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Lysozyme activity in donkey milk

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ABSTRACT

Lysozyme activity of donkey milk in relation to the lactation stage, age of the animals and pasteurisation treatment was studied. Raw and pasteurised 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 pasteurisation 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 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-tumour 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 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-

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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 and 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 with other sources such as other mammalian milk or hen egg white, and factors that affect its activity have been poorly investigated.

In this work we expanded knowledge on the activity of LZ in donkey milk in relation to the lactation stage, age of the animals and pasteurisation 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 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. 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 pasteurised 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 h.

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 PerkinElmer Clarus 480 (PerkinElmer, Norwalk, CT, USA) equipped with a flame ionisation detector and a capillary column (ThermoScientific TR-FAME 60 m × 0.25 mm ID; film thickness 0.25 μm, Fisher Scientific, Loughborough, Leicestershire, UK) were used. The peak areas of individual FAs were identified using a FAs standard injection (Food Industry FAME Mix – Restek Corporation, Bellefonte, PA, USA) and quantified as the percentage of total FAs.

2.3. Lysozyme 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 that 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}.

2.4. Statistical analysis

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

3. Results

Table 1 reports the average chemical composition of individual donkey milk samples evaluated throughout the entire lactation. Despite being low in lipids, donkey milk provides a good contribution of unsaturated fatty acids (over 46%) and Omega 3. Table 2 shows the individual LZ activity during lactation and between the different age classes of animals. The lactation stage influenced the LZ activity of donkey milk. A statistically higher value was found at three months of lactation and a lower value at six months, while an increasing trend was observed at nine months. LZ activity was also influenced by the age of the animals, and higher values were found as the age of animals increased. In particular, a significantly higher LZ activity was found in donkeys older than 15 years compared with those younger than 9 years. With regard to the pasteurisation treatment, no significant differences were found in the LZ activity between the raw and the pasteurised milk (1340.8 ± 371.1 and

Table 1
Gross composition and fatty acids classes of individual donkey milk.

Parameter	Mean	Standard deviation
Gross composition (g 100 mL^{-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 fat^{-1})		
SFAs	53.16	7.650
MUFAs	27.07	6.767
PUFAs	19.77	2.766
SCFAs (≤C10)	12.90	3.221
MCFAs (C11–C17)	42.45	4.594
LCFAs (≥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

Abbreviations are: SFAs, saturated fatty acids; MUFAs, monounsaturated fatty acids; PUFAs, polyunsaturated fatty acids; SCFAs, short chain fatty acids; MCFAs, medium chain fatty acids; LCFAs, long chain fatty acids.

Table 2
Average lysozyme (LZ) activity in relation to lactation stage and age of the animals.[a]

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

[a] Within a data set, means without a common lowercase superscript letter differ at $P < 0.05$.

1402.5 ± 294.7 U mL^{-1} respectively, with a SEM equal to 154.3). Significant correlations between LZ activity and the chemical composition of donkey milk were found during lactation: the most numerous and significant correlations were found at the third month 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.

Table 3
Correlations between milk composition and lysozyme activity at three months of lactation.[a]

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	0.868**
PUFAs	−0.727*
SCFAs (≤C10)	0.677*
MCFAs (≥C11 ≤ C17)	0.857**
LCFAs (≥C18)	−0.877***
Total n-3	0.738*
Total n-6	−0.884***

[a] Abbreviations are: SFAs, saturated fatty acids; PUFAs, polyunsaturated fatty acids; SCFAs, short chain fatty acids; MCFAs, medium chain fatty acids; LCFAs, long chain fatty acids. Only significant correlations are shown; asterisks indicate significance levels: *$P < 0.05$; **$P < 0.01$, ***$P < 0.001$.

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 characterised 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 with the results reported for bulk milk of the Ragusano donkey (Conte, Foti, Malvisi, Giacopello, & Piccinini, 2012). Higher values (11,531 U mL^{-1}) have been reported by Addo and 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 and Enbergs (2012) reported that donkeys older than 10 years exhibited significantly higher LZ activity values compared with 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 with 5–9 year-old mares (Sarwar, Enbergs, & Klug, 2001). Comparing LZ activity in raw and pasteurised milks, our results showed that pasteurisation treatment did not affect the LZ activity. Similarly, other studies applying similar temperature treatments (Chiavari et al., 2005; Coppola et al., 2002) or higher temperatures and shorter times (70–90 °C for 1 min) did not find changes in the LZ activity of donkey milk (Addo & Ferragut, 2015; Coppola et al., 2002).

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 stabilise 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 (Desbois & Smith, 2010; Sprong, Hulstein, & Van der Meer, 2001). 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 lipophilisation 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 pasteurisation 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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References

Addo, C. N. A., & Ferragut, V. (2015). Evaluating the ultra-high pressure homogenization (UHPH) and pasteurization effects on the quality and shelf life of donkey milk. *International Journal of Food Studies, 4*, 104–115.
Alhazmi, A., Stevenson, J. W., Amartey, S., & Qin, W. (2014). Discovery, modification and production of T4 lysozyme for industrial and medical uses. *International Journal of Biology, 6*, 45–63.
Altomonte, I., Salari, F., Licitra, R., & Martini, M. (2019). Donkey and human milk: Insights into their compositional similarities. *International Dairy Journal, 89*, 111–118.
AOAC. (1990). *Official methods of analysis* (15th ed.). Arlington, VA, USA: Association of Official Analytical Chemists.
AOAC. (2000). *Official methods of analysis* (17th ed.). Gaithersburg, MD, USA: Association of Official Analytical Chemists.
Benkerroum, N. (2008). Antimicrobial activity of lysozyme with special relevance to milk. *African Journal of Biotechnology, 7*, 4856–4867.
Brumini, D., Criscione, A., Bordonaro, S., Vegarud, G. E., & Marletta, D. (2016). Whey proteins and their antimicrobial properties in donkey milk: A brief review. *Dairy Science & Technology, 96*, 1–14.
Caroli, A. M., Bulgari, O., Gigliotti, C., Altomonte, I., Salari, F., & Martini, M. (2015). Profilo lattoproteico e attività proteasica totale del latte di asina. *Scienza e Tecnica Lattiero-Casearia, 66*, 11–16.
Chiavari, C., Coloretti, F., Nanni, M., Sorrentino, E., & Grazia, L. (2005). Use of donkey's milk for a fermented beverage with lactobacilli. *Le Lait, 85*, 481–490.
Christie, W. W. (1982). A simple procedure of rapid transmethylation of glycerolipids and cholesteryl esters. *Journal of Lipid Research, 23*, 1072–1075.
Conte, F., Foti, M., Malvisi, M., Giacopello, C., & Piccinini, R. (2012). Valutazione dell'azione antibatterica del lisozima del latte d'asina. Considerazioni igienico-sanitarie. *Large Animal Review, 18*, 13–16.
Coppola, R., Salimei, E., Succi, M., Sorrentino, E., Nanni, M., Ranieri, P., et al. (2002). Behaviour of *Lactobacillus rhamnosus* strains in ass's milk. *Annals of Microbiology, 52*, 55–60.
Cosentino, C., Paolino, R., Freschi, P., & Calluso, A. M. (2013). Jenny milk as an inhibitor of late blowing in cheese: A preliminary report. *Journal of Dairy Science, 96*, 3547–3550.
Desbois, A. P., & Smith, V. J. (2010). Antibacterial free fatty acids: Activities, mechanisms of action and biotechnological potential. *Applied Microbiology and Biotechnology, 85*, 1629–1642.
Gubić, J. M., Tomić, J., Torbica, A., Iličić, M., Tasić, T., Šarić, L.Ć., et al. (2016). Characterization of several milk proteins in domestic Balkan donkey breed during lactation, using lab-on-a-chip capillary electrophoresis. *Chemical Industry and Chemical Engineering Quarterly, 22*, 9–15.
Guo, H. Y., Pang, K., Zhang, X. Y., Zhao, L., Chen, S. W., Dong, M. L., et al. (2007). Composition, physiochemical properties, nitrogen fraction distribution, and amino acid profile of donkey milk. *Journal of Dairy Science, 90*, 1635–1643.
Huang, G., Li, X., Lu, D., Liu, S., Suo, X. M., Li, Q., et al. (2018). Lysozyme improves gut performance and protects against enterotoxigenic *Escherichia coli* infection in neonatal piglets. *Veterinary Research, 49*, 1–11.
Lee, W., Ku, S. K., Na, D. H., & Bae, J. S. (2015). Anti-inflammatory effects of lysozyme against HMGB1 in human endothelial cells and in mice. *Inflammation, 38*, 1911–1924.
Liu, S., Sugimoto, T., Azakami, H., & Kato, A. (2000). Lipophilization of lysozyme by short and middle chain fatty acids. *Journal of Agricultural and Food Chemistry, 48*, 265–269.
Mao, X., Gu, J., Sun, Y., Xu, S., Zhang, X., Yang, H., et al. (2009). Anti-proliferative and anti-tumour effect of active components in donkey milk on A549 human lung cancer cells. *International Dairy Journal, 19*, 703–708.
Martini, M., Altomonte, I., Licitra, R., & Salari, F. (2018). Nutritional and nutraceutical quality of donkey milk. *Journal of Equine Veterinary Science, 65*, 33–37.
Martini, M., Altomonte, I., Salari, F., & Caroli, A. M. (2014). Monitoring nutritional quality of Amiata donkey milk: Effects of lactation and productive season. *Journal of Dairy Science, 97*, 6819–6822.
Nazzaro, F., Orlando, P., Fratianni, F., & Coppola, R. (2010). Isolation of components with antimicrobial property from the donkey milk: A preliminary study. *The Open Food Science Journal, 4*, 43–47.
Niro, S., Fratianni, A., Colavita, G., Galassi, L., Zanazzi, M., & Salimei, E. (2017). Technological use of donkey milk in cheesemaking. *International Journal of Dairy Technology, 70*, 439–442.
Pilla, R., Daprà, V., Zecconi, A., & Piccinini, R. (2010). Hygienic and health characteristics of donkey milk during a follow-up study. *Journal of Dairy Research, 77*, 392–397.
Polidori, P., & Vincenzetti, S. (2010). Differences of protein fractions among fresh, frozen and powdered donkey milk. *Recent Patents on Food, Nutrition & Agriculture, 2*, 56–60.
Qureshi, A. S., & Enbergs, H. (2012). Studies on the lysozyme activity in the milk of female donkeys (*Equus asinus*) in relation to reproductive physiology. *Journal of Animal and Plant Sciences, 22*, 70–74.
Ragland, S. A., & Criss, A. K. (2017). From bacterial killing to immune modulation: Recent insights into the functions of lysozyme. *PLoS Pathogens, 13*, 1–22.
Ragona, G., Corrias, F., Benedetti, M., Paladini, I., Salari, F., Altomonte, I., et al. (2016). Amiata donkey milk chain: Animal health evaluation and milk quality. *Italian Journal of Food Safety, 5*, 173–178.
Sarwar, A., Enbergs, H., & Klug, E. (2001). Influences of parity, age and mineral and trace element mixture on lysozyme activity in mare's milk during early lactation period. *Veterinarsky Arhiv, 71*, 139–147.
SAS Institute. (2002). *JMP user's guide. Version 5.0*. Cary, NC, USA: SAS Institute Inc.
Silvetti, T., Morandi, S., Hintersteiner, M., & Brasca, M. (2017). Use of hen egg white lysozyme in the food industry. In P. Y. Hester (Ed.), *Egg innovations and strategies for improvements* (pp. 233–242). London, UK: Academic Press.
Sprong, R. C., Hulstein, M. F. E., & Van der Meer, R. (2001). Bactericidal activities of milk lipids. *Antimicrobial Agents and Chemotherapy, 45*, 1298–1301.
Tidona, F., Criscione, A., Devold, T. G., Bordonaro, S., Marletta, D., & Vegarud, G. E. (2014). Protein composition and micelle size of donkey milk with different protein patterns: Effects on digestibility. *International Dairy Journal, 35*, 57–62.
Venema, K. (2012). Intestinal fermentation of lactose and prebiotic lactose derivatives, including human milk oligosaccharides. *International Dairy Journal, 22*, 123–140.
Vincenzetti, S., Polidori, P., Mariani, P., Cammertoni, N., Fantuz, F., & Vita, A. (2008). Donkey's milk protein fractions characterization. *Food Chemistry, 106*, 640–649.
Vincenzetti, S., Savini, M., Cecchini, C., Micozzi, D., Carpi, F., Vita, A., et al. (2011). Effects of Lyophilization and use of probiotics on donkey's milk nutritional characteristics. *International Journal of Food Engineering, 7*, 1–16.