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PAPER

A multi-approach study of the performance of dairy donkey during lactation: preliminary results

Federica Salari[a], Roberta Ciampolini[a], Chiara Mariti[a], Francesca Millanta[a], Iolanda Altomonte[a], Rosario Licitra[a], Barbara Auzino[a], Carlo D' Ascenzi[a], Carlo Bibbiani[a], Lorella Giuliotti[a], Roberto Amerigo Papini[a] and Mina Martini[a,b]

[a]Dipartimento di Scienze Veterinarie, University of Pisa, Pisa, Italy; [b]Centro Interdipartimentale di Ricerca Nutraceutica e Alimentazione per la Salute (NutraFood), University of Pisa, Pisa, Italy

ABSTRACT

Donkey milk is known for some nutritional and nutraceutical peculiarities compared to the milks traditionally used for human nutrition. Moreover although the number of studies on donkey milk production has increased the asinine species still remains little investigated. This is the first study providing a multiple assessment approach in order to extend the knowledge on the production of donkey milk, on the haematological and milk cytological parameters during the whole span of lactation. Furthermore, this study characterised the LYZ and OXT genes. Twenty two individual milk and blood samples from Amiatina donkey were taken at one, six and ten months after parturition. Milk total proteins and caseins and ash were significantly higher at the 1 month of lactation while the urea was lower (1.88% 0.95%, 0.41% and 26.08 mg/mL respectively). Whereas lactose and fat did not significantly changed and showed average values of 6.84 ± 0.145% and 0.67 ± 0.546% respectively. pH and titratable acidity were respectively higher (7.20) and lower (0.10 g/l of lactic acid) at 10th month. The mean values of somatic cell count/mL and of milk macrophages were low with maximum value at tenth month. The haematological parameters were stable during lactation except for mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration and platelet count with maximum at sixth month (respectively 20.22 pg, 36.1 g/dl, 281.16 x 10^9/l). This study also provides for the first time a characterisation of the LYZ gene in the Amiata donkey and describes a first polymorphism at the promoter level.

HIGHLIGHTS

- This study extends the knowledge on the production of donkey milk, on the haematological and milk cytological parameters during the whole span of lactation.
- The results may have economic, health and social impacts since donkey milk is primarily targeted at sensitive consumers.
- Lysozyme polymorphisms could have association with anti-bacterial activity in milk and the inflammatory response in the mammary gland.

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Donkey milk quality; lactation phase; milk cytological analysis; complete blood count; genetic analysis

Introduction

With the advent of industrialisation, the donkey (*Equus asinus*) population declined drastically throughout the world. However, in recent years there has been increased interest in this species especially in relation to its milk production. In fact, donkey milk is now known to have particular nutritional and nutraceutical characteristics compared to the milk traditionally used for human nutrition (Altomonte et al. 2019).

Compared to ruminant milk, donkey milk has on average lower fat, protein and casein content, a smaller average diameter of fat globules, lower quantity of saturated fatty acids, and a higher C18:3 n3 content of lactose (71.2 g/l) and vitamin D (Martini et al. 2018a, 2018b).

The increased interest in donkey milk has led to changes in the farming management of donkeys from traditional to more organised farming systems

CONTACT Dr Iolanda Altomonte altomonte@vet.unipi.it Department of Veterinary Science, University of Pisa, Pisa, Italy
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(Bibbiani et al. 2017). Since donkey milk production is primarily targeted at sensitive consumers such as children (Barni et al. 2018), the breeding system not only has to guarantee a quantitatively sufficient milk production, but also a safe product.

Management practices to improve animal welfare and aimed at breeding rustic breeds with high genetic resistance to diseases have guaranteed the limited use of drug treatments and safe products.

Although the number of studies on donkey milk production has increased in the last decade, especially in Italy and China, this species remains scarcely investigated. In fact, no selective actions have been taken to improve the qualitative and quantitative characteristics of its milk production.

Recent knowledge in the field of molecular genetics has made it possible to identify single genes, whose polymorphisms are associated with or responsible for a significant proportion of the variability in the quanti-qualitative characteristics of milk in the different species (McEwan et al. 1991; Ceriotti et al. 2005). Donkey milk is rich in Lysozyme c-type (LYZ), a natural antimicrobial that inhibits the development of some pathogen bacteria (Martini et al. 2019). In donkey breeding, one of the major future research challenges is to increase the quantity of milk produced. Oxytocin polymorphisms have been associated with milk yield and flow (Pauciullo et al. 2012), thus decreasing the milking time in ruminants. Oxytocin is a peptide hormone involved in the contraction of smooth muscles leading to milk ejection (Gimpl and Fahrenholz 2001). The oxytocin-neurophysin I encoding gene (OXT) has been sequenced and fully characterised in many species (Cosenza et al. 2007, 2017; Pauciullo et al. 2018). However, although the genome sequencing has been completed in the donkey (GeneBank NW_014638180.1), the annotation is still incomplete and no studies have been carried out to investigate genetic polymorphisms of the OXT gene.

To the best of our knowledge, this is the first study providing a multiple assessment approach in order to extend the knowledge on the production of donkey milk, the variation of haematological and milk cytological parameters during the whole span of lactation. Furthermore, this study is aimed to characterise the LYZ and OXT genes in order to highlight genetic polymorphisms that might affects quanti-qualitative characteristics of milk.

Material and methods

Animals and sampling

Pluriparous dairy jennies of autochthonous Amiata breed were selected for the study. The donkeys were all bred in an individual farm located in central Italy (42°53′52.59 N 10°47′05.52 E, WGS84) and reared outdoors in a semi-intensive system. In the farm, more than 200 donkeys are housed, of which 50 are lactating jennies. They were fed with mixed hay *ad libitum* and about 2.5 kg/day/head of concentrate for dairy donkeys. Composition of hay and concentrate are reported in Supplementary Tables 1 and 2. Their body condition score was 3.5 (measured on a 1 to 5 scale, following the Guidelines of the Italian Ministry of Labour, Health and Social Policies). The farm produced pasteurised milk for human consumption in accordance with the requirements of Regulation (EC) No 853/2004. During the first month of lactation, all the milk was left for the foal. Starting from 30 days after delivery (1 month), the jennies were routinely machine-milked once per day. Four hours before being machine-milked, the foals were separated from the jennies. All the jennies delivered in the spring.

Individual samples from 22 animals were taken at three different moments of lactation (1, 6 and 10 months after parturition) for a total of 66 samples.

The biological matrices used for the current study were:

- Blood: peripheral blood samples were collected in EDTA tubes to be evaluated for genetic parameters and complete blood cell count (CBC).
- Milk: individual milk yield of the morning milking was measured for each individual by a mechanic milk metre from which homogeneous sample of 100 mL of milk were collected from each jenny.

All the samples were refrigerated at +4 °C immediately after the sampling and processed within 24 h of collection.

Approval for this study was obtained from the Ethical Committee on Animal Experimentation of the University of Pisa, and the protocol was sent to the Ministry of Health.

Milk, genetic and haematological analyses

To determine the chemical quality of the milk, the following parameters were evaluated in all individual raw fresh milk samples: dry matter, fat, lactose, and urea content were measured by infra-red analysis (MilkoScan 7 RM; Italian Foss Electric, Padova, Italy); somatic cell count (SCC) was evaluated by the fluor-optoelectronic method (Fossomatic Italian Foss Electric, Padova, Italy).

Protein was calculated as total nitrogen (N) (determined by Kjeldahl) multiplied by 6.38; caseins was precipitated from milk at pH 4.6 using acetate solution; the acidified solution, which contains the non-casein N components was separated from casein precipitate by filtration. Nitrogen contents of casein precipitate were determined by Kjeldahl. Protein, casein ashes content were measured using methods of the Association of Official Analytical Chemists (AOAC 1990).

DNA for genetic analysis was extracted from leukocytes, following Goossens and Kan (1981). The genomic DNA of the whole LYZ encoding gene (5136 bp) and OXT gene, was amplified and sequenced for four randomly chosen subjects. In addition, the sequencing was also extended to the promoter (342 nucleotides) and the 3′ flanking region (1017 nucleotides) of LYZ and to the proximal promoter region of the OXT gene.

All primers for amplification (PCR) and sequencing of donkey LYZ gene were designed using DNAsis-Pro (Hitachi), using the donkey genome sequence (GeneBank NW_014638180.1, complement 1781536.1786728) as templates (Supplementary Table S3). For the primers for OXT, the whole genome horse (EMBL acc. no. NC_009165.3) and donkey (EMBL NW_014638254.1) sequences were used as templates (Supplementary Table S4).

For the analysis of OXT gene, PCR reaction mixture and thermal conditions were established according to Cosenza et al. (2017). PCR products were sequenced on both strands at CEINGE – Biotecnologie Avanzate (Naples, Italy).

To evaluate the role of the single nucleotide polymorphisms (SNP) g.203C>T on the promoter activity of the LYZ gene, the following protocol was used according to Cosenza et al 2018:

1. Construction of reporter plasmids: firstly, a DNA region of 1102 bp was amplified and cloned upstream of the reporter in the pGL3 basic vector (Promega), from four homozygous individuals (g.203CC and g.203T).
2. Transient transfections: vectors carrying the two different constructs, g.203C and g.203T, were then used to transiently transfect HEK293 cells. Transfection with a pGL control vector was also performed as a control.
3. Luciferase assays: after 24 h, the reporter activity of the variants was measured by luminometry using the Luciferase assay system (Promega).

Analysis of the complete blood count (CBC) was carried out by Auto Haematology Analyser BC-2800Vet® (Mindray, Shenzhen, P.R. China). This included the erythrocyte count (RBC), leukocyte count (WBC), haemoglobin concentration (Hgb), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), and platelet count (PLT).

Cytological examination for the presence of leukocytes from each milk sample was obtained from 1 mL of milk by centrifugation at 1078 x g in order to obtain a pellet. Twenty µl of cytocentrifuge smears were air-dried and stained with Dif-Stain-Quik (Titolchimica, RO, Italy). The evaluation of the leukocytes subpopulations and, if present, of aetiologic agents, cellular debris and somatic cells was performed by light microscopy, on at least 10 representative high power fields, as previously described by Conte et al. (2003).

Statistical analysis and bioinformatics

The results of the milk composition and blood count were analysed using ANOVA for repeated measurements, considering the three sampling times (1, 6 and 10 months of lactation) as fixed effects. Least significance means were compared by the *t*-test. Significant differences were considered at $p < .05$. Statistical analysis was carried out using JMP software (JMP 2002). For milk cytology, means and standard deviations were calculated for the three sampling periods. The results of LYZ and OXT gene sequencing were analysed by BLAST (www.ncbi.nlm.nih.gov/BLAST). Homology searches, comparisons among nucleotides and deduced amino acid sequences and multiple alignments were carried out using DNAsis-Pro (Hitachi Software Engineering Co., Japan). The analysis of putative regulatory sites was performed in silico using the Transfact 7.0 database.

Results

Table 1 shows the average milk yield and quality during the three phases of lactation. The average daily production of the morning milking was 536.42 ± 281.921 mL. During lactation, the milk production decreased progressively and significantly ($p < .01$) from 1 month to 10 months of lactation.

Regarding the physicochemical composition of the milk, dry matter and ash percentages (on average 10.40 ± 1.354 and 0.36 ± 0.063, respectively), significantly decreased ($p < .01$) at 10 months of lactation.

The mean percentage of total proteins and caseins during lactation were 1.75 ± 0.243 and 0.79 ± 0.178,

Table 1. Average milk yield and quality during the three phases of lactation.

	Lactation				
	1st month	6th month	10th month	SEM	*p*-value
Morning milking, mL	771.11[a]	585.66[b]	303.02[c]	200.59	.0001
Fat, %	0.67	0.80	0.49	0.46	.7790
Protein, %	1.88[a]	1.75[b]	1.66[b]	0.22	.0040
Casein, %	0.95[a]	0.71[b]	0.79[b]	0.16	.0020
Lactose, %	6.81	6.90	6.83	0.15	.1540
Urea, mg/mL	26.06[b]	39.21[a]	39.33[a]	8.04	.0001
Dry Matter, %	10.59[a]	10.52[a]	9.73[b]	1.00	.0090
Ash, %	0.41[a]	0.35[b]	0.31[c]	0.05	.0001
pH	6.97[b]	6.92[b]	7.20[a]	0.10	.0030
Titratable acidity, g/L of lactic acid	0.13[a]	0.12[a]	0.10[b]	0.01	.0003
Log_{10} SCC	0.75[a]	0.55[b]	0.89[a]	0.26	.0070

SCC: somatic cell count.
[a,b,c]Values within a row with different superscripts differ significantly at $p < .01$.

respectively, and were both significantly higher at 1 month ($p < .01$). Throughout the experimental period, the average content of urea was 35.16 ± 9.818 mg/mL with the highest values starting from 6 months of lactation ($p < .01$).

Titratable acidity (on average 0.12 ± 0.014) and pH values (on average 7.01 ± 0.155) changed significantly ($p < .01$) at 10 months of lactation. The percentage of lactose (on average 6.84 ± 0.145) and fat (on average 0.67 ± 0.546) did not show significant variations during lactation, however an increasing tendency -although not significant -was found for lipid at 6 months of lactation.

The mean value of log_{10} SCC/mL during lactation was on average 0.72 ± 0.283, with significantly higher values ($p < .01$) at the beginning and in the last lactation phase.

The sequences obtained for LYZ showed genetic variability at this locus, while a comparison of the sequences for OXT did not show genetic variability.

The set-up of a Luciferase assay confirmed that the transition g.203C>T influences the promoter activity of LYZ gene in perfect agreement with Cosenza et al. (2018).

Of the haematological parameters analysed (Table 2), no differences were observed comparing the three periods of sampling for the values of HCT (mean 47.97%±14.742), RBC (mean $7.88*10^{12}$/l ± 2.452), MCV (mean 61.20 fl ± 3.727), hgb (mean 15.30 g/dl ± 5.217), WBC (mean $12.78*10^{9}$/l ± 4.273).

The mean values for PLT, MCH, MCHC were 233.67$*10^{12}$/l ± 100.974, 19.58 pg ± 1.393 and 33.07 g/dl ± 1.619 respectively; statistically significant ($p < .01$) lower values were observed at 10 months of lactation compared to six for PLT (186.70$*10^{12}$/l), while higher values were found at sixth month for MCH (20.22 pg), and MCHC (36.10 g/dl).

The main leukocyte subpopulation in all the milk samples consisted of macrophages (66%), with a mean count of 2.5 ± 5 cells/10 HPF (High Power Field). A total of 16% of specimens showed the presence of rare neutrophils (2.2 ± 4.8 cells/10 HPF) and in 17.5% of samples, rare lymphocytes were observed (1.2 ± 1.6). When comparing leukocyte composition at different stages of lactation, the most pronounced difference was detected in the macrophage population. At 1 month of lactation, the mean macrophage count was 1.7/HPF, at 6 months it was 1.1/HPF, and the mean increased to 4.5/HPF at 10 months. No aetiological agents were noticed using routine staining.

Discussion

In terms of the quantitative milk production, previous studies on a few mainly European and Chinese donkey breeds, have highlighted a high variability (D'alessandro and Martemucci 2012; Martini et al. 2014a; Muhatai et al. 2017). Several of these authors have also found that a higher milk yield occurs in the first 3 months of lactation. Similarly, in the current study it was found that the highest milk production was in the first month of lactation, as found in the Ragusana donkey breed (Giosuè et al. 2008); while at 10 months the production was reduced by half.

Regarding the average gross composition, similar values were found in a previous study on the Amiatina breed (Martini et al. 2014b).

Little information is available regarding the trend in nitrogen fractions during lactation. However, the significant decrease in the percentage of total proteins and caseins at 6 months of lactation and the increase in urea content during lactation have also been detected by Fantuz et al. (2009) in the Martina Franca

Table 2. Donkey haematological parameters during the three phases of lactation.

	Lactation				
	1st month	6th month	10th month	SEM	*p*-value
MCH, pg	19.360[b]	20.220[a]	19.050[b]	1.202	.009
MCHC, g/dL	32.040[a,b]	36.100[a]	31.240[b]	1.499	.008
PLT, number x 10^9/L	227.140[a,b]	281.160[a]	186.700[b]	92.568	.009
HCT, %	46.220	49.170	48.190	14.871	.838
MCV, fl	60.660	61.600	61.250	3.287	.695
hgb, g/dL	14.830	17.750	15.180	5.297	.866
RBC, number x 10^12/L	7.670	7.980	7.940	2.422	.921
WBC, number x 10^9/L	12.770	13.920	11.460	4.218	.208

MCH: mean corpuscular haemoglobin; MCHC: mean corpuscular haemoglobin concentration; PLT: platelet count; HCT: haematocrit; MCV: mean corpuscular volume; hgb: haemoglobin concentration; RBC: erythrocyte count; WBC: leukocyte count.
[a,b]Values within a row with different superscripts differ significantly at $p < .01$.

donkey; this trend could be due to a nutritional imbalance of the diet from 6 months of lactation.

With regard to fat, the stability of its content during lactation confirmed previous studies on dairy donkeys (Salimei et al. 2004; Martini et al. 2014a, 2015). In any case, the increasing trend recorded at 6 months has also been reported by Guo et al. (2007) in the Jiangyue breed.

In donkey milk, the average pH value tends to basic values compared to cow milk, while it is similar to human milk probably due to the low content of casein and phosphate (Altomonte et al. 2019). The stability of the pH and titratable acidity found in the first 6 months of lactation is in agreement with other findings (Salimei et al. 2004; Guo et al. 2007). Moreover, the pH increase in the late lactation stage was associated with a lower titratable acidity; these results may be explained by the decrease of casein and the increase of urea content.

Despite the low content of SCC, in line with other studies conducted on Amiatina donkey milk (Ragona et al. 2016), the increased presence at the beginning and at the end of lactation is consistent with Pilla et al. (2010). In the final phase of lactation, this trend is associated with the simultaneous increase in the pH and the decrease in milk yield indicating a physiological involution of the mammary gland.

In addition, with respect to the cytological analysis of milk, the percentage of leukocytes recorded was very low at 1 and 6 months of lactation. A slightly higher presence of macrophages was observed at 10 months, while in all three sampling times, the other subpopulations of inflammatory cells were rarely detected. Our results are in agreement with previous data (Conte et al. 2003) and confirm that mastitis in donkeys is rare. The increased leukocyte count recorded at 10 months suggests that a decline of the immunity function of the mammary gland may occur in the late phases of lactation, with a possible increase in subclinical mastitis.

Similarly to our results, a functional promoter polymorphism in the LYZ gene has been identified for the Ragusana and Sicilian Grey asinine breeds (Cosenza et al. 2018). At the promoter level of the LYZ gene, the SNP g.203C>T can alter a putative binding site of the transcription factor NF-1 (Nuclear Factor 1). NF-1 has been shown to be involved in the regulation of milk protein genes (Kannius-Janson et al. 2002). The reduced activity of homozygote TT suggests that this promoter-binding site transcription factor NF-1 (Nuclear Factor 1) plays a key role in the expression of the LYZ gene in donkey milk.

The results of this study suggest that the SNP g.203C>T might be useful to promote association studies with all the traits linked to the qualitative-quantitative variability of donkey milk. Given the antibacterial actions of the Lys enzyme, the polymorphism detected represents a good opportunity to carry out studies to identify significant association with the occurrence of mastitis, anti-bacterial activity, and modulation of the inflammatory response.

The OXT organisation observed in the donkey samples of this study showed similarities with those described by Cosenza et al. (2007, 2017) for the homologous gene in buffalo, goat and sheep.

Regarding the haematological parameters, reference values are not yet set in lactating jennies and in general there only a few studies on blood values in donkeys. The knowledge of the blood values of the animal during lactation is important in the evaluation of animal health and in order to diagnose potential pathologies.

Our results on WBC, MCH, MCHC are in the range observed by Dezzutto et al. (2018) in crossbreed donkeys at different stages of lactation. MCV values are also in agreement with the results on female adult donkeys (Dezzutto et al. 2018) and higher compared to reference values for horses. PLT is in agreement with the study of Burden et al. (2016) on donkey in maintenance, whereas HCT, RBC, and hgb are higher compared the value available in the literature for

donkey (Dezzutto et al. 2018; Burden et al. 2016) but in the range of the reference values for horses.

We found higher values for MCH, MCHC at 6 months and lower PLT at the tenth month. MCH, MCHC are related to the degree of haemoglobinization of red blood cells and negative variations are linked to the diagnosis of anaemia. However, in our study these variations were not accompanied by changes in the MCV and the hgb content, thus we hypothesised they would be non-pathological changes likely related to the paraphysiological changes in lactation.

Conclusions

The current study is the first study providing a multiple assessment approach on the production of donkey milk, on the haematological and milk cytological parameters during the whole span of lactation providing further insights into this little explored field. In addition, the first polymorphism we found in Amiata donkey LYZ could have association with anti-bacterial activity in milk and the inflammatory response in the mammary gland and could be useful to promote association studies related to all the traits linked to the qualitative-quantitative variability of donkey milk.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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ORCID

Roberta Ciampolini http://orcid.org/0000-0001-5676-1798
Roberto Amerigo Papini http://orcid.org/0000-0002-5928-0663

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