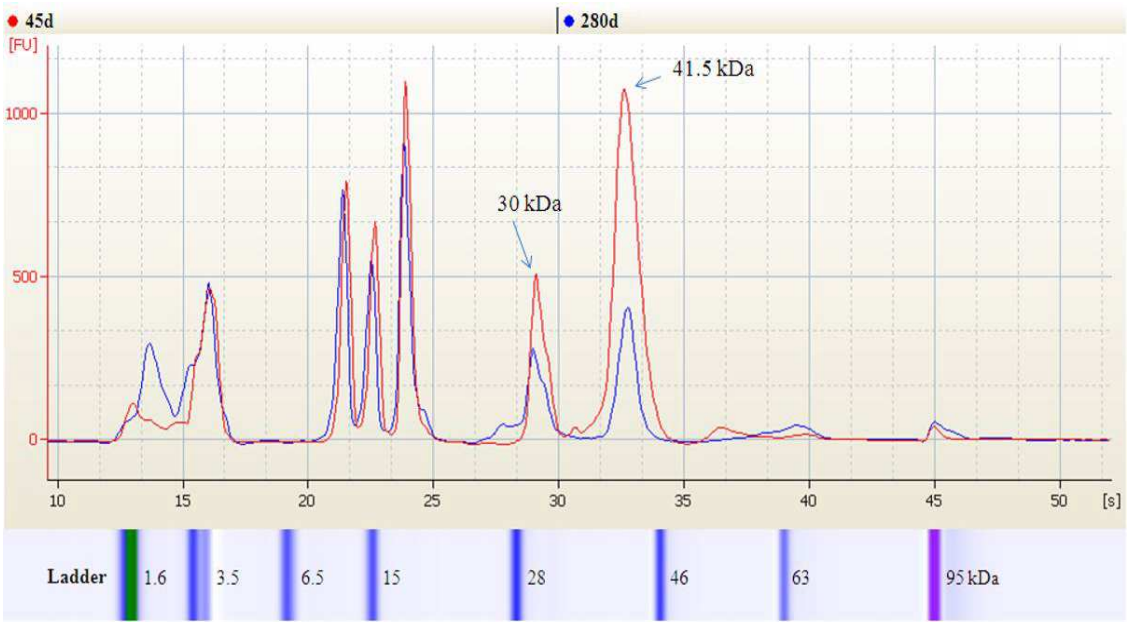

Fig 1

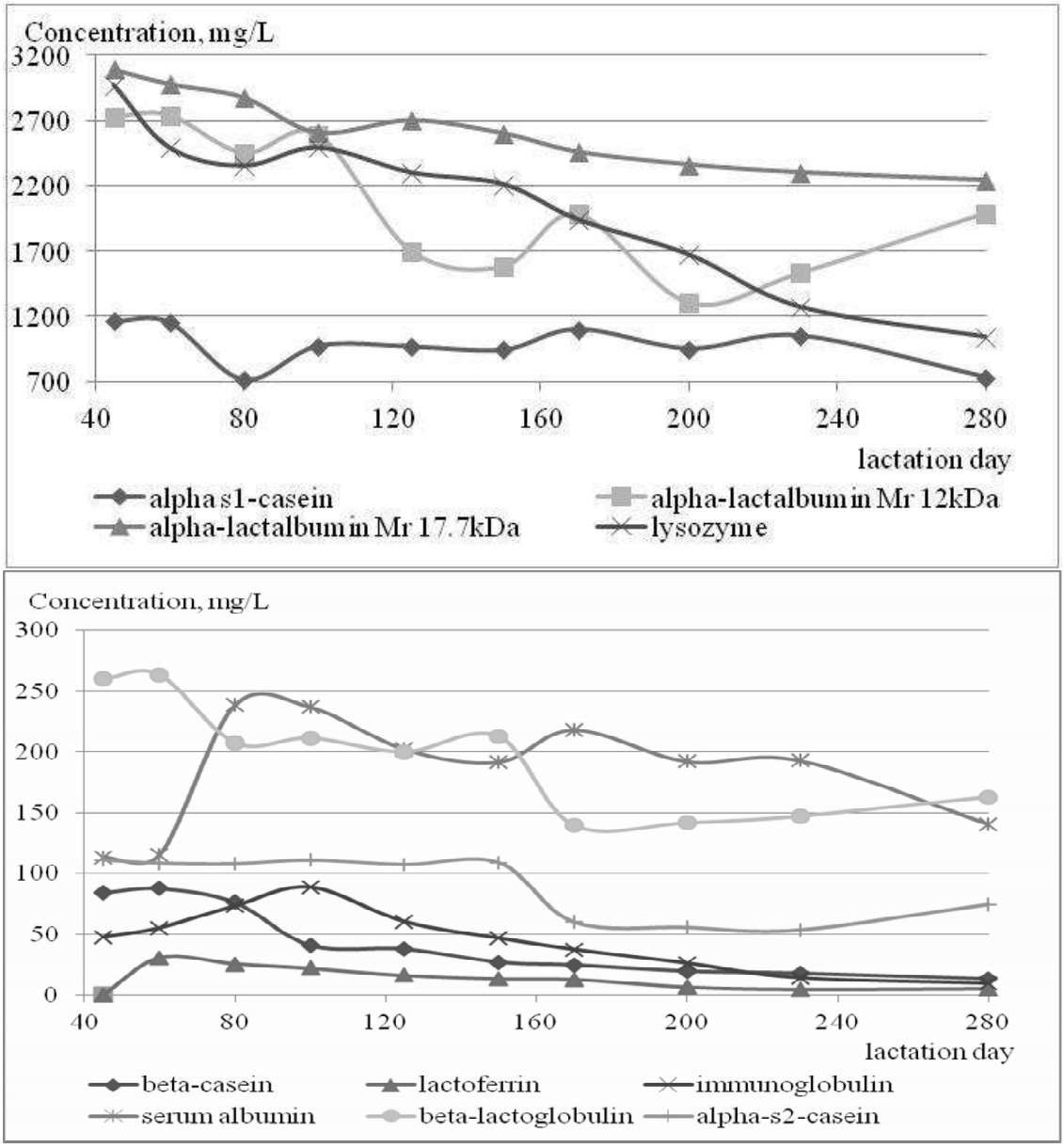
Concentration, mg/L
3200
2700
2200
1700
1200
700
40
80
120
160
200
240
280
lactation day
alpha s1-casein
alpha-lactalbumin Mr 12kDa
alpha-lactalbumin Mr 17.7kDa
lysozyme
Concentration, mg/L
300
250
200
150
100
50
0
40
80
120
160
200
240
280
lactation day
beta-casein
lactoferrin
immunoglobulin
serum albumin
beta-lactoglobulin
alpha-s2-casein

Fig. 2