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Colostrum and Milk Characteristics in Murgese Breed Mares

Carlo Cosentino[1], Giovanni Notarnicola[2], Rosanna Paolino[1], Carmine D'Adamo[1], Cristiana Labella[1], Mauro Musto[1], Pierangelo Freschi[1]*

[1]School of Agricultural, Forest, Food, and Environmental Sciences, University of Basilicata, Potenza, Italy

[2]State Forestry Corps, Territorial Office for Biodiversity, Taranto, Italy

**Corresponding author: carlo.cosentino@unibas.it*

ABSTRACT

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The physicochemical composition and antioxidant activity of Murgese mare's colostrum and milk during lactation were analyzed. Colostrum and milk samples from 14 lactating mares were collected on the 1st and 2nd day after foaling, and then at 15, 90, and 150 days postpartum. The results showed that the values of most of the chemical parameters in colostrum were higher. During lactation, the content of proteins, fat, dry matter as well as pH values increased, while lactose and lysozyme content decreased. The total antioxidant capacity (TAC) was evaluated by 2,2'-azinobis (3-ethylbenzthiazoline-6-acid) (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) assays. For both tests, colostrum showed a greater TAC. In milk TAC decreased over time to remain constant in the last months of lactation.

Keywords: horse; lactation; total antioxidant capacity; biodiversity

Autochthonous breeds of farm animals have strongly contributed over the centuries to the shaping of most of the traditional landscapes of Mediterranean area, and, in recent years, their preservation and/or reintroduction have been still more recommended as effective strategies for biodiversity conservation, especially in anthropogenic habitats (Cosentino et al. 2015; Freschi et al. 2015). The Mediterranean steppic grasslands of Murgia (Apulia, South of Italy), featuring a typical dry karst landscape, are a representative example in this regard, as their extension and composition are strictly linked to grazing activities (Terzi et al. 2001). Moreover, here the autochthonous breeds of livestock have become a featuring part of the landscape. This is particularly true for the black, and sometimes irongrey, horses kept under extensive conditions in the Murgia, and from which their breed takes the name. Indeed, the so-called Murgese is the oldest Italian horse breed still extant (Porter et al. 2016), with its origins dating back to the 15th and 19th centuries (Dario et al. 2006).

During the past decades, mechanization in agriculture has led to a considerable contraction of population of several native breeds of livestock, with the Murgese horse having a large risk for extinction (Pieragostini et al. 2005; Caroprese et al. 2007). Although the actual number of registered horses (5776 according to Equidae Registry 2016) exceeds the threshold value (1500) established by

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the Rare Breeds Survival Trust to define a horse breed as "at risk", the probability of extinction may remain high without effective conservation efforts and valorization strategies.

Consuming mare's milk is a well-established habit in Asia, and it is becoming more and more widespread in Europe, which has led to focus on mare's milk composition (Malacarne et al. 2002; Potocnik et al. 2011). Summarizing what has been found so far it can be stated that mare's milk composition is quite different from that of other farm animal species (Dankow et al. 2006); it is rather more similar to human milk (Pietrzak-Fiecko et al. 2009; Markiewicz-Keszycka et al. 2013), which makes it an effective substitute for mother's milk for premature newborns, as well as the best alternative of cow milk in allergic children (Cosentino et al. 2015). Besides, the composition of milk and the nutritive value of its components may vary according to several factors, such as lactational stage, age, parity, nutrition.

Although valorising the milk from Murgese mares has been described as a promising strategy to promote this horse breed, too (Pinto et al. 2001), few studies have been conducted on the milk composition. A preliminary description of chemical composition and lysozyme content was presented by Pinto et al. (2001) and Faccia et al. (2001), respectively, who analyzed milk samples from a little group of Murgese mares reared on different farms in Apulia. More recent data were provided by Caroprese et al. (2007), who evaluated the milking ability of the Murgese mares utilizing machine milking. In particular, the authors compared milk yield and composition, as well as behavioural activities, in two groups (one subjected to hand milking and the other to machine milking) of 4 mares from an extensively managed herd. Although the above mentioned studies are valuable contributions, they suffer from some limitations, such as the small number of sampled mares (which were, moreover, kept under different faming conditions) in Pinto et al. (2001) and Faccia et al. (2001), whereas in Caroprese et al. (2007) the mares were monitored for only 25 days during lactation. Moreover, there has been no detailed investigation on colostrum, the mare's first milk rich in antibodies providing an important source of immunity for the newborn foal. Therefore, the purpose of this study is to: (1) describe the chemical composition of Murgese mare's colostrum and milk; (2) determine changes in the composition of milk throughout lactation.

MATERIAL AND METHODS

Animals and sampling procedures. The experiment involved 14 pluriparous Murgese mares (age: 8–9 years; body weight: 500–650 kg), reared in the Natural Biogenetic Reserve "Eastern Murge" (755 ha overall, ~700 m a.s.l., Gorgofreddo, Martina Franca, Apulia) and never subjected to any milking procedures.

After foaling (from March 2015 to May 2015), the animals were kept under identical environmental conditions: they were on a natural pasture during the day together with their foals and had free access to fresh water from troughs.

Collection of colostrum and milk samples was performed as described in previous studies (Martuzzi et al. 2004; Caroprese et al. 2007; Pikul and Wojtowski 2008). Briefly, the mares were provided with concentrate and their udder was hand milked in presence of the foal, without oxytocin injection.

Colostrum samples (250 ml) were collected between 12–24 h after foaling, whereas milk samples (350 ml) at 15, 90, and 150 days of lactation, always at the same time of the day (between 10 and 11 h in the morning). After collection, samples were stored at 4°C and transported to the laboratory for analytical determinations.

Chemical analysis. On colostrum and milk samples, we measured pH (ph meter HI931410; Hanna Instruments, Italy), protein, fat, and lactose content according to the International Dairy Federation standard (ISO 9622: 2013/IDF 141:2013), as well as dry matter (DM) content (AOAC 1990). All determinations were carried out in triplicate.

Antioxidant assays. The 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) assays were used to determine the radical scavenging activity (RSA, %) of colostrum and milk samples according to the following formula (Cosentino et al. 2015):

$$RSA\ (\%) = (1 - Ai/A0) \times 100\%$$

where:
Ai = absorbance of sample
A0 = absorbance of colorimetric radical substance without sample

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Both tests were carried out in triplicate on each sample.

DPPH assays. The stock radical solution of DPPH was prepared by dissolving 20 mg of 2,2-diphenyl-1-picrylhydrazyl in 15 ml of methanol. After 1 min with Vortex, 1 ml of stock DPPH solution was diluted with 29 ml of methanol. 50 µl of colostrum/milk were added to 950 µl of DPPH solution in a microcentrifuge tube and left in the dark for 30 min. After centrifuging (5 min, 8000 rpm), absorbance was measured at 515 nm against the reference sample (methanol) by using a UV-Vis spectrophotometer (Ultrospec II 4050 UV/VIS; LKB Biochrom Ltd., UK).

ABTS assay. This assay is based on the reduction of ABTS + radicals by antioxidants of tested milk. The stock solution of the ABTS radicals was prepared by dissolving 38 mg of 2,2'-azinobis (3-ethylbenzthiazoline-6-acid) in 10 ml of a sodium persulphate solution (2.45 mM), and the mixture was dark stored for 12–16 h. For the analysis, 1 ml of stock ABTS + solution was diluted with 29 ml of methanol. 20 µl of colostrum/milk were added to 980 µl of ABTS + solution. Samples were reacted with ABTS + working solution for 2 h in incubation in the dark at room temperature. After centrifuging (5 min, 8000 rpm), absorbance was measured at 734 nm against the reference sample (methanol) by using the Ultrospec II 4050 UV/VIS (LKB Biochrom Ltd.) spectrophotometer. The solutions were prepared fresh for the analysis.

Lysozyme determination.

Sample preparation. Lysozyme extraction was carried out according to a modified Pellegrino and Tirelli's (2000) protocol. Samples were prepared by mixing 10 ml of colostrum/milk with 30 ml of 1M NaCl. After heating under magnetic stirring at 40°C for 10 min, the samples were stirred on rotary shaker (100 rpm) at room temperature for 1 h. Afterwards, some drops of 1M HCl were added in order to decrease the pH value from 6.0 to 2.2, and hence, to provide the caseins precipitate. Finally, the samples were first filtered over a paper filter, and then through a 0.20 µm syringe cellulose acetate (CA) filter (Minisart NML; Sartorius, Germany). Only for colostrum analysis, after paper filtration, the sample was centrifuged in a 15-ml tube for 10 min at 3000 rpm, and then the liquid was filtered through a 0.20 µm syringe CA filter.

Liquid chromatographic condition. HPLC analysis was performed according to the method of Labella et al. (2016) using a chromatographic system Agilent 1200 Series quaternary pump furnished with vacuum degasser and auto-injector (Agilent Technologies, USA). The chromatographic separations were run on a Synergi MAX-RP 80 Å column (150 × 4.6 mm, 4 µm particle size) (Phenomenex, USA) with a MAX-RP guard column (4 mm × 2 mm id). Injection volume was 20 µl and flow rate was 0.8 ml/min. The mobile phase consisted of water (A) and acetonitrile (B) both containing 0.1% trifluoroacetic acid (v/v). The following eluting conditions were used: 0 min 80% A and 20% B; 9 min 60% A and 40% B; 15 min 60% A and 40% B; 20 min 80% A and 20% B. UV detection was carried out using the Agilent 1200 series diode array detector set at 280 nm. All determinations were carried out in triplicate.

Statistical analysis. The results of chemical analyses of colostrum and milk were presented as mean values ± standard deviations (SD). Data on milk determinations were submitted to analysis of variance (ANOVA) and comparisons among the days (15, 90, and 150) of lactation were evaluated by least significant difference (LSD). Differences at $P < 0.05$ were considered significant.

RESULTS AND DISCUSSION

The physicochemical characteristics of colostrum and milk from Murgese mares are shown in Table 1. Colostrum was characterized by a higher content of fat, protein, lysozyme, and dry matter than milk. By contrast, milk had a lower pH value and a lower content of lactose. Besides, colostrum showed the highest TAC as measured by RSA%, in both DPPH and ABTS tests (93.6% and 62.8%, respectively).

These data trends, i.e. the increasing or the decreasing of the values during the lactation, are in agreement with those reported in literature (Santos and Silvestre 2008; Centoducati et al. 2012).

The total protein content of colostrum was 7.79 g/100 g. This value was higher than that (6.65 g/100 g) reported for the colostrum at 12 h post foaling in Polish mare breed (Markiewicz-Keszycka et al. 2015).

The protein content of milk significantly decreased ($P < 0.001$) during lactation: the highest value (2.83 g/100 g) was observed at 15 days, whereas no significant differences were observed

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between the remaining sampling periods. A similar trend was observed in a study on the Italian Heavy Draft mare breed (Centoducati et al. 2012). As shown in Table 1, the fat content of colostrum was 2.95 g/100 g. During lactation, the fat content significantly decreased ($P < 0.001$): the values ranged from 1.20 (15 days) to 0.22 (150 days) g/100 g. Mare's milk is characterized by a very low fat content and the trend observed in this study is in line with previous studies (Caroprese et al. 2007; Markiewicz-Keszycka et al. 2013). Pikul and Wojtowski (2008), in a study on mares in the fifth and sixth months of lactation, reported a fat content over 1.0 g/100 g. Hand milking may be responsible for the low fat content of milk from Murgese mares, since it did not allow a complete milk removal from the udder (Caroprese et al. 2007). In fact, the cisternal component of the mare's udder has a very low capacity and the main part of milk is alveolar, and milking routines are needed to obtain the maximal recovery of milk (Markiewicz-Keszycka et al. 2013). The dry matter content of colostrum was 15.19 g/100 g, whereas significant differences ($P < 0.001$) were observed during lactation. In milk, the highest value (10.94 g/100 g) was observed at 15 days, whereas there were no significant differences between the remaining sampling periods. A similar trend was observed for protein content, with values ranging from 2.83 (15 days) to 1.75 (150 days) g/100 g. These data are in agreement with previous studies (Caroprese et al. 2007; Markiewicz-Keszycka et al. 2013). The lactose content observed in colostrum was 4.02 g/100 g. In milk samples it significantly increased, reaching the highest value at 150 days. Similar trends in lactation of 150 days were recorded in Murgese breed (Caroprese et al. 2007), in Lusitano breed (Santos and Silvestre 2008), in Heavy Draft breed (Centoducati et al. 2012), and in Polish breed (Markiewicz-Keszycka et al. 2013). The value of pH was 6.69 in colostrum, and it changed significantly during lactation ($P < 0.001$): the value observed at 15 days (6.85) was significantly lower than those observed at 90 and 150 days.

Concerning lysozyme content, the value was 526.62 mg/l in colostrum and averaged 273.49 mg/l in milk during lactation. This value is higher than that found in cow (traces), in human (0.12 mg/ml), and in goat milk (traces), but is close to asinine milk (1.0 mg/ml). The lysozyme content varied significantly ($P < 0.01$) during lactation: the highest content (303.69 mg/l) was observed at 15 days, whereas no significant differences were found between the remaining two sampling periods. Our data are in agreement with Sarwar et al. (2001), who studied the lysozyme activity in mare's milk during early lactation period. When a foal is born, it passes from the sterile uterus into an environment where it is immediately exposed to several pathogen microorganisms. Because the placenta of mare is classified as epitheliochorial with six strata (endometrial capillaries, e. interstitium, e. epithelium, chorionic epithelium, c. interstitium, c. capillaries) and diffuse, so at birth the foals are totally deprived of immunity. The newborn is provided with specific and non-specific immune factors through mother's milk for survival (Uniacke-Lowe et al. 2010). Thanks to its antimicrobial activity, a higher lysozyme content during the postpartum period can be attributed to immune mechanisms.

In the present study, two different assays were performed in order to verify the antioxidant scavenging activity of colostrum and milk. As showed by Frankel and Meyer (2000), antioxidant capacity

Table 1. Physicochemical parameters and lysozyme content of colostrum and milk from Murgese mares during lactation (mean ± SD)

Parameters	Colostrum 12–24 h	Milk		
		15 days	90 days	150 days
Dry matter (g/100 g)	15.19 ± 3.17	10.94^{a} ± 0.34	9.86^{b} ± 0.56	9.68^{b} ± 0.18
Protein (g/100 g)	7.79 ± 4.37	2.83^{a} ± 0.21	1.72^{b} ± 0.16	1.75^{b} ± 0.13
Fat (g/100 g)	2.95 ± 1.61	1.20^{a} ± 0.29	0.65^{b} ± 0.29	0.22^{c} ± 0.20
Lactose (g/100 g)	4.02 ± 1.58	6.22^{a} ± 0.19	6.88^{b} ± 0.17	7.05^{c} ± 0.23
pH	6.69 ± 0.31	6.85^{a} ± 0.08	7.18^{b} ± 0.11	7.13^{b} ± 0.07
Lysozyme (mg/l)	526.62 ± 90.09	303.69^{a} ± 47.46	264.02^{b} ± 41.47	252.75^{b} ± 32.95

$^{a–c}$means within each column with different superscripts differ significantly ($P < 0.05$)

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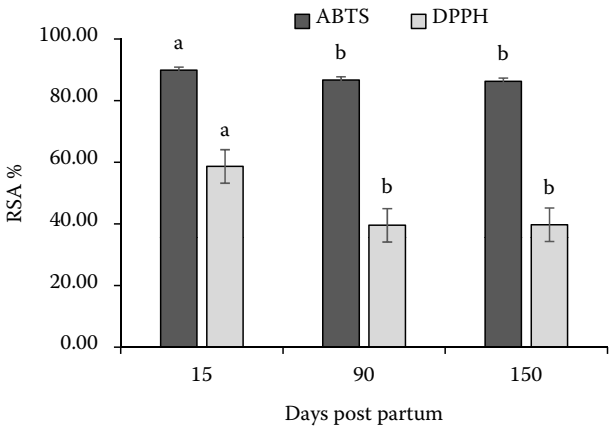

Figure 1. Total antioxidant capacity of milk from Murgese mares during lactation obtained by 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) (black bars) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) (grey bars) assays and expressed in radical scavenging antioxidant (RSA) % (means ± standard deviation)

[a,b]means with different superscripts differ significantly ($P < 0.05$)

of food is affected by several features: the composition of the test system, the substrate, and the mode of inducing oxidation. For this reason, it is necessary to employ several methods to measure total antioxidant capacity. In this case, we choose ABTS and DPPH tests, the most popular methods of evaluating TAC. The antioxidant capacity of colostrum was the highest (93.69 ± 3.02 RSA% and 62.76 ± 7.84 RSA% for ABTS and DPPH test, respectively). At birth, the high antioxidant activity in colostrum can defend the foal from the abrupt exposure to an environment rich in oxygen unlike the intrauterine environment and can avoid an oxidative stress (Zarban et al. 2009).

The total antioxidant levels of milk decreased during lactation in both tests: from 89.89 ± 1.02% at 15 days to 86.27 ± 1.09% after five months of study in ABTS test, and from 58.73 ± 5.82% to 39.77 ± 5.43% after five months of study in DPPH test. Between the 3rd and the 5th month, the RSA% remained constant in both tests (Figure 1). In both tests, the milk TAC was significantly different between days 15 and 150 postpartum ($P < 0.01$).

For all parameter values, the standard deviation for colostrum was always greater than that of the same parameter but measured in milk. For example, the error about protein content in colostrum was 4.37 g/100 g equal to 56.1%, indeed in milk the standard deviation was in the range 8.2–7.4%. The same for the lactose content: the standard deviation was 39.3% for the colostrum lactose content, and it was between 2.6–3.4% in milk during the lactation. And so on for the other parameters, except for fat content, for which the standard deviation became high at the end of lactation with an error value of quite 100%. The high values of standard deviation observed in colostrum are strictly related to its chemistry composition, which is affected by individual characteristics of mares (Doreau et al. 1990).

CONCLUSION

The preservation of an autochthonous breed such as the Murgese mare may be achieved by valorising its colostrum and milk. Understanding the chemical composition of both these types of milk may be the first step to achieve this goal. Our results showed that the milk from Murgese mares has important nutritional properties, and its chemical composition is significantly affected by the stage of lactation. Colostrum was characterized by a high dry matter, protein, and lysozyme content and a greater antioxidant activity. In fact, the survival of foals is guaranteed only through the intake of colostrum in the first 12–48 hours of life. Foals often do not take colostrum in necessary quantity or quality for several reasons: rejection by mother; orphans; premature separation; unreliability of mare; poor production or poor immunological quality of colostrum. For these reasons, the study was carried out with the Territorial Office for Biodiversity managing the Natural Biogenetic Reserve Eastern Murge as it will be the driver to create a "public bank of colostrum" for national farms and all horse breeders.

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