

RESEARCH ARTICLE

Major whey proteins in donkey's milk: effect of season and lactation stage. Implications for potential dietary interventions in human diseases

Angela G. D'Alessandro¹, Giovanni Martemucci¹, Emilio Jirillo², and Vincenzo De Leo¹

¹Department PRO.GE.S.A., University of Bari, Bari, Italy, and ²Immunology, Faculty of Medicine, University of Bari, Bari, Italy

Abstract

According to current literature, donkey's milk has been suggested as a hypoallergenic substitute in children affected by cow's milk protein allergy as well as a promising nutraceutical for aged people. However, the biologically active components of donkey's milk have not yet completely elucidated. In this framework this study is aimed at measuring α -lactalbumin (α -LA), β -lactoglobulin (β -LG), and lysozyme (LYS), the principal whey proteins in donkey's milk, in relation to lactation stage and production season. Analysis were performed by reversed-phase high-performance liquid chromatography. α -LA, β -LG, and LYS resulted to be affected by lactation stage ($P < 0.01$) and production season ($P < 0.01$). Overall, the protein content was higher ($0.01 > P < 0.05$) during the first four lactation's months and decreased until the month 8. The β -LG was the major protein (1.75 mg mL^{-1} as mean; peak $2.24 \pm 0.09 \text{ mg mL}^{-1}$), while the α -LA had a mean concentration of 1.32 mg mL^{-1} and peaked at month 1 ($1.57 \pm 0.09 \text{ mg mL}^{-1}$) and LYS (0.66 mg mL^{-1} as mean) showed the highest value equal to $0.76 \pm 0.03 \text{ mg mL}^{-1}$. The highest ($P < 0.01$) concentration of all proteins was recorded at spring (α -LA: 1.69 mL^{-1} ; β -LG: 2.07 mL^{-1} ; LYS: 0.76 mL^{-1}).

Keywords: Donkey milk, α -lactalbumin, β -lactoglobulin, lysozyme, human health implications

Introduction

In the last years, considerable interest has been focusing on donkey's milk for human feeding and it has also been suggested as a hypoallergenic substitute in children affected by cow's milk protein allergy or multiple food intolerance.^(1,2) Moreover, donkey's milk seems to be a promising nutraceutical for aged people for its capacity to upregulate the elderly immune response.^(3,4) According to current literature, donkey's milk differs from that of other species traditionally employed for human feeding⁽⁵⁾ also being very close to human milk.^(6,7) Thus, it is characterized by a low casein index (43% as average)⁽⁸⁾ while the whey proteins represent an important component of the nitrogenous fraction (35–50%).⁽⁹⁾ Basically, it is known that whey proteins with their high body value, which exceeds egg protein by 15%, exhibit a great nutritional and functional properties.^(10–15) The principal whey proteins in milk from ruminants consist

of α -lactalbumin (α -LA), β -lactoglobulin (β -LG), bovine serum albumin, casein-derived peptides, lactoferrin (LF), lactoperoxidase (LP), and immunoglobulins.⁽⁵⁾ On the other hand, in domesticated equid milk, the major whey proteins are α -LA, β -LG, and lysozyme (LYS).^(16,17) α -LA and β -LG are the two main whey proteins,^(18,19) both synthesized in the mammary gland. In mare's milk three molecular types of α -LA have been isolated⁽²⁰⁾ while only one genetic variant was found in donkey's milk.⁽⁹⁾ In cow's milk α -LA has a molecular weight (MW) of 14,175 Da and is constituted by 123 amino acid residues.⁽²¹⁾ This protein acts as a coenzyme for the biosynthesis of lactose, which is an important source of energy for the newborn.⁽²²⁾ Its functional properties have been studied in the field of cancer prevention.^(22–24) Moreover, it has been reported that α -LA contains well-digestible amino acids and the α -LA peptides are able to stimulate proliferation and maturation of T- and natural killer cells,

thus contributing to the defence of children against some pathogenic bacteria, mainly of the intestinal tract.⁽²⁵⁾

β -LG presents several genetic variants, among that the β -LG A is the most common one. It consists of 162 amino acid residues⁽²⁶⁾ and is dimeric (MW 36,000 Da)⁽⁵⁾ in milk of ruminants, while it is predominantly present as a monomer in equine milk.^(19,27,28) In donkey's milk, two molecular forms with their respective amino acid sequences have been identified (β -LG I and β -LG II), β -LG I representing the major form (80% of the total).^(29,30) β -LG is also characterized by the presence of free thiolic groups which represent a rich source of cysteine,⁽³¹⁾ an essential amino acid. In rats, cysteine appears to stimulate glutathione synthesis, an anticarcinogenic tripeptide, produced by the liver and protective against intestinal tumors.⁽³²⁾ However, β -LA biological function is not completely known. Its role is thought to be related to a transport action for acquisition of passive immunity in newborn, to regulation of phosphorous metabolism at mammary gland,⁽³³⁾ to its capability to exert an antioxidative activity,⁽³¹⁾ and to bind and transport retinol to small intestine.⁽³⁴⁾ All these biological functions appear to be less important to human babies, which may explain why β -LA does not occur in human milk.⁽²²⁾

LYS is a muramidase of 14,000–18,000 Da MW⁽²⁶⁾ which is present in body fluids (serum, tears, saliva) and secreted by mononuclear phagocytes.^(35,36) In milk, LYS seems to be of leukocyte origin.⁽⁵⁾ LYS enzymatically acts on the peptidoglycan of bacterial cell walls hydrolyzing the 1–4 glycosidic linkages between η -acetylmuramic acid and η -acetylglucosamine.^(37,38) Moreover, LYS exhibits a synergic antimicrobial activity together with LF and LP, constituting the non-immunological protective system in milk.⁽³⁹⁾ In human milk together with LF and secretory immunoglobulin A, LYS plays a key role in affording passive protection to infants.⁽⁴⁰⁾

In donkey's milk, two genetic variants for LYS, as LYS A and LYS B, have been identified,^(9,30) while limited data are available on the composition of whey proteins and factors which influence their concentration.

This study was undertaken with the aims to determine in donkey's milk the amounts of the major whey proteins— α -LA, β -LG, and LYS—and their variations in relation to lactation stage and production season. Potential benefits to human health deriving from donkey's milk consumption will be highlighted.

Materials and methods

Animal

A total of 27 pluriparous, healthy lactating asses of Martina Franca breed, an autochthonous breed of Apulia region (Southern Italy), were used. The animals were reared at an experimental private farm located in the typical breeding area for this breed (Alberobello, South Eastern Murgia; 40°37'20"latitude, 60°17'44"longitude). The animals were bred following the traditional semi-extensive breeding system which is based

on the use of natural scrub pasture and an integration with oats (2.0 kg/day/head). According to the routine used by us for asses milking, during the trials animals were subjected to a twice daily milking by machine in the presence of foals, after a 3 h interval from foal separation by dams.^(41,42)

Source of milk

A trial aimed at evaluating the milk content in α -LA, β -LG, and LYS throughout a long-term lactation of 8 months. Milk samples were collected from 9 asses selected according to a close foaling date. Individual milk samples from morning milking were collected. The first sampling was carried out at 25 ± 5 days after foaling, and then every 28 ± 2 days in correspondence of each lactation month until to the month 8. Milk samples were frozen and stocked (-20°C) until analysis.

An experiment was designed to investigate how the concentration of α -LA, β -LG, and LYS in donkey's milk could change in relation to the season. The trial lasted a whole year, throughout the four seasons in Europe: Spring (April to June), Summer (July to August), Autumn (September to December), and Winter (January to March). Milk was drawn from nine asses at different lactation stages: five asses were at 1st to 3rd month of lactation and four asses were at fourth to sixth month. At the end of the 6 months lactation the asses were gradually substituted with other asses for a total of 18 animals. Individual milk samples from morning milking were collected at 3 weeks interval for each season. Milk samples were frozen and stocked (-20°C) until analysis.

Preparation of milk samples

After thawing, one 10 mL aliquot from each milk sample was skimmed by centrifugation (Allegra X22R, Beckmann Coulter, Brea, CA) at 9,500 rpm for 15 min at 4°C . Caseins were then precipitated by adjusting the pH to 4.6 according to the procedure of Aschaffenburg and Drewry.⁽⁴³⁾

RP-HPLC

The analysis of the whey proteins— α -LA, β -LG, and LYS—was performed by reversed phase-high-performance liquid chromatography (RP-HPLC), using a DIONEX Ultimate™ 3000 System (Dionex Corporation, Sunnyvale, CA), according to the procedure of Vincenzetti et al.⁽⁴⁴⁾ slightly modified. Each serum sample was filtered with a cartridge in cellulose acetate (pore 0.45 μm , Teknokroma, Barcelona, Spain) and then diluted 1:1 with water for HPLC (Carlo Erba Reagents, Milan, Italy). 20 μL serum were then injected into a RP-HPLC column, type Waters Symmetry300™ (C_4 , 4.6 mm $\times$ 150 mm, pore size 3.5 μm ; Waters Corporation, Milford, MA). The column was conditioned in trifluoroacetic acid (TFA; Fluka, Buchs, Switzerland)/ H_2O 1:1000 v/v (buffer B) and the elution was conducted with TFA/ H_2O /acetonitrile (Carlo Erba Reagents) 1:100:900 v/v (buffer A), according to the following gradient: %B = 100, time = 2 min; %B = 80, time = 10 min; %B = 60, time = 0.1 min; %B = 45, time = 19.9 min; %B = 45,

time = 3 min; %B = 100, time = 2 min. The flow was 1 mL/min and the proteins eluted were detected at 280 nm. For each protein concentration analysis five dilutions of the standards—2.0 mg mL⁻¹, 1.0 mg mL⁻¹, 0.5 mg mL⁻¹, 0.25 mg mL⁻¹, 0.1 mg mL⁻¹—were also included in each assay series. The standards used were the following: for LYS, white egg LYS (Sigma-Aldrich, St Louis, MO) dissolved in acetonitrile/H₂O 1:1 (v/v); for α -LA, bovine milk α -LA (Sigma-Aldrich) dissolved in acetonitrile/H₂O 1:1 (v/v); for β -LG, bovine milk β -LG (Sigma-Aldrich) dissolved in H₂O. The peak area was measured using the method of integration valley to valley and calibration curves were linear in the concentration range achieved. Each fraction eluted in correspondence of the three chromatographic peaks was collected separately and then analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE).

SDS-PAGE

The fractions corresponding to each protein were collected separately. The eluate was deprived of acetonitrile using a rotary evaporator (Buchi, Flawil, Switzerland), frozen and then freeze-dried. The powders (5 μ g) were then dissolved in 50 μ L of running buffer and 2 μ L of β -mercaptoethanol (Sigma-Aldrich). After 5 min of incubation, the samples were analyzed by SDS-PAGE (8–18% gradient). Electrophoresis was performed at 12°C and 10 mA. The proteins were visualized by Coomassie Blue staining. SDS and MW standard (low range) for SDS-PAGE were from Bio-Rad Laboratories (Hercules, CA).

The RP-HPLC chromatogram of donkey whey proteins and the SDS-PAGE analysis of the eluted peaks are shown in Figure 1. Peak A with a MW of 15.0 kDa was identified as LYS, peak B with a MW of 14.2 kDa was associated to α -LA and peak C with a MW of 19.0 kDa was identified as β -LG. MW found for the three proteins overlap those described in literature.^(9,44)

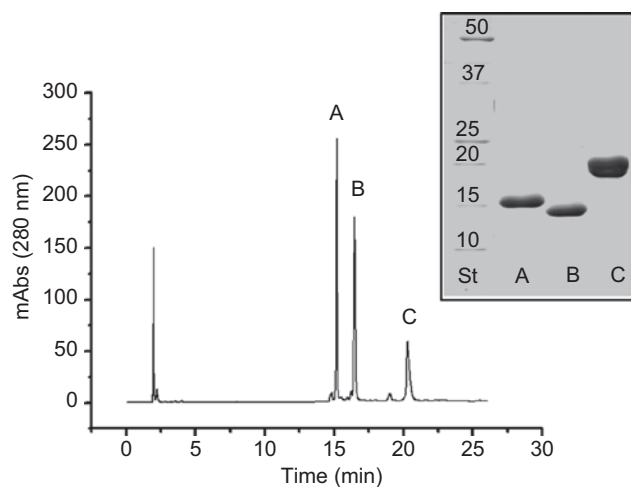


Figure 1. Separation of donkey whey proteins by RP-HPLC. Inset: SDS-PAGE analysis of peaks A, B, and C eluted from RP-HPLC and identified as LYS (MW 15.0 kDa), α -LA (MW 14.2 kDa), and β -LG (MW of 19.0 kDa), respectively. St indicate molecular weight standard (low range).

Statistical analysis

Data were analyzed by least square analysis of variance using the GLM procedure of the SAS.⁽⁴⁵⁾

Data were analyzed considering a linear model. In experiment I, the model included the fixed effects of month of lactation and animal within month of lactation, their interaction and the residual error. In experiment II, the model included the fixed effects of season and animal within season, their interaction and the residual error. Means were compared by Student's *t*-test with the predicted difference (pdiff) option of the GLM.

Results

Effects of lactation period

Month of lactation exerted a significant ($P < 0.01$) effect on all the three whey proteins at study.

Overall, the mean content of α -LA during the 8 months of lactation ($\pm$ SD) was 1.32 ± 0.22 mg mL⁻¹, while an α -LA to β -LG ratio of 3:4 (data not shown). Considering the variations during the lactation, α -LA showed the highest value at the first month (mean $\pm$ SE: 1.57 ± 0.09 mg mL⁻¹) with a tendency ($P > 0.05$) to decrease until the fourth month (Figure 2). From the fifth month, its concentration underwent a progressive marked decreasing ($0.01 > P < 0.05$), reaching the lowest value at the month 8 of lactation (0.94 ± 0.09 mg mL⁻¹).

Among the three whey proteins studied, β -LG showed the highest concentration with a mean value (mean $\pm$ SD) during lactation of 1.75 ± 0.27 mg mL⁻¹ (data not shown). The peak was recorded at month 4 of lactation (mean $\pm$ SE: 2.24 mg mL⁻¹ ± 0.09 ; $0.01 > P < 0.05$), after which the β -LG concentration began to decrease markedly ($0.01 > P < 0.05$) to the lowest value at month 8 (1.38 ± 0.09 mg mL⁻¹) (Figure 3).

During lactation, the mean concentration of LYS resulted to be 0.69 ± 0.09 mg mL⁻¹ (mean $\pm$ SD; data not shown). By analogy to β -LG, the peak was observed at month 4 of lactation (mean $\pm$ SE: 0.76 ± 0.03 mg mL⁻¹) (Figure 4). This value resulted to be significantly ($P < 0.01$) different compared to 0.67 ± 0.03 mg mL⁻¹, recorded at months 4 and 7.

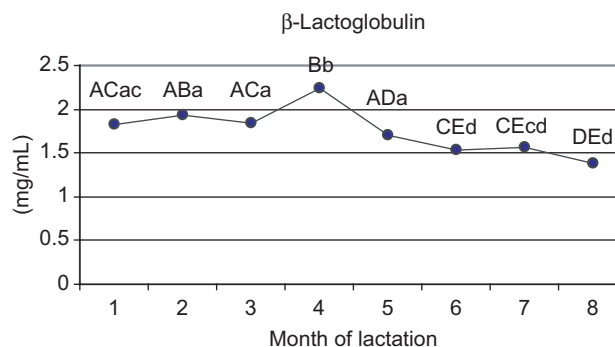


Figure 2. Effect of lactation stage on α -lactalbumin concentration in donkey's milk (MSE = 0.09). Different letters indicate significant differences (A, B, C: $P < 0.01$; a, b, c: $P < 0.05$).

The lowest LYS concentration was recorded at the month 8 ($0.47 \pm 0.03 \text{ mg mL}^{-1}$).

Effect of season

In Figure 5 the influence of the season on α -LA, β -LG, and LYS concentrations is shown. Overall, the highest

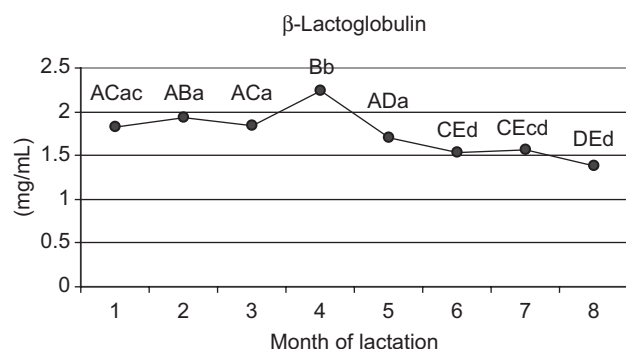


Figure 3. Effect of lactation stage on α -lactalbumin concentration in donkey's milk (MSE = 0.09). Different letters indicate significant differences (A, B, C, D: $P < 0.01$; a, b, c, d: $P < 0.05$).

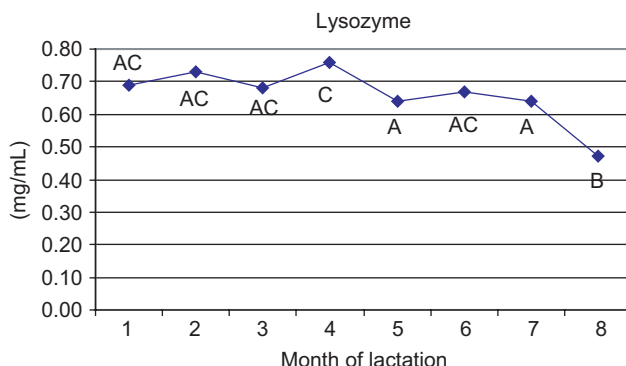


Figure 4. Effect of lactation stage on lysozyme concentration in donkey's milk (MSE = 0.03). Different letters indicate significant differences (A, B, C: $P < 0.01$).

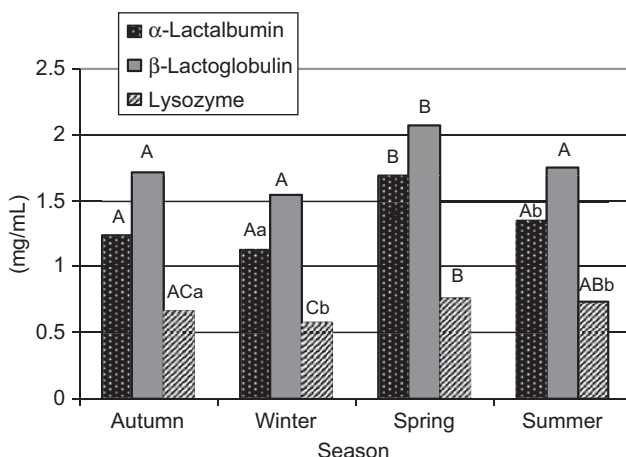


Figure 5. Effect of season on α -lactalbumin (MSE = 0.08), β -lactoglobulin (MSE = 0.09), and lysozyme (MSE = 0.03) concentration in donkey's milk. Different letters indicate significant differences (A, B, C: $P < 0.01$; a, b: $P < 0.05$).

concentrations for all the three whey proteins at study were recorded in spring. The highest α -LA content was observed in spring (mean $\pm$ SE: $1.69 \pm 0.08 \text{ mg mL}^{-1}$; $P < 0.01$). In summer it reached the level of $1.35 \pm 0.08 \text{ mg mL}^{-1}$, resulting higher in comparison to the value recorded during the winter ($1.13 \pm 0.08 \text{ mg mL}^{-1}$; $P < 0.05$) and similar to that of the autumn ($1.24 \pm 0.08 \text{ mg mL}^{-1}$; $P > 0.05$).

β -LG showed a trend similar to that of α -LA. In spring, it showed the highest concentration (mean $\pm$ SE: $2.07 \pm 0.09 \text{ mg mL}^{-1}$) with a significant difference ($P < 0.01$) compared to the other seasons. The lowest value of β -LG occurred in winter ($1.54 \pm 0.09 \text{ mg mL}^{-1}$) and no differences were found in comparison with summer ($1.75 \pm 0.09 \text{ mg mL}^{-1}$) and autumn ($1.71 \pm 0.09 \text{ mg mL}^{-1}$) contents.

Also LYS showed the lowest ($P < 0.01$) values in the winter—autumn period (0.57 – 0.66 mg mL^{-1}), while the highest concentration was recorded in the spring—summer period (0.76 – 0.74 mg mL^{-1}).

Discussion

It is well known that milk is the best food for the newborn of the mammalian species. It evolves with their nutritional requirement, from colostrum to milk, and changes its composition during lactation. In this respect, it could be stressed out that also donkey's milk production varies in terms of both quantitative and qualitative aspects.^(46–48) Therefore, the study of milk protein variations in this species is of particular interest for a developing industrial exploitation, also considering its increasing consumption in human nutrition mainly addressed to particular consumers as children with cow's milk protein allergy^(49–53) and elderly people.^(3,4) With special reference to aged people, donkey's milk administration in these subjects promoted a significant increase in interleukin (IL)-8 and IL-6 (an acute phase response mediator) and a moderate upregulation of serum levels of IL-1 β and tumor necrosis factor.⁽⁴⁾ In this framework, it should be mentioned that previous experiments of our group have demonstrated an *in vitro* modulating activity of colostrum and milk from donkey on normal human peripheral blood mononuclear cells.⁽³⁾ Both colostrum and milk were able to increase innate and adaptive immunity in terms of nitric oxide, cytokine and antibody production, thus affording potential protection to the host in *in vivo* conditions.

Donkey's milk is characterized by a low protein content, ranging from $1.36 \text{ g}/100 \text{ g}$ milk to $1.9 \text{ g}/100 \text{ g}$ ⁽⁴⁶⁾ with the whey protein content ranging from 0.49 to $0.80 \text{ g}/100 \text{ g}$ ⁽⁷⁾.

Owing to the biological and functional properties of α -LA, β -LA, and LYS^(24,25,31,38) and their major concentration in donkey's milk,⁽⁷⁾ in this study we have analyzed their content in relation to lactation stage and season. Overall, all these whey proteins are markedly influenced by both month of lactation and season.

Considering the effect of lactation, α -LA, β -LA, and LYS show a similar trend with higher values during the first half (4 months) of lactation period. This finding substantially agrees with that reported by Vincenzetti et al.⁽⁴⁴⁾ while it differs from the results of Guo et al.⁽⁷⁾ who found a more consistent trend during lactation. The different breeds and breeding conditions of the animals might explain such a discrepancy. In mares⁽⁵⁴⁾ and human milk,^(55,56) it was found that whey protein concentrations are very high early in lactation and decrease subsequently, while the concentration of milk casein increases.

Here, the highest α -LA concentration was recorded at the first month after foaling but without significant changes until the fourth month after which it decreases. Of note, in Ragusana donkey breed during 60–190 days lactation period, the highest α -LA value was found at the month 3.⁽⁴⁴⁾

The overall β -LG content was 1.75 mg mL⁻¹ on average, which results similar to that observed by Greppi et al.⁽⁵⁷⁾ (1.12 g L⁻¹) but much lower than that recorded in Ragusana donkey by Vincenzetti et al.⁽⁴⁴⁾ (3.80 mg mL⁻¹). This difference might be attributable to the different genotype of the animals. In our study, the β -LG concentration was also much lower than that reported in cow's milk (1.8–5.0 g L⁻¹).⁽⁵⁸⁾ All that assumes a considerable meaning in nutritional terms, taking into account that β -LG protein is absent in human milk,⁽⁶⁾ while in cow's milk it has been identified as the main allergen together with casein.^(59–61) β -LG as allergen is considered mainly important in infants and small children.^(49,62,63) Also the balance between casein and whey proteins may determine allergenicity of cow's milk.⁽⁶⁴⁾ Therefore, the low content in β -LG of Martina Franca donkey's milk found in this study might assume a considerable interest for its use in babies feeding, as maternal milk substitute, since it appears to be a hypoallergenic food. In fact, some recent studies have documented its better tolerance in children with cow's milk allergy.^(53,65)

Also the content of LYS in donkey's milk has to be evaluated in terms of side effects since it is present in traces in cow's as well as in goat's milk,^(26,66,67) while it is one of the major components of the human milk whey fraction.⁽⁵⁶⁾ None allergic responses have been detected to mammalian LYS in patients with cow's milk allergy.⁽⁶⁸⁾

The mean LYS content was 0.66 mg mL⁻¹. This value results to be very close to the content in human milk (0.50 mg mL⁻¹),⁽⁶⁷⁾ comparable to that found in other studies (0.50–0.76 g L⁻¹)^(58,69) but lower to that found in Ragusana milk (1.00 mg mL⁻¹).⁽⁴⁴⁾ In the present study, LYS shows the highest value at month 4 of lactation and a marked decreased at month 8. A more consistent trend for this protein was found by Guo et al.⁽⁷⁾ in Jiangyue donkey's milk. In conclusions, other and our own data demonstrate that all milk whey protein content varies among different breeds of donkey.

As far as the influence of production season on the milk protein fraction synthesis is concerned, spring affects the highest concentration of the all three whey

proteins at study. Autumn and even more winter affects the lowest values of α -LA, β -LA, and LYS. During spring, a more favorable availability of pasture for the animals bred under semi-extensive conditions could have influenced positively the milk content of the whey proteins. A different trend of protein content was observed in Ragusana donkey's milk.⁽⁴⁷⁾ In this study, the lowest percentage of protein corresponded to summer and autumn. In cow milk, no difference in relation to the hot season was reported on total whey fraction⁽⁷⁰⁾ as well as on α -LA and β -LG, in particular.⁽⁷¹⁾

Conclusions

In donkey's milk the content of the major whey proteins— α -LA, β -LA, and LYS—is affected by lactation stage and season. Overall, the best milk quality corresponds to the first 4 months of lactation, after that all the three whey proteins at study decrease consistently, reaching about 40% of their highest value at month 8. This indicates a decreasing capability of whey proteins synthesis by the mammary gland with advancing of lactation, and, therefore, in order to maintain a good nutritional quality of milk, it is useless to extend too much the lactation of jennies. The highest concentration of the major whey proteins found in spring leads to hypothesize that donkey's milk quality is affected by availability and/or quality of food. Moreover, the findings of this study outlines some differences among the studies carried out in other breeds. In particular, β -LG showed a lower concentration compared to Ragusana donkey breed.⁽⁴³⁾ Further researches to deepen the knowledge about the factors influencing the nutritional quality are important to better characterize donkey's milk and define the optimal breeding system for market development. Finally, the role of major whey proteins in the modulation of the immune response in allergic children to cow's milk and elderly will be object of investigation for a better understanding of the beneficial effects exerted by this aliment consumption in humans.

Acknowledgement

The authors thank Dr Marianna La Guardia for her support in the experimental work.

Declaration of interest

Paper supported by a grant from the University of Bari Aldo Moro (Bari, Italy).

References

1. Iacono, G., Carroccio, A., Cavataio, F., Montalto, G., Soresi, M., Balsamo, V. Use of ass' milk in multiple food allergy. *J. Pediatr. Gastroenterol. Nutr.* 1992, 14, 177–181.
2. Carroccio, A., Cavataio, F., Montalto, G., D'Amico, D., Alabrese, L., Iacono, G. Intolerance to hydrolysed cow's milk proteins in infants: clinical characteristics and dietary treatment. *Clin. Exp. Allergy* 2000, 30, 1597–1603.

3. Tafaro, A., Magrone, T., Jirillo, F., Martemucci, G., D'Alessandro, A.G., Amati, L., Jirillo, E. Immunological properties of donkey's milk: its potential use in the prevention of atherosclerosis. *Curr. Pharm. Des.* 2007, 13, 3711-3717.
4. Amati, L., Marzulli, G., Martulli, M., Tafaro, A., Jirillo, F., Pugliese, V., Martemucci, G., D'Alessandro, A.G., Jirillo, E. Donkey and goat milk intake and modulation of the human aged immune response. *Curr. Pharm. Des.* 2010, 16, 864-869.
5. Alais C. *Scienza del latte*. III Edition. Ed. Tecniche Nuove, Milan, Italy, 1984.
6. Malacarne, M., Martuzzi, F., Summer, A., Mariani, P. Protein and fat composition of mare's milk: some nutritional remarks with reference to human and cow's milk. *Int Dairy J* 2002, 12, 869-877.
7. Guo, H.Y., Pang, K., Zhang, X.Y., Zhao, L., Chen, S.W., Dong, M.L., Ren, F.Z. Composition, physicochemical properties, nitrogen fraction distribution, and amino acid profile of donkey milk. *J. Dairy Sci.* 2007, 90, 1635-1643.
8. Fantuz, F., Polidori, P., Maglieri, C., Varisco, G., Vincenzetti, S., Salimei, E. Ass's milk nitrogen distribution: effects of different dietary fibre source. In: *Proc 3rd European Workshop on Equine Nutrition*, Campobasso, Italy, 20-22 June, 2006, 179-180.
9. Herrouin, M., Mollé, D., Fauquant, J., Ballestra, F., Maubois, J.L., Léonil, J. New genetic variants identified in donkey's milk whey proteins. *J. Protein Chem.* 2000, 19, 105-115.
10. Moatsou, G., Hatzinaki, A., Samolada, M., Anifantakis, E. Major whey proteins in ovine and caprine acid wheys from indigenous greek breeds. *Int Dairy J* 2005, 15, 123-131.
11. Smithers GW. Whey and whey proteins - From "gutter-to-gold". *Int Dairy J* 2008;18:695-704.
12. Gill, H.S., Cross, M.L. Anticancer properties of bovine milk. *Br. J. Nutr.* 2000, 84 Suppl 1, S161-S166.
13. Parodi, P.W. A role for milk proteins in cancer prevention. *Australian J Dairy Technol* 1998, 53, 37-47.
14. Clare, D.A., Catignani, G.L., Swaisgood, H.E. Biodefense properties of milk: the role of antimicrobial proteins and peptides. *Curr. Pharm. Des.* 2003, 9, 1239-1255.
15. Hall, W.L., Millward, D.J., Long, S.J., Morgan, L.M. Casein and whey exert different effects on plasma amino acid profiles, gastrointestinal hormone secretion and appetite. *Br. J. Nutr.* 2003, 89, 239-248.
16. Conti, A., Napolitano, L., Lai, P., Pinna, W., Godovac-Zimmermann, J. Isolation of donkey whey proteins and N-terminal amino acid sequence of α -lactalbumins A and B, b-lactoglobulins I and II and lysozyme. *Milchwissenschaft* 1989, 44, 138-141.
17. Paglarini, E., Solaroli, G., Peri, C. Chemical and physical characteristics of mare's milk. *Ital J Food Sci* 1993, 1, 323-332.
18. Pérez, M.D., Calvo, M. Interaction of beta-lactoglobulin with retinol and fatty acids and its role as a possible biological function for this protein: a review. *J. Dairy Sci.* 1995, 78, 978-988.
19. Doreau, M., Gaillard, J.L., Chobert, J.M., Léonil, J., Egito, A.S., Haertlé, T. Composizione in acidi grassi ed in proteine del latte di cavalla e di asina. Implicazioni per l'utilizzazione del latte. In: *Proc 4th Cong S.I.D.I., Campobasso, Italy, July 11-13, 2002*, 51-71.
20. Godovac-Zimmermann, J., Shaw, D., Conti, A., McKenzie, H. Identification and the primary structure of equine alpha-lactalbumin B and C (*Equus caballus*, *Perissodactyla*). *Biol. Chem. Hoppe-Seyler* 1987, 368, 427-433.
21. Madureira, A.R., Pereira, C., Gomes, A.M.P., Pintado, M.E., Xavier, M.C., F. Bovine whey proteins - Overview on their main biological properties. *Food Research International*, 2007, 40, 1197-1211.
22. de Wit, J.N. Marschall Rhône-Poulenc Award Lecture. Nutritional and functional characteristics of whey proteins in food products. *J. Dairy Sci.* 1998, 81, 597-608.
23. Ganjam, L.S., Thornton, W.H. Jr, Marshall, R.T., MacDonald, R.S. Antiproliferative effects of yogurt fractions obtained by membrane dialysis on cultured mammalian intestinal cells. *J. Dairy Sci.* 1997, 80, 2325-2329.
24. Sternhagen, L.G., Allen, J.C. Growth rates of a human colon adenocarcinoma cell line are regulated by the milk protein alpha-lactalbumin. *Adv. Exp. Med. Biol.* 2001, 501, 115-120.
25. Clare, D.A., Swaisgood, H.E. Bioactive milk peptides: a prospectus. *J. Dairy Sci.* 2000, 83, 1187-1195.
26. Park, Y.W., Haenlein, Y.W. *Handbook of milk of non-bovine mammals*. Ames, Iowa, USA: Blackwell Publishing, 2006.
27. Sélo, G., Clément, H., Bernard, J.-M., Chatel, C., Créminon, C., Peltre, G. Allergy to bovine β -lactoglobulin: specificity of human IgE to tryptic peptides. *Clin Exp Allergy* 1999, 29, 1055-1063.
28. Fessas, D., Iametti, S., Schiraldi, A., Bonomi, F. Thermal unfolding of monomeric and dimeric beta-lactoglobulins. *Eur. J. Biochem.* 2001, 268, 5439-5448.
29. Godovac-Zimmermann, J., Conti, A., Sheil, M., Napolitano, L. Covalent structure of the minor monomeric beta-lactoglobulin II component from donkey milk. *Biol. Chem. Hoppe-Seyler* 1990, 371, 871-879.
30. Godovac-Zimmermann, J., Conti, A., Sheil, M., Napolitano, L. Covalent structure of the minor monomeric beta-lactoglobulin II component from donkey milk. *Biol Chem Hoppe-Seyler* 1990, 369, 171-179.
31. Liu, H.C., Chen, W.L., Mao, S.J. Antioxidant nature of bovine milk beta-lactoglobulin. *J. Dairy Sci.* 2007, 90, 547-555.
32. McIntosh, G.H., Regester, G.O., Le Leu, R.K., Royle, P.J., Smithers, G.W. Dairy proteins protect against dimethylhydrazine-induced intestinal cancers in rats. *J. Nutr.* 1995, 125, 809-816.
33. Farrell, H.M. Jr, Behe, M.J., Enyeart, J.A. Binding of p-nitrophenyl phosphate and other aromatic compounds by beta-lactoglobulin. *J. Dairy Sci.* 1987, 70, 252-258.
34. McLeod, A., Fedio, W., Chu, L., Ozimek, L. Binding of retinoic acid to β -lactoglobulin variants A and B: effect of hepatic and tryptic digestion on the protein-ligand complex. *Milchwissenschaft* 1996, 51, 3-7.
35. Osserman, E.F., Lawlor, D.P. Serum and urinary lysozyme (muramidase) in monocytic and monomyelocytic leukemia. *J. Exp. Med.* 1966, 124, 921-952.
36. Gordon, S., Todd, J., Cohn, Z.A. In vitro synthesis and secretion of lysozyme by mononuclear phagocytes. *J. Exp. Med.* 1974, 139, 1228-1248.
37. Unanue, E.R. Secretory function of mononuclear phagocytes: a review. *Am. J. Pathol.* 1976, 83, 396-418.
38. Vannini, L., Lanciotti, R., Baldi, D., Guerzoni, M.E. Interactions between high pressure homogenization and antimicrobial activity of lysozyme and lactoperoxidase. *Int. J. Food Microbiol.* 2004, 94, 123-135.
39. Wever, R., Kast, W.M., Kasinodien, J.H., Boelens, R. The peroxidation of thiocyanate catalysed by myeloperoxidase and lactoperoxidase. *Biochim. Biophys. Acta* 1982, 709, 212-219.
40. Welsh, J.K., May, J.T. Anti-infective properties of breast milk. *J. Pediatr.* 1979, 94, 1-9.
41. D'Alessandro, A.G., Martemucci, G. Influence of milking number and frequency on milk production in Martina Franca breed asses. In: *Proc XVII Nat Cong ASPA, Alghero, Italy, 29th May - 1st June, 2007*, 643-645.
42. D'Alessandro, A.G., De Petro, R., Claps, S., Pizzillo, M., Martemucci, G. Yield and quality of milk and udder health in Martina Franca ass: effects of daily interval and time of machine milking. In: *Proc XVIII Nat Cong ASPA, Palermo, Italy, June 9-12, 2009*, 697-699.
43. Aschaffenburg, R., Drewry, J. New procedure for the routine determination of the various non-casein proteins of milk. In: *Proceedings of the 15th International Dairy Congress, London, United Kingdom, 1959*, 3, 1631-1637.
44. Vincenzetti, S., Polidori, P., Mariani, P., Cammertoni, N., Fantuz, F., Vita, A. Donkey's milk protein fractions characterization. *Food Chemistry* 2008, 106, 640-649.
45. SAS. *User's Guide Statistics*. Version 8.2. SAS.Institute, Inc. Cary, NC, USA, 2000.
46. Salimei, E., Chiofalo, B. Asses: milk yield and composition. In: N. Miraglia and W. Martin-Rosset, ed. *Nutrition and feeding of the broodmare*, Wageningen Academy Press, Wageningen, The Netherlands, 2006, 117-132.

47. Giosuè, C., Alabiso, M., Russo, G., Alicata, M.L., Torrisi, C. Jennet milk production during the lactation in a Sicilian farming system. *Animal* 2008, 2, 1491-1495.
48. D'Alessandro, A.G., Tesse, R., Montagna, C., De Leo, V., Armenio, L., Martemucci, G. Production of donkey milk for human feeding: changes of gross composition and energetic value during lactation. In: *Proc IV Int Symposium of Livestock Production*, Struga, Republic of Macedonia, September 9-11, 2009, 236.
49. Businco, L., Giampietro, P.G., Lucenti, P., Lucaroni, F., Pini, C., Di Felice, G., Iacovacci, P., Curadi, C., Orlandi, M. Allergenicity of mare's milk in children with cow's milk allergy. *J. Allergy Clin. Immunol.* 2000, 105, 1031-1034.
50. Monti, G., Bertino, E., Muratore, M.C., Coscia, A., Cresi, F., Silvestro, L., Fabris, C., Fortunato, D., Giuffrida, M.G., Conti, A. Efficacy of donkey's milk in treating highly problematic cow's milk allergic children: an in vivo and in vitro study. *Pediatr. Allergy Immunol.* 2007, 18, 258-264.
51. Vita, D., Passalacqua, G., Di Pasquale, G., Caminiti, L., Crisafulli, G., Rulli, I., Pajno, G.B. Ass's milk in children with atopic dermatitis and cow's milk allergy: crossover comparison with goat's milk. *Pediatr. Allergy Immunol.* 2007, 18, 594-598.
52. Alessandri, C., Mari, A. Efficacy of donkey's milk in treating cow's milk allergic children: major concerns. *Pediatr. Allergy Immunol.* 2007, 18, 625-626.
53. Tesse, R., Paglialunga, C., Braccio, S., Armenio, L. Adequacy and tolerance to ass's milk in an Italian cohort of children with cow's milk allergy. *Ital. J. Pediatr.* 2009, 35, 19.
54. Csapò-Kiss, Zs., Stefler, J., Martin, T.G., Makray, S., Csapò, J. Composition of mares' colostrum and milk. Protein content, amino acid composition and contents of macro- and micro-elements. *Int Dairy J.* 1995, 5, 403-415.
55. Kunz, C., Lönnerdal, B. Re-evaluation of the whey protein/casein ratio of human milk. *Acta Paediatr.* 1992, 81, 107-112.
56. Lönnerdal, B. Nutritional and physiologic significance of human milk proteins. *Am. J. Clin. Nutr.* 2003, 77, 1537S-1543S.
57. Greppi, G.F., Iametti, S., Bonomi, F., Sessa, F., Feligni, M., Enne, G. Minor whey components in horse and donkey milk. In: *Proc XXXI Int Symposium of the Società Italiana per il Progresso della Zootecnia*, Milano, Italy, 13 settembre, 1996, 149-154.
58. Butler, J.E. Immunoglobulins of mammary secretion. Lactation. Ed. Larson and Smith: Academic Press, NY, USA, 1974, 217-256.
59. Goldman, A.S., Anderson, D.W. Jr, Sellers, W.A., Saperstein, S., Kniker, W.T., Halpern, S.R. Milk allergy. i. oral challenge with milk and isolated milk proteins in allergic children. *Pediatrics* 1963, 32, 425-443.
60. Docena, G.H., Fernandez, R., Chirido, F.G., Fossati, C.A. Identification of casein as the major allergenic and antigenic protein of cow's milk. *Allergy* 1996, 51, 412-416.
61. Järvinen, K.M., Chatchatee, P., Bardina, L., Beyer, K., Sampson, H.A. IgE and IgG binding epitopes on alpha-lactalbumin and beta-lactoglobulin in cow's milk allergy. *Int. Arch. Allergy Immunol.* 2001, 126, 111-118.
62. Businco, L., Bellanti, J. Food allergy in childhood. Hypersensitivity to cows' milk allergens. *Clin. Exp. Allergy* 1993, 23, 481-483.
63. Carroccio, A., Cavataio, F., Iacono, G. Cross-reactivity between milk proteins of different animals. *Clin. Exp. Allergy* 1999, 29, 1014-1016.
64. Lara-Villoslada, F., Olivares, M., Xaus, J. The balance between caseins and whey proteins in cow's milk determines its allergenicity. *J. Dairy Sci.* 2005, 88, 1654-1660.
65. Paglialunga, C., Tesse, R., D'Alessandro, A.G., Rizzi, D., Martemucci, G., Armenio, L. Efficacia del latte di asina nel trattamento di bambini con allergia alle proteine del latte vaccino: un'esperienza pugliese. In: *Proc 10° Nat Congr of the Società Italiana di Allergologia ed Immunologia Pediatrica*. Salsomaggiore, Italy, September 3-5, 2008, 344.
66. Solaroli, G., Pagliarini, E., Peri, C. Composition and nutritional quality of mare's milk. *Ital J Food Sci* 1993, 1:3-10.
67. Miranda, G., Mahé, M.F., Leroux, C., Martin, P. Proteomic tools to characterize the protein fraction of Equidae milk. *Proteomics* 2004, 4, 2496-2509.
68. Aalberse, R.C., Stapel, S.O. Structure of food allergens in relation to allergenicity. *Pediatr. Allergy Immunol.* 2001, 12 Suppl 14, 10-14.
69. Faccia, M., Pinto, F., Veronico, M., Liuzzi, V.A. Il lisozima nel latte di cavalle di razza Murgesa e Agricola Italiana t.p.r. e di asine di Martina Franca. In: *Proc 3rd Cong SIDI*, Campobasso, Italy, July 12-14, 2001, 149-154.
70. Muroya, S., Terada, F., Shioya, S. Influence of heat stress on distribution of nitrogen in milk. *Anim Sci Technol* 1997, 68, 297-300.
71. Bernabucci, U., Lacetera, N., Ronchi, B., Nardone, A. Effects of the hot season on milk protein fractions in Holstein cows. *Anim Res* 2002, 51, 25-33.

Copyright of Immunopharmacology & Immunotoxicology is the property of Taylor & Francis Ltd and its content may not be copied or emailed to multiple sites or posted to a listserv without the copyright holder's express written permission. However, users may print, download, or email articles for individual use.