Lysozyme activity in donkey milk

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ABSTRACT

The aim of this work was to expand the knowledge on the lysozyme activity of donkey milk in relation to the lactation stage, age of the animals and pasteurization treatment. Raw and pasteurized bulk milk samples were collected monthly for one year and were analysed in relation to lysozyme activity. Individual raw milk samples were collected from 12 jennies, at three lactation stages: three, six and nine months. The results showed that the lactation stage and the age of the animals influenced the lysozyme activity of donkey milk. The highest lysozyme activity values were found at early lactation and in animals older than 15 years. We confirm that low-temperature long-time pasteurization of donkey milk does not reduce the activity of this enzyme. In addition, calcium, ash and some fatty acids, and in particular the saturated fatty acids, are linked to a higher lysozyme activity of donkey milk at early lactation.

1. Introduction

Lysozyme (LZ) is a glycoside hydrolase, which has shown antimicrobial activity against a

great variety of bacteria and is able to inhibit viruses, parasites and fungi (Benkerroum, 2008). This powerful enzyme is part of the innate immune system and is present in many secretions (tears, saliva, urine, mucus and milk) and is also produced by macrophages, neutrophils and dendritic cells (Ragland & Criss, 2017). Regarding the LZ content in milk, LZ concentrations of between 0.3 and 1.1 g L^{-1} have been reported in humans, while in the milk of ruminants, LZ is only present in trace amounts (Altomonte, Salari, Licitra, & Martini, 2019; Benkerroum, 2008). Surprisingly, LZ represents one of the main whey protein fractions in donkey milk and its presence is one of the key components of interest in asinine milk (Martini, Altomonte, Licitra, & Salari, 2018). Among different donkey breeds, the LZ content ranges from 1 to 3.7 g L^{-1}. In particular, the LZ content has been reported to be about 1 g L^{-1} in Ragusano (Vincenzetti et al., 2008), 1.5 g L^{-1} in Amiata (Caroli et al., 2015), 2.2 g L^{-1} in Martina Franca and in Jiangyue (Guo et al., 2007; Vincenzetti et al., 2011) and 1-3 g L^{-1} in the Serbian Balkan breed (Gubić et al., 2016). Analysis of the LZ content of milk obtained by four cross-breed jennies has shown mean values of 3.7 g L^{-1} (Chiavari, Coloretti, Nanni, Sorrentino, & Grazia, 2005). In donkey milk, the LZ content seems to decrease during lactation (Gubić et al., 2016; Polidori & Vincenzetti, 2010).

LZ activity is linked to its ability to catalyse the hydrolysis of the 1,4-β-linkages between N-acetylmuramic acid and N-acetylglucosamine in peptidoglycan, which is the major component of the gram-positive bacterial cell wall, causing bacterial lysis (Alhazmi, Stevenson, Amartey, & Qin, 2014). By virtue of its activity, LZ contributes to the low bacterial count of donkey milk and seems to exert antimicrobial functions synergistically with other milk components, such as lactoferrin, lactoperoxidase, N-acetyl-β-D glucoaminidase, immunoglobulins and some fatty acids (FAs) (Brumini, Criscione, Bordonaro, Vegarud, & Marletta, 2016; Nazzaro, Orlando, Fratianni, & Coppola, 2010). In vitro, LZ has shown resistance to acid pH and human gastrointestinal enzymes (Tidona et al., 2014) and anti-inflammatory and anti-tumor actions (Lee, Ku, Na, & Bae, 2015; Mao et al., 2009). On animal models, LZ has also been found to limit bacterial infections and to promote the growth of probiotic bacteria associated with gut health (Huang et al. 2018).

An interesting aspect of LZ antibacterial activity is its use in the food industry. Traditionally LZ from hen egg white has been widely used as a natural preservative in order to extend the shelf life of food products (Silvetti, Morandi, Hintersteiner, & Brasca, 2017). However recently, donkey milk has been tested as an antimicrobial additive in dairy products and has shown excellent results in preventing cheese-blowing during ripening as an alternative to hen egg white LZ, which may cause allergic reactions in egg allergic patients (Cosentino, Paolino, Freschi, & Calluso, 2013; Niro et al., 2017). Understanding the potential application of donkey milk LZ in the food industry entails knowledge of which factors affect its variability and its resistance to technological processes. Addo & Ferragut (2015) reported the resistance of LZ activity in donkey milk to moderate heat treatments and storage, however only a few studies have been carried out on the effects of thermal treatments. In addition, LZ activity in donkey milk is less studied compared to other sources such as other mammalian milk or hen egg white, and factors that affect its activity have been poorly investigated. The aim of this work was to expand the knowledge on the activity of LZ in donkey milk in relation to the lactation stage, age of the animals and pasteurization treatment. A possible correlation between LZ activity and the chemical composition of donkey milk was also evaluated.

2. Material and methods

2.1 Animals and samples collection

The research was conducted on a herd rearing a total of about 160 Amiata donkey, an Italian breed native to Mount Amiata, in Tuscany (central Italy). Animals were reared in a semi-intensive system and were fed with mixed hay ad libitum and about 2.5 kg per day per head of commercial pelleted concentrate formulated for dairy jennies. The farm produced pasteurized 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. One month after delivery, the jennies were routinely machine-milked once per day and four hours before being milked, the foals were separated from the jennies. Bulk milk samples from 40 jennies (of which 8 primiparous, 7 secondiparous and

25 multiparous; aged on average 9.5 years) were collected once a month for one year and at each sampling, two raw and two pasteurized samples (low-temperature long-time: 65 °C for 30 min) were analysed in terms of LZ activity. In addition, individual raw milk samples were collected from 12 healthy jennies, at three lactation stages: three, six and nine months. The animals were homogeneous in terms of foaling date and were aged between 9 and 20 years. All the samples were immediately refrigerated at 4 °C and then analysed within 24 hours.

2.2 Milk analysis

Individual milk samples were analysed for dry matter, fat and lactose by infrared analysis (MilkoScan; Italian Foss Electric, Padova, Italy) and for total protein, casein, ash and calcium content, using methods of the Association of Official Analytical Chemists (AOAC, 1990). Milk fat extraction was performed following Rose-Gottlieb's method (AOAC, 2000) and methyl esters of fatty acids were prepared according to Christie (1982). A Perkin Elmer Clarus 480 (Perkin Elmer, Norwolk, CT, USA) equipped with a flame ionization detector and a capillary column (ThermoScientific TR-FAME 60 m × 0.25 mm ID; film thickness 0.25 μm, Fisher Scientific UK) were used. The peak areas of individual FAs were identified using a FAs standard injection (Food Industry FAME Mix – Restek Corporation, 110 Benner Circle, Bellefonte, PA16823) and quantified as the percentage of total FAs.

2.3 LZ activity analysis

The LZ activity was evaluated in both bulk and individual milk samples using a commercial fluorimetric method on a microplate (EnzChek Lysozyme Assay Kit, Thermo Fisher Scientific, Waltham, MA, USA). The test uses a suspension of *Micrococcus lysodeikticus* labelled with fluorescein. This microorganism is sensitive to the lithic activity of LZ which leads to a variation in the intensity of the fluorescence measured at ~485/530 nm (excitation/emission). Milk was diluted and no defatting methods were used. The results were compared with a LZ standard curve and expressed in U ml^{-1}.

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106 *2.4 Statistical analysis*

107 The results of the LZ activity on individual samples were analysed using ANOVA for repeated
108 measurements, considering the three sampling times (three, six and nine months of lactation) and the
109 age of the jennies (< 9 years, between 9 to 15 years and > 15 years) as fixed effects; mean and standard
110 deviation of milk composition were also calculated. The LZ activity of bulk milk samples was
111 analysed using ANOVA considering the milk heat treatment as the fixed effect (raw or pasteurized
112 milk). Least significance means were compared by the t-test. Pearson's correlations were also carried
113 out for each stage of lactation. The significance level was set at $P < 0.05$. Statistical analysis was
114 carried out using JMP software (SAS Institute, 2002).

115

116 **3. Results**

117 Table 1 reports the average chemical composition of individual donkey milk samples
118 evaluated throughout the entire lactation. Despite being low in lipids, donkey milk provides a good
119 contribution of unsaturated fatty acids (over 46%) and Omega 3. Table 2 shows the individual LZ
120 activity during lactation and between the different age classes of animals. The lactation stage
121 influenced the LZ activity of donkey milk. A statistically higher value was found at three months of
122 lactation and a lower value at six months, while an increasing trend was observed at nine months. LZ
123 activity was also influenced by the age of the animals, and higher values were found as the age of
124 animals increased. In particular, a significantly higher LZ activity was found in donkeys older than
125 15 years compared to those younger than 9 years. With regard to the pasteurization treatment, no
126 significant differences were found in the LZ activity between the raw and the pasteurized milk
127 (1340.82±371.078 and 1402.50±294.722 U mL^{-1} respectively, with a SEM equal to 154.295).
128 Significant correlations between LZ activity and the chemical composition of donkey milk were
129 found during lactation: the most numerous and significant correlations were found at the third month
130 of lactation (Table 3).

In the first stage of lactation, significant correlations were found between LZ activity and both the calcium and ash contents. In addition, the results of the correlation analysis between LZ activity and FAs showed that the content of some saturated fatty acids (SFAs) (C6:0, C10:0, C12:0, C14:0 and C16:0), total SFAs, short chain fatty acids (SCFAs) and medium chain fatty acids (MCFAs) was correlated positively with the LZ activity. In addition, another two unsaturated fatty acids C18:2n6(t9,t12) and C18:3n3(c9,c12,c15) showed positive correlations with LZ activity. Differently, the content of the following unsaturated fatty acids: C18:1(t9), C18:2n6(c9,c12), C20:1 and C20:4n6, total polyunsaturated fatty acids (PUFAs) and long chain fatty acids (LCFAs) was correlated negatively with LZ activity. Finally, LZ activity was correlated positively with the total n-3 and negatively with the total n-6 fatty acids.

4. Discussion

The results on the gross composition are in agreement with our previous studies on Amiata donkey (Martini, Altomonte, Salari, & Caroli, 2014; Ragona et al., 2016). As shown in Table 1, the donkey milk was characterized by a low total protein content and in particular by a low casein content. These characteristics contribute to the hypoallergenic properties of donkey milk which make it a valid alternative for people with an allergy to cow milk proteins (Martini et al., 2018). The high content of lactose, in addition to making it more palatable, contributes to the development of intestinal flora (Venema, 2012).

Our data on the LZ activity in Amiata milk were slightly lower than those reported in previous studies on individual milk samples from the same breed (Pilla, Daprà, Zecconi, & Piccinini, 2010; Ragona et al., 2016) and similar compared to the results reported for bulk milk of the Ragusano donkey (Conte, Foti, Malvisi, Giacopello, & Piccinini, 2012). Higher values (11531 U ml^{-1}) have been reported by Addo & Ferragut (2015) in a pool of milk from donkeys between the first and third month of lactation. The apparent variability of results found in the literature is due to differences in sampling methods and analyses. In our study, the trend of LZ activity during lactation is similar to

that reported by Pilla et al. (2010). Regarding the age of the animals, Qureshi & Enbergs (2012) reported that donkeys older than 10 years exhibited significantly higher LZ activity values compared to donkeys younger than 10 years, in agreement with our findings. Similar results were also found in a study carried out on horses, in which 10-14 year-old mares showed significantly higher LZ activity values compared to 5-9 year-old mares (Sarwar, Enbergs, & Klug, 2001). Comparing LZ activity in raw and pasteurized milks, our results showed that pasteurization treatment did not affect the LZ activity. Similarly, other studies applying similar temperature treatments (Coppola et al., 2002; Chiavari et al., 2005) or higher temperatures and shorter times (70-90 °C for 1 min) did not find changes in the LZ activity of donkey milk (Coppola et al., 2002; Addo & Ferragut, 2015).

The positive correlation between LZ activity and calcium content found in this study was in agreement with the findings of Sarwar et al. (2001) in mare milk. These results may be related to the ability of equid milk LZ to bind calcium, which seems to stabilize the LZ structure and increase the antimicrobial activity (Brumini et al., 2016). The strong positive correlations found between LZ activity and SFAs and FAs with less than 17 carbon atoms strengthen the hypothesis of Brumini et al. (2016) regarding the synergistic activity of LZ with some FAs known to be antibacterial such as C12:0. In addition, we found other positive correlations with LZ activity and C10:0, C14:0 and C16:0 FAs, the latter having already been reported to be bactericidal FAs (Sprong, Hulstein, & Van der Meer, 2001; Desbois & Smith, 2010). This relationship could strengthen the milk antibacterial activity when the immature immune system of the offspring still needs protective factors. Further confirmation of the synergistic action between LZ and milk FAs is provided by a study on egg white LZ which showed that the lipophilization of LZ with SCFAs improves the bactericidal action against gram-negative bacteria without decreasing its effect on gram-positive bacteria (Liu, Sugimoto, Azakami, & Kato, 2000). Further studies are needed to clarify the negative relationships we observed between lysozyme and PUFAs and LCFAs.

5. Conclusions

In Amiata donkey milk, LZ activity was found to be influenced by the lactation stage and the

age of the animals. In particular, the highest LZ activity values were found at early lactation and in animals older than 15 years. This study confirms that the low-temperature long-time pasteurization treatment of donkey milk does not impact on the variability in the activity of this enzyme. In addition, both calcium, ash and some fatty acid contents, and in particular the SFAs, are linked to a higher activity of donkey milk LZ at early lactation.

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International Journal of Food Engineering, 7, 1-16.

289 Table 1. Gross composition and fatty acids classes of individual donkey milk. Data are expressed as
290 mean value and standard deviation.

	Mean	Standard deviation
Gross composition (g 100mL^{-1})		
Dry matter	9.80	1.095
Total Protein	1.65	0.201
Casein	0.81	0.099
Fat	0.33	0.193
Lactose	6.97	0.194
Ash	0.33	0.055
Calcium	0.09	0.035
Fatty acids classes (g 100 g of fat^{-1})		
SFAs[1]	53.16	7.650
MUFAs[2]	27.07	6.767
PUFAs[3]	19.77	2.766
SCFAs[4] (≤C10)	12.90	3.221
MCFAs[5] (C11-C17)	42.45	4.594
LCFAs[6] (≥C18)	44.65	6.789
Total n-3	5.16	1.453
Total n-6	14.54	3.514
Lysozyme activity (U mL^{-1})	1670.11	411.454

291 [1]SFAs: saturated fatty acids; [2]MUFAs: monounsaturated fatty acids; [3]PUFAs: polyunsaturated fatty
292 acids; [4]SCFAs: short chain fatty acids; [5]MCFAs: medium chain fatty acids; [6]LCFAs: long chain fatty
293 acids.
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295 Table 2. Average lysozyme (LZ) activity in relation to lactation stage and age of the animals.

Month of lactation	3	6	9	SEM
LZ activity (U mL^{-1})	1842.21a	1433.84b	1733.46ab	446.058
Age of the animals	<9	9-15	>15	SEM
LZ activity (U mL^{-1})	1547.83b	1610.57ab	1824.11a	446.058

296 Within a row, means without a common lowercase letter differ at $P < 0.05$.

297

298 Table 3. Correlations between milk composition and lysozyme activity at three months of lactation.

Component	Lysozyme activity (U mL^{-1})
Ash	0.684*
Calcium	0.613*
C6:0	0.813**
C10:0	0.666*
C12:0	0.816**
C14:0	0.789**
C16:0	0.793**
C18:1(t9)	0.726*
C18:2n6(t9,12)	0.828**
C18:2n6(c9,12)	0.884***
C18:3n3(c9,12,15)	0.732*
C20:1	-0.696*
C20:4n6	-0.787**
SFAs[1]	0.868**
PUFAs[2]	-0.727*
SCFAs[3] (≤C10)	0.677*
MCFAs[4] (≥C11≤C17)	0.857**
LCFAs[5] (≥C18)	-0.877***
Total n-3	0.738*
Total n-6	-0.884***

299 [1]SFAs: saturated fatty acids; [2]PUFAs: polyunsaturated fatty acids; [3]SCFAs: short chain fatty acids;

300 [4]MCFAs: medium chain fatty acids; [5]LCFAs: long chain fatty acids.

301 Only significant correlations are shown

302 Significance levels: *P < 0.05; **P < 0.01, ***P < 0.001

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