

Effect of farming system on donkey milk composition

Emanuela Valle^a Luisa Pozzo^b, Marzia Giribaldi^c, Domenico Bergero ¹, Maria Silvia Gennero^d, Daniela Dezzutto^d, Amy McLean^e, Giorgio Borreani^f, Mauro Coppa ^f, Laura Cavallarin^c.

^a Dipartimento di Scienze Veterinarie, Università di Torino, Largo P. Braccini 2, 10095 Grugliasco (Turin), Italy

^b Istituto di Biologia e Biotecnologia Agraria, Consiglio Nazionale delle Ricerche, Via Moruzzi 1, 56124 Pisa, Italy

^c Istituto di Scienze delle Produzioni Alimentari, Consiglio Nazionale delle Ricerche, Largo P. Braccini 2, 10095 Grugliasco (Turin), Italy

^d Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta, Via Bologna 148, Turin, Italy.

^e UC Davis, University of California at Davis, Davis 95616.

^f Dipartimento di Scienze Agrarie, Forestali e Alimentari, Università di Torino, Largo P. Braccini 2, 10095 Grugliasco (Turin), Italy.

Corresponding author. Laura Cavallarin. E-mail: laura.cavallarin@ispa.cnr.it

Short title: Farming system affects the composition of donkey milk

ABSTRACT

BACKGROUND: Donkey milk is considered a functional food for sensitive consumers, such as children allergic to cow milk. No information is available regarding the effect of farming systems on the quality of donkey milk. The study was aimed at evaluating the

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/jsfa.8777

effect of the farming system and lactation stage on gross composition, fat-soluble vitamins and fatty acids (FA) in donkey milk.

RESULTS: Individual milk samples were collected from lactating jennies (n=53) on the six of the largest farms located in North West Italy. The performance of lactating jennies, the herd characteristics, and feeding strategies were recorded at each milk sampling. The gross composition of the milk along with the fat-soluble vitamin content differed according to the farming system. The lactation stage had limited effects on milk quality. A higher milk fat content corresponded to a higher amount of fresh herbage proportion in the diet, with the highest polyunsaturated (PUFA) FA, C18:1c9, C18:3n-3, n-3 FA, retinol and α -tocopherol content and the lowest concentrations of the FA less favorable for human health in the milk of animals fed on only forage diets.

CONCLUSIONS: Extensive farming of dairy donkeys increased fat content and fat-soluble vitamin concentration of milk and altered the FA composition to a more favorable profile for human nutrition.

Keywords: *Equus asinus*; Donkey milk; Feeding system; Fatty acids; Vitamins.

INTRODUCTION

Donkey milk consumption is widespread in the Mediterranean area and, the EU production is estimated to be about 300 tons per year (Eurolactis, 2016, personal communication). In the last 10 years, breeding donkeys for milk production has increased, along with the number of published research papers on the use of donkey milk.¹ Dairy donkey farms in the EU are mainly located in Italy, France, Spain and Belgium.² Clinical studies have indicated that donkey milk can be used successfully as an alternative to the available hypoallergenic formulas for infants suffering from cow milk protein allergy.³ It has recently been demonstrated *in vivo* that dietary supplementation with donkey and human milk is associated with a decrease in inflammatory status, and this decrease is in turn associated with an improvement in the lipid and glucose metabolism, compared to a diet with a cow milk supplementation.⁴ The composition of donkey and human milk are similar, in terms of average total solid, crude protein, lactose and ash content. However, the fat content of donkey milk is lower than the fat content of human milk, as it is in the 0.03 to 0.12 g L⁻¹ range. This difference is associated with low energy content^{5,6} a main limit to its use as a nutritional source for children allergic to cow milk protein, during the first year of life. However, the lipid fraction of donkey milk has shown a more favorable fatty acid (FA) composition than that of the milk fat of ruminants, as it is richer in polyunsaturated FA (PUFA).⁶ Fat in donkey milk has shown a higher concentration of C18:3n-3 and n-3 FA, and a lower saturated FA (SFA) content than cow milk, as well as a lower n-6 to n-3 FA ratio.⁶ On the other hand, equid milk appears to have a lower fat-soluble vitamins content (α -tocopherol and retinol), than ruminant milk.^{7,8} The variables that are significantly associated with changes in donkey milk composition are (1) the lactation

stage; (2) daily rhythms; and (3) the interval between mechanical milkings.² However, feeding is also believed to play a relevant role in milk yield and composition, since nutrient absorption in equines precedes the ceco-colic fermentations of feeds.⁹ The composition of the diet has been shown to be the main factor that affects the FA composition of milk in ruminants.^{10,11} In particular, it is known that pasture feeding increases in cow's milk the concentrations of FA that are more favorable for human nutrition, such as C18:3n-3, n-3 FA and conjugated linoleic acids (CLA). Pasture feeding also decreases the n-6 to n-3 ratio and the concentrations of FA less favorable for human nutrition, such as C14:0, C16:0, and SFA.^{12, 13} However, the effect of the diet composition on donkey milk characteristic has only been studied so far in experimental conditions for a restricted group of FA,¹⁴ and no information is available regarding the effect of the diet on the FA composition of donkey milk according to the feeding strategies adopted on commercial farms. Furthermore, changes in donkey milk fat-soluble vitamins, because of the feeding system, have never been investigated. The aim of this study was to evaluate the effect of the farming system and lactation stage on milk composition of dairy asses, based on observational data collected during a survey on six commercial farms located in North-West Italy.

EXPERIMENTAL

Milk Sampling and Survey

Individual milk samples were collected (0.5 L) from 53 lactating jennies reared on the six biggest commercial farms located in the Piedmont Region, in North West Italy, during Spring of 2014. All milking jennies were sampled per farm. All samples were collected from jennies by hand milking. Performance and herd characteristics of all lactating jennies were recorded. Herd demographics included: number of jennies, breed,

milk yield, body condition scores (BCS), milking management (milking system and timing were similar in all the studied farms), feeding strategies, forage type and conservation methods adopted were recorded at each milk sampling and characterized through a detailed on farm survey. The BCS were determined as described by Burden¹⁵ and body weight according to Pearson and Ouassat.¹⁶ The farm characteristics, herd composition and diets of the jennies are reported in Table 1. The milk samples were immediately refrigerated, stored at -20°C and lyophilized within 72 h. The lyophilized samples were then stored at -20°C.

Milk Gross Composition Analyses

The donkey milk samples were analyzed for fat, proteins, lactose and total solids content. The fat, protein, and total solids were assessed as described by Cavallarin et al.¹⁷ The lactose content was determined by means of spectrophotometric absorbance at 340 nm (Cary 60 UV-Vis, Agilent Technologies, Santa Clara, CA), according to the AOAC 984.15 Official Method (2005).

Milk Fat-soluble Vitamin Analysis

Retinol and α -tocopherol in the milk samples were extracted according to the Kuhl et al.¹⁸, with the following adaptations. The retinol and α -tocopherol concentrations were quantified according to Prola et al.¹⁹ by means of a HPLC system (Dionex, Sunnyvale, CA, USA). The analytical column was an XTerra RP18 column (250-mm $\times$ 4.6-mm, 5 μ m particles) (Waters, Milford, MA).

A calibration curve was obtained with two determinations of six concentration levels of α -tocopherol and retinol standard solutions (Sigma-Aldrich, St. Louis, MO) between 0.07 and 10 μ g L⁻¹. The linearity was excellent ($R^2 = 0.999$). Recovery experiments were performed by spiking blank donkey milk samples with retinol and with α -tocopherol. The recoveries of the method were good, ranging from 91.1% to 96.8%.

The average recovery was 93.2% (standard deviation 3.16) for retinol and 90.7 (standard deviation 13.4) for α -tocopherol and the average relative standard deviations were 3.39 and 14.8%, respectively.

Milk Fatty Acid Analysis

Milk samples were analyzed for FA composition by gas chromatography (GC), as described by Coppa et al.²⁰ The method was adapted to donkey milk, because of the lower lipid content and its larger variation in donkey milk than in cow milk. The lipids in 0.7 g of the lyophilized milk samples were methylated directly using 4 mL of 0.5 M sodium methanolate plus 1.5 mL of hexane for 15 min at 50°C. This was followed, after cooling, by the addition of 2 mL of 12 M HCl at 50°C for 15 min. Six mL of 6% K₂CO₃ water solution was added after cooling. The FA methyl esters were separated as a supernatant after centrifugation and injected into a GC equipped with a flame ionization detector, separating and identifying the FA methyl esters as described by Coppa et al,²⁰ with the sole adaptation of the split ratio to the lower fat content of donkey milk. A volume of 1 μ L was injected into the column at a split ratio ranging from 2.5:1 to 100:1, according to the fat content of the sample. The atherogenic and thrombogenic indexes were calculated as follows: $AI=(C12:0+4\times C14:0+C16:0)/[\Sigma MUFA+\Sigma(n-6)+\Sigma(n-3)]$; $TI=(C14:0+C16:0+C18:0)/[0.5\times \Sigma MUFA+0.5\times \Sigma(n-6)+3\times \Sigma(n-3)+\Sigma(n-3)/\Sigma(n-6)]$ ²¹

Statistics

Statistical analyses were performed using the SPSS for Windows software package (version 17.0; SPSS Inc., Chicago, IL). The milk composition data were processed using the general linear model (GLM) of ANOVA, in which the farm was the fixed factor and the lactation stage (DIM) was the covariate. Fisher's F-distribution of the two variables included in a model was used as an indicator of the relative weight of the variables in determining the model itself. The Bonferroni test was used as the *post-hoc*

test. To have an overview of the differences of milk FA profile among farms, a principal components analysis (PCA) was performed on the main FA: the sum of even chain-saturated FA (ECSFA); the odd chain-FA (OCFA) to branched chain-FA (BCFA) ratio; the sum of monounsaturated FA (MUFA); the sum of PUFA; the sum of n-3 FA; the sum of n-6 FA to sum of n-3 FA ratio; the sum of de novo synthesis FA (even-chain SFA from C4:0 to C14:0); the sum of CLA isomers; and the Atherogenicity and Trombogenicity indexes.

RESULTS

Milk gross composition and fat-soluble vitamin content

The fat-soluble vitamin content of donkey milk differed significantly for all parameters over the different farms (Table 2), with the exception of lactose concentration. The highest protein content was found in milk collected on Farm 5, while the highest fat content was found in milk from Farm 4. Only the protein content was affected by the lactation stage, with the highest protein content corresponding to the beginning of the lactation period (Table 2). However, Fischer's F for the farm effect was far higher for the farm effect than for the DIM (Table 4).

The retinol content was within the 0.089 to 0.466 $\mu\text{g L}^{-1}$ range, and α -tocopherol was within the 0.214 to 3.840 $\mu\text{g L}^{-1}$ range. A farm effect was seen for both vitamins, with the highest levels being found in the milk on Farm 3 (Table 2).

Milk fatty acid composition

The FA composition of the donkey milk differed significantly over the farms (Table 3). The milk from Farm 3 showed the highest concentrations of C18:1c9, total C18:1cis isomers, C22:5n-3, CLA c9t11 and total CLA, and the lowest concentrations of C8:0,

C12:0, C14:0, total *de novo* synthesis FA, and ECSFA. The highest concentration of C18:3n-3, PUFA, and n-3 FA and the lowest value of the Atherogenicity and Thrombogenicity indexes were observed in the milk from Farms 3, 4 and 5. The OCFA and BCFA concentrations were highest in the milk from Farm 2 and the lowest in the milk from Farms 3 and 6, with intermediate values in the milk from Farms 4 and 5 for BCFA. The OCFA/BCFA ratio showed the lowest value in the milk from Farm 2 and the highest in the milk from Farms 3 and 4.

Only a few FA were affected to a great extent by DIM. An increase in the concentrations of C14:1c9, C15:0, isoC16:0, C17:0, C18:1t11, C18:2c9t12, C18:2n-6, C18:3n-3, C22:0, C20:3n-3+C22:1c13, OCFA, PUFA, total C18:1trans isomers and n-3 FA increased with increasing DIM, whereas the concentrations of C8:0, C20:4n-6, then-6/n-3 ratio and the Trombogenicity Index decreased with increasing DIM. However, Fischer's F for those FA that showed a significant effect of both DIM and farm were far higher for the farm effect than for the DIM (Table 4).

The results of the PCA performed on the main FA concentrations are given in Fig. 1. The PCA separated samples according to the farm in which milk was produced on both the first principal component (PC1) and the second PC (PC2) (Figure 1). The milk samples from Farm 3 were clearly separated from those of the other farms on PC1, whereas the samples from Farm 4 was separated for Farm 3 and from Farms 1, 2 and 6 on both PC1 and PC2. The samples from Farm 5 were in an intermediate position between those from Farm 4 and from Farms 1, 2, and 6, which were not separated by the PCA (Figure 1). The first principal component (PC 1, 46.4% of variance) was positively and closely correlated to ECSFA, the total *de novo* synthesis FA, the Atherogenicity index and the Trombogenicity index (correlation coefficients > 0.88), while PUFA, n-3FA and total CLA were negatively correlated to PC1 (correlation

coefficients < -0.76). The PC2 (33.6% of variance) was highly positively correlated with C16:0, C18:1c9 and MUFA (correlation coefficients > 0.80) and negatively correlated with n-3 FA, total *de novo* synthesis FA and PUFA (correlation coefficients < -0.53). The n-6/n-3 ration and the OCFA/BCFA texture also made significant and positive contribution to PC2 and negative contribution to PC1, respectively (correlation coefficients > 0.51 and < -0.46).

DISCUSSION

Effect of Lactation Stage on Donkey Milk Gross Composition

The mean protein content of milk observed in the present study is in accordance with previous data reported for donkey milk in Italy.^{5,17} The decrease in the protein content of the donkey milk during lactation is in agreement with the findings of Salimei et al.⁵ Giosuè et al.,²² Salimei and Fantuz² who reported overall values ranging from a maximum of 2.1 g/100 mL, at the beginning of lactation, to a minimum of 1.6 g/100 mL in late lactation.klh,

Effect of Lactation Stage on the Fatty Acid Composition of Donkey Milk

The effect of lactation on the FA composition of donkey milk was studied by Martemucci and D'Alessandro,²³ Gubić et al.²⁴ and Martini et al.²⁵ These authors highlighted an increase in concentration of long chain FA and a decrease in concentrations of short chain FA from *de novo* synthesis in the mammary gland, with the development of the lactation stage. These results are in agreement with the significant increase observed for several long-chain FA during lactation in the present study, even if the differences found in literature in donkey milk FA composition during lactation were larger than those observed in the present study. However, the aforementioned studies followed the evolution of the

FA composition of milk collected from individual animals throughout the entire lactation period in controlled condition and with a constant diet.^{23,25} On the other hand, the effect of animal related factors, such as breed and lactation stage, are known to have a negligible effect on the FA composition of milk in dairy cows on farms, compared to animal diet.²⁰ The results of the present study have shown a greater effect on milk FA of the farming system, with a limited effect of the lactation stage, which is pointed out by the higher ANOVA Fisher's F coefficients for the farm effect than for DIM.

Effect of farming system on the gross composition of milk

To the best of our knowledge, the effect of the farming system on donkey milk quality has never been studied before. The higher content of fat in the milk collected on Farm 3 and 4 could be associated to a higher pasture proportion in the diet than on the other farms. In addition, the hay sampled on Farm 4 in two different periods (data not shown) resulted to be of high quality, in terms of protein and ADF content. This indicates that forage quality could have played an important role in the fat concentration of donkey milk. On the other hand, it is well known that, in ruminants, genetics may also account for differences in protein and fat content of milk.¹⁰ No evidence is available for the equine species. However, it can be hypothesized that the higher content of milk protein from Farm 2 and 5 could also depend on the fact that a homogenous breed is reared on these farms (Martina Franca and Ragusana, respectively), unlike the other farms, where crossbreeds animal were found. It can be speculated that genetics and dietary factors interactions may determine donkey milk gross composition.

Effect of farming system on the fat-soluble vitamin content of the milk

Little is known about the fat-soluble vitamin content in donkey milk. Gentili et al.⁷ and Claeys et al.²⁶ reported the average contents of α -tocopherol and retinol in donkey milk, and compared them with milk from other species. However, the variations in fat-soluble vitamins in donkey milk reared under different farming system have never been studied before. Differences between breeds in fat soluble vitamins transfer efficiency from feedstuffs to milk were observed for ruminants,²⁷ thus a possible breed effect cannot be excluded also for donkeys. However, Álvarez et al.⁸ reported a concentration of retinol in milk from mares grazing from a pasture was double that reported by other authors for mares fed hay.¹⁸ Similarly, the amount of α -tocopherol and retinol in cow milk was shown to approximately double when cows were fed pasture instead of conserved forages.²⁷ Pro-vitamin carotenoids originate from β -carotene through enzymatic oxidation. As β -carotene is highly sensitive to ultraviolet light, it is degraded into forages during herbage wilting in the field, and this results in the hay having lower β -carotene contents than the fresh herbage or hay.²⁷ Thus, the higher concentrations of α -tocopherol and retinol in the milk from Farm 3 than in milk from the other farms are coherent with the high proportion of fresh herbage in the donkey diet.

Effect of farming system on the fatty acid composition of the milk

The present results are the first evidence suggesting FA profile maybe effected by the farming system found at commercial donkey dairy farms. The effect of farming systems on milk FA composition have been widely studied for ruminants, both in controlled and on farm experiments.^{20,28} Breed along with farming system can affect the concentration of some FA in ruminant milk. Breed was shown to effect the FA concentration in relationship to the Δ -9 desaturase activity in the mammary gland (i.e. C14:1c9, C16:1c9

and C18:1c9).^{28,29} However, the breed effect resulted negligible when operating on commercial farms, where the feeding system was the most important factor of variation.²⁰ Similarly, in our study the PCA based on donkey milk FA composition grouped together samples from farms applying similar feeding strategies, but rearing different breeds. However, samples from the Martina Franca or Ragusana breeds were not separated from the other breeds reared in similar conditions. Indeed, a great effect of feeding composition was demonstrated on donkey milk FA composition in a controlled experiment on the total concentration of main groups of FA (i.e SFA, MUFA, PUFA)¹⁴. Thus, a relevant effect of feeding system on farm can be hypothesized. Consequently, our study points out the probable influence of animal diets on the FA profile of donkey milk. The milk collected in Farm 3 was clearly separated by the PCA from those of the other farms and showed the highest concentration of FA that are favorable for human nutrition, such as C18:1c9, C18:3n-3, n-3 FA and PUFA, and the lowest concentration of FA less favorable for human health, such as ECSFA, and *de novo* synthesis FA.²⁶ An important factor that can coherently explain FA pattern of milk from Farm 3 is related to the diets, which were exclusively constituted by fresh forage from pastures. The higher concentration of C18:3n-3, compared to that in the milk from the other farms, could be derived from a direct transfer of this FA from the ingested pasture¹⁴, as C18:3n-3 is the most abundant FA in fresh herbage.²⁰ A higher transfer of C18:3n-3 in the milk of equids than that of ruminants is allowed by the lack of biohydrogenation²⁶, which conversely occurs for most of the ingested long-chain PUFA in ruminants.¹⁰ The C18:3n-3 has been shown to be a valuable indicator of pasture feeding for dairy cows^{13, 30}, and its concentration has been shown to increase with increasing fresh herbage proportions in cow diets.¹² The increase in the C18:3n-3 concentration in donkey milk, with increasing proportions of fresh herbage in the diet, is also consistent with the

intermediate concentration of this FA in the milk from Farms 4 and 5, which had 50 and 40% of fresh herbage in the diets, respectively. For the same reason the milk from the farm has an intermediate position between those of Farm 3 and those of Farm 1, 2 and 6 in the PCA.

The higher C18:3n-3, C22:3n-3 and PUFA proportions in the donkey milk for Farm 3, due to the full grazing diet, could also have partially inhibited the *de novo* synthesis process in the mammary gland^{10,26} thus resulting in lower concentrations in the milk of C8:0, C10:0, C12:0, C14:0, total *de novo* synthesis FA, and ECSFA. A lower concentration of SFA in the milk from donkeys fed fresh herbage than those from donkeys fed hay was also observed by Chiofalo et al.¹⁴ as observed for ruminants.¹⁰ Small concentrations of CLAc9t11 have been observed in horse milk, but have never been detected in donkey milk.^{31,6} However, the same authors reported concentrations of C18:1t11 in donkey milk. This FA is known to be the substrate for CLAc9t11 desaturation by Δ^9 -desaturase activity in the mammary gland in ruminants¹⁰, and to be responsible for the desaturation in the mammary gland of donkey²³, thus suggesting a possible similar origin in donkey milk. The CLAc9t11 in ruminants can also originate from dietary C18:2n-6 biohydrogenation by *Butyrivibrio* sp. bacteria, as well as C18:1t11 from C18:3n-3.³² *Butyrivibrio* sp. bacteria were also identified as main components of equine gastrointestinal compartments.^{33, 34} This would seem to suggest that a small part of ingested C18:3n-3 may have been biohydrogenated, by these bacteria to C18:1t11, which could have been desaturated to CLAc9t11 in the mammary gland. This hypothesis also seems to be supported by the higher concentrations of both C18:1t11 and CLAc9t11 in the milk of Farm 3, in which the donkeys were fed at pasture. The sum of CLA clearly separated the milk sample from Farm 3 compared to

the other farms on the PCA. In fact, C18:1t11 and CLAc9t11 have been identified as indicators of pasture proportion in cow diets for ruminants.^{30,12}

The variations in OCFA and BCFA in donkey milk, according to the feeding system, are more difficult to interpret, as little is known about the mechanism that determines their concentration for equines. Only Devle et al.³¹ and Medhammar et al.⁶ reported the average OCFA and BCFA concentrations in donkey milk. The OCFA and BCFA in the milk of ruminants are mainly derived from the lipid membrane of ruminal bacteria.³⁵ Their concentration in cow milk varies according to the shift in ruminal population due to the changes in ruminal substrate, as a function of different diets.³⁵

In particular, forage-based diets favor the cellulolytic bacteria population in rumen, and determine an increase in BCFA in milk.^{35,11} On the other hand, the substitution of hay or pasture with corn silage or cereal based-concentrates, which are rich in starch, favor the rumen population of amylolytic bacteria, with a resultant increase in the milk concentration of OCFA and OCFA/BCFA ratio.³⁶ In addition, the concentration of BCFA in cow milk has also been negatively related to dietary protein and total FA contents that arise from legume and oilseed supplementations.³⁵ The main cellulolytic bacteria in the rumen are *Ruminococcus flavescentis*, *R. albus*, *Fibrobacter succinogenes* and *Butyrivibrio* sp. These bacteria are also the main cellulolytic bacteria in the equine gastrointestinal compartments. Similarly, *Megasphaera elsdenii* and *Streptococcus bovis*, which are among the main amylolytic bacteria of the cow's rumen, are also important components of the gastrointestinal flora of equine.^{34, 37} *Streptococcus bovis* also plays a proteolytic role in the rumen.³⁵ The changes in microbiota population in the gastrointestinal compartments of equine fed grass or concentrate diets³³ are also in line with the findings observed for the rumen population in cows³⁵, which would therefore suggest a similar regulation mechanism of the microbiota in cow rumen and equine

gastrointestinal compartments. Even though the de novo synthesis of small amounts OCFA and BCFA cannot be excluded, as for ruminants ³⁵, the results on the milk OCFA and BCFA concentrations in donkey milk also seem to support the hypothesis of their bacterial origin in equids. In fact, the OCFA concentrations were the highest in the farms in which the donkeys were supplemented with cereal-based concentrates. The OCFA/BCFA ratio showed the lowest values in the milk from Farms 3 and 4, in which the donkeys were fed at pasture (with a higher protein content and lower fiber content than hay) and with pasture and hay, respectively, without any cereal-based concentrate. This ratio separates the milk from farms in which pasture was an important ingredient of the diet (in particular Farm 3) and from those where PCA was the main diet. The concentrate supplementation on Farm 5, which had a slightly lower fresh herbage proportion than Farm 4, could have reduced the effect of the diet on the OCFA/BCFA ratio. Farm 2 showed the lowest OCFA/BCFA ratio, which could be explained by the presence of oilseeds in the concentrate composition.

CONCLUSIONS

The present research has highlighted the effect of farming systems on the composition of donkey milk, which has shown to be more relevant than the effect of stage of lactation. The study suggests that differences in FA composition despite the effect of breed not being removed is related to the feeding system. Pasture feeding dairy donkeys produced similar improvement in milk composition and components as seen and previously reported for ruminants such as improved milk fat content, fat-soluble vitamin concentration and a more suitable FA composition for human nutrition.

ACKNOWLEDGEMENTS

Dr. Amy McLean was recipient of a Short-Mobility Program Fellowship of CNR (Contract Ref. N. AMMCNT-CNR 35203/2014). The present study was partially supported by a grant from the University of Turin (Funding Program ex 60% Linea B, 2013). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

REFERENCES

- [1] Valle E, Raspa F, Giribaldi M, Barbero R, Bergagna S, Antoniazzi S, Mc Lean AK, Minero M and Cavallarin L, A functional approach to the body condition assessment of lactating donkeys as a tool for welfare evaluation. *PeerJ* **5**:e3001; (2017).
- [2] Salimei E and Fantuz F, Equid milk for human consumption. *Int Dairy J* **24**: 146–152 (2012).
- [3] Monti G, Bertino E, Muratore MC, Coscia A, Cresi F, Silvestro L, Fabris C, Fortunato D, Giuffrida MG and 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* **18**: 258–264 (2007).
- [4] Trinchese G, Cavaliere G, Canani RB, Matamoros S, Bergamo P, De Filippo C, Aceto S, Gaita M, Cerino P, Negri R, Greco L, Cani PD and Mollica MP, Human, donkey and cow milk differently affects energy efficiency and inflammatory state by modulating mitochondrial function and gut microbiota. *J Nutr Biochem* **26**: 1136–1146 (2015).
- [5] Salimei E, Fantuz F, Coppola R, Chiofalo B, Polidori P and Varisco G, Composition and characteristics of ass's milk 1. *Anim Res* **53**: 67–78 (2004).

- [6] Medhammar E, Wijesinha-Bettoni R, Stadlmayr B, Nilsson E, Charrondiere UR and Burlingame B, Composition of milk from minor dairy animals and buffalo breeds: A biodiversity perspective. *J Sci Food Agric* **92**: 445–474 (2012).
- [7] Gentili A, Caretti F, Bellante S, Ventura S, Canepari S and Curini S, Comprehensive profiling of carotenoids and fat-soluble vitamins in milk from different animal species by LC-DAD-MS/MS hyphenation. *J Agric Food Chem* **61**: 1628–1639 (2013).
- [8] Álvarez R, Meléndez-Martínez AJ, Vicario IM and Alcalde MJ, Carotenoids and fat-soluble vitamins in horse tissues: a comparison with cattle. *Anim* **9**: 1230–1238 (2015).
- [9] Doreau M, Gaillard JL, Chobert JM, Léonil J, Composition of mare and donkey milk fatty acids and proteins and consequences on milk utilization, in *Proceedings of the 4th Meeting New findings in equine practice*, ed. by Università degli Studi del Molise, Campobasso, Italy, pp. 51–71 (2002).
- [10] Shingfield KJ, Bonnet M and Scollan ND, Recent developments in altering the fatty acid composition of ruminant-derived foods. *Anim* **7**: 132–162 (2013).
- [11] Coppa M, Farruggia A, Ravaglia P, Pomiès D, Borreani G, Le Morvan A, and Ferlay A, Frequent moving of grazing dairy cows to new paddocks increases the variability of milk fatty acid composition. *Anim* **9**: 604–13 (2015).
- [12] Coppa M, Gorlier A, Lonati A, Martin B, Russo EM and Lombardi G, The management of the transition from hay to pasture-based diets affects milk fatty acid kinetics. *Dairy Sci Technol* **92**: 279–295 (2012).
- [13] Coppa M, Ferlay A, Borreani G, Revello Chion A, Tabacco E, Tornambé G, Pradel P and Martin B, Effect of phenological stage and proportion of fresh herbage in cow diets on milk fatty acid composition. *Anim Feed Sci Technol* **208**: 66–78

- (2015).
- [14] Chiofalo B, Polidori M, Costa R and Salimei E, Fresh forage in dairy ass's ration : effect on milk fatty acid composition and flavours. *Ita J anim Sci* **4**: 433–435 (2005).
- [15] Burden F, Practical feeding and condition scoring for donkeys and mules *Equine Vet Educ* **24**: 589–596 (2012).
- [16] Pearson A and Ouassat M, *A guide to live weight estimation and body condition scoring of donkeys*. ed. by Centre for Tropical Veterinary Medicine, Edinburgh, UK pp 21. (2000).
- [17] Cavallarin L, Giribaldi M. Soto-Del Rio MD, Valle E, Barbarino G, Gennero MS and Civera T, A survey on the milk chemical and microbiological quality in dairy donkey farms located in NorthWestern Italy. *Food Contr* **50**: 230–235 (2015).
- [18] Kuhl J, Aurich J, Wulf M, Hurtienne A, Schweigert F and Aurich C, Effects of oral supplementation with β -carotene on concentrations of β -carotene, vitamin A and α -tocopherol in plasma, colostrum and milk of mares and plasma of their foals and on fertility in mares. *J Anim Physiol Anim Nutr* **96**: 376–384 (2012).
- [19] Prola L, Nery J, Lauwaerts A, Bianchi C, Sterpone L, De Marco M, Pozzo L and Schiavone A, Effects of N,N-dimethylglycine sodium salt on apparent digestibility, vitamin E absorption, and serum proteins in broiler chickens fed a high- or low-fat diet. *Poult. Sci.* **92**:1221–1226 (2013).
- [20] Coppa M, Chassaing C, Ferlay A, Agabriel C, Laurent C, Borreani G, Barcarolo R, Baars T, Kusche D, Harstad OM, Verbic J, Golecky J, Delavaud C, Chilliard Y, and Martin B, Potential of milk fatty acid composition to predict diet composition and authenticate feeding systems and altitude origin of european bulk milk. *J Dairy Sci* **98**: 1539–1551 (2015).

- [21] Ulbricht TLV, and Southgate DAT, Coronary heart disease: seven dietary factors. *Lancet* **338**: 985–992 (1991)
- [22] Giosuè C, Alabiso M, Russo G, Alicata ML and Torrisi C, Jennet milk production during the lactation in a Sicilian farming system. *Animal* **2**: 1491-1495 (2015).
- [23] Martemucci G and D'Alessandro AG, Fat content, energy value and fatty acid profile of donkey milk during lactation and implications for human nutrition. *Lipid Health Dis* **11**: 113–126 (2012).
- [24] Gubić J, Milovanović I, Ilić M, Tomić J, Torbica A, Šarić L and Ilić N, Comparison of the protein and fatty acid fraction of Balkan donkey and human milk. *Mljekarstvo* **65**: 168-176 (2015).
- [25] Martini M, Altomonte I, Manicac E and Salari F, Changes in donkey milk lipids in relation to season and lactation. *J Food Comp Anal* **41**: 30–34 (2015).
- [26] Claeys WL, Verraes C, Cardoen S, De Block J, Huyghebaert A, Raes K, Dewettinck K and Herman L, Consumption of raw or heated milk from different species: An evaluation of the nutritional and potential health benefits. *Food Contr* **42**: 188–201 (2014).
- [27] Nozière P, Graulet B, Lucas A, Martin B, Grolier P and Doreau M, Carotenoids for ruminants: From forages to dairy products. *Anim Feed Sci Technol* **131**: 418–450 (2006).
- [28] Ferlay A, Martin B, Pradel P, Coulon JB, and Chilliard Y, Influence of grass-based diets on milk fatty acid composition and milk lipolytic system in Tarentaise and Montbéliarde cow breeds. *J Dairy Sci* **89**: 4026–4041 (2006).
- [29] Chilliard Y, Glasser F, Ferlay A, Bernard L, Rouel J, and Doreau M, Diet, rumen biohydrogenation and nutritional quality of cow and goat milk fat. *Eur J Lipid Sci Technol* **109**: 828-855 (2007).

- [30] Hurtaud C, Dutreuil M, Coppa M, Agabriel C and Martin B, Characterization of milk from feeding systems based on herbage or corn silage with or without flaxseed and authentication through fatty acid profile, *Dairy Sci Technol* **94**: 103–123 (2014).
- [31] Devle H, Vetti I, Naess-Andresen CF, Rukke EO, Vegarud G and Ekeberg D, A comparative study of fatty acid profiles in ruminant and non-ruminant milk *Eur J Lipid Sci Technol* **114**: 1036–1043 (2012).
- [32] Kemp P and Lander D, Hydrogenation in vitro of α -linolenic acid to stearic acid by mixed cultures of pure strains of rumen bacteria *J Gen Microbiol* **130**: 527–533 (1984).
- [33] Daly K, Proudman CJ, Duncan SH, Flint HJ, Dyer J and Shirazi-Beechey SP, Alterations in microbiota and fermentation products in equine large intestine in response to dietary variation and intestinal disease. *Br J Nutr* **107**: 989–995 (2012).
- [34] Sadet-Bourgeteau S and Julliand V, La diversité de l'écosystème microbien du tractus digestif équin, *Animales* **25**: 407–418 (2012).
- [35] Vlaeminck B, Fievez V, Cabrita ARJ, Fonseca AJM and Dewhurst RJ, Factors affecting odd- and branched-chain fatty acids in milk: A review, *Anim Feed Sci Technol* **131**: 389–417 (2006).
- [36] Comino L, Righi F, Coppa M, Quarantelli A, Tabacco E, and BorreaniG, Relationships among early lactation milk fat depression, cattle productivity and fatty acid composition on intensive dairy farms in Northern Italy. *Italian J Anim Sci* **14**: 350:361 (2015).
- [37] Costa MC, Silva G, Ramos RV, Staempfli HR, Arroyo RG, Kim P and Weese JS, Characterization and comparison of the bacterial microbiota in different gastrointestinal tract compartments in horses, *Vet J* **205**: 74–80 (2015).

Table 1 Farm characteristics obtained from on farm survey

	Farm 1	Farm 2	Farm 3	Farm 4	Farm 5	Farm 6
Total donkeys ¹ (n.)	53	63	150	60	130	48
Jennies ¹ (n.)	44	40	7	40	80	32
Milking jennies ¹ (n.)	6	12	9	6	10	10
BW ² of milking jennies(kg)	213	353	307	276	321	337
BCS of milking jennies	2.3	2.8	2.3	3.3	1.8	3.4
Breed	Crossbreeds	Martina Franca	Crossbreeds	Crossbreeds	Ragusana	Crossbreeds
Milk yield (L/animal×d)	0.5	0.7	0.8	1.1	2.0	1.0
Feeding (% of dry matter diet)	Pasture 0% Hay 90% Cereal mix A ⁴ 10%	Pasture 0% Hay 90% Cereal mix B ⁴ 10%	Pasture 100%	Pasture 50% Hay 50%	Pasture 40% Hay 50% Cereal mix A ⁴ 10%	Pasture 0% Hay 100%

¹Counted during the visit.²BW = Body weight³BCS = Body condition score on 5 point scale

⁴Cereal Mix A = 60% cereals, 30% cereal by-products, 10% legumes; Cereal mix B = 40% cereals, 40% cereal by-products, 10% legumes, 10% oilseeds.

Table 2. Composition and fat-soluble vitamins contents of the donkey milk on the studied farms

Milk constituents	Farm										Effect and significance ¹	
	1	2	3	4	5	6				SEM	DIM	Farm
Fat (g kg ⁻¹ milk)	0.013 ^b	0.017 ^b	0.036 ^{ab}	0.065 ^a	0.025 ^b	0.026 ^b				0.003	NS	**
Protein (g kg ⁻¹ milk)	0.176 ^b	0.196 ^a	0.184 ^b	0.165 ^b	0.203 ^a	0.193 ^b				0.004	***	***
Lactose (g kg ⁻¹ milk)	0.790	0.749	0.687	0.639	0.760	0.668				0.023	NS	NS
Retinol (µg L ⁻¹)	0.091 ^b	0.136 ^{ab}	0.304 ^a	0.278 ^{ab}	0.284 ^{ab}	0.182 ^{ab}				0.021	NS	**
α-Tocopherol (µg L ⁻¹)	0.313 ^b	0.580 ^b	2.579 ^a	1.931 ^{ab}	0.857 ^b	0.558 ^b				0.223	NS	*

¹ DIM = days in milk; NS = not significant.

^{a-b} Values within a row with different superscripts differ significantly at: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Table 3. Fatty acid composition of the donkey milk on the studied farms

Fatty acids (g kg ⁻¹ FA)	Farm												SEM	Effect and significance ^a	
	1	2	3	4	5	6								DIM	Farm
C4:0	6.0	5.7	3.1	5.3	8.5	10.2	0.9							NS ¹	NS
C6:0	3.8	ab	5.0	a	3.3	b	3.2	b	4.1	ab	3.3	ab	0.2	NS	**
C8:0	45.3	ab	52.5	a	35.4	b	40.4	ab	44.8	ab	43.1	ab	1.8	**	**
C10:0	97.1	ab	94.2	ab	64.4	b	84.4	ab	94.5	ab	100.1	a	3.7	NS	*
C10:1c9	15.7	ab	12.6	ab	07.5	c	16.8	a	10.4	bc	12.6	ab	0.6	NS	***
C12:0	92.0	a	77.6	a	48.8	b	87.4	a	83.0	a	92.8	a	3.5	NS	**
C12:1c9	1.8	a	1.2	b	0.7	c	2.1	a	1.1	bc	1.4	ab	0.1	NS	***
isoC14:0	1.2	a	1.1	ab	0.7	bc	0.7	bc	1.2	a	0.6	c	0.1	NS	**
C14:0	75.2	a	66.0	a	41.0	b	75.2	a	66.1	a	74.4	a	2.5	NS	***
isoC15:0	1.1	ab	1.3	a	0.6	c	0.8	bc	0.8	bc	0.8	bc	0.1	NS	***
anteisoC15:0	1.0	b	1.3	a	0.6	c	0.5	c	0.8	bc	0.6	c	0.1	NS	***
C14:1c9	4.0	ab	2.9	bc	1.9	c	4.8	a	2.6	bc	4.0	ab	0.2	*	***
C15:0	4.4	a	3.8	b	2.9	cd	3.3	bc	3.4	bc	2.5	d	0.1	†	***
isoC16:0	2.3	a	2.4	a	1.5	b	1.5	b	1.6	ab	1.6	ab	0.1	*	***
C16:0	207.2		205.0		189.9		190.0		187.6		192.5		2.9	NS	NS
C16:1c9	37.8		33.2		37.9		32.8		24.5		44.7		2.0	NS	NS
anteisoC17:0	2.2	b	2.7	a	1.7	bc	1.9	bc	2.1	b	1.5	c	0.1	NS	***
C17:0	3.5	ab	4.7	a	2.2	ab	2.2	ab	3.0	ab	1.9	b	0.2	*	*
C17:1c9	4.3	a	4.1	a	3.5	ab	4.3	a	2.8	b	3.0	b	0.1	NS	**
C18:0	15.5	bc	18.6	ab	19.0	ab	10.1	c	20.2	a	14.9	bc	0.6	NS	***
C18:1t11	1.0	b	0.9	b	3.0	a	1.4	b	2.1	ab	1.1	b	0.2	**	***
C18:1c9	171.9	ab	176.4	ab	207.4	a	125.9	b	147.0	b	168.4	ab	6.7	NS	*
C18:1c11	12.8		13.1		13.8		8.9		10.2		14.5		0.5	NS	†
C18:2c9t12	0.31	ab	0.27	b	0.47	a	0.27	b	0.34	ab	0.26	ab	0.02	*	***
C18:2n-6	60.3	b	68.7	b	87.7	a	54.3	b	90.5	a	54.8	b	3.0	NS	***
C18:3n-6	0.61	b	0.71	b	0.97	ab	1.11	a	0.76	bc	0.76	bc	0.03	**	***
C18:3n-3	96.8	b	106.9	b	179.7	a	205.4	a	147.0	ab	119.9	b	7.0	***	***
C20:1c11	2.2	ab	2.3	ab	2.5	a	1.6	b	1.8	ab	2.0	ab	0.1	NS	*
CLAc9t11	0.6	b	0.6	b	0.9	a	0.7	b	0.7	b	0.7	b	0.1	NS	**

C20:2n-6	1.6	bc	1.5	bc	1.8	ab	1.1	c	2.1	a	1.1	c	0.1	NS	***
C22:0	0.22	a	0.18	ab	0.23	a	0.16	ab	0.20	a	0.11	b	0.01	**	**
C20:3n-3+C22:1c13	2.9	b	3.2	b	4.6	a	4.7	a	3.8	ab	3.3	b	0.1	*	***
C20:4n-6	0.43	bc	0.31	c	0.66	a	0.31	c	0.64	a	0.54	ab	0.03	†	***
C24:0	0.15	b	0.18	b	0.39	a	0.10	b	0.28	ab	0.20	b	0.02	NS	***
C22:5n-3	0.77		0.47	b	1.13	a	0.76	ab	0.97	ab	0.77	ab	0.06	NS	**
ECSFA	543.1	a	526.0	a	406.2	b	496.5	ab	510.0	a	532.0	ab	10.3	NS	***
OCFA	14.1	a	15.3	a	10.9	b	12.4	ab	12.0	ab	10.0	b	0.5	*	*
BCFA	9.3	ab	11.0	a	6.3	c	6.6	bc	7.7	b	6.3	c	0.4	NS	***
MUFA	261.6		256.6		291.0		207.6		212.5		261.0		8.9	NS	NS
PUFA	167.1	b	185.6	b	281.1	a	271.4	a	249.8	a	184.5	b	8.9	**	***
∑ cis18:1	185.6	ab	190.6	ab	222.5	a	135.7	b	158.1	b	184.0	ab	7.2	NS	*
∑ trans18:1	1.8	b	1.9	b	4.6	a	2.2	b	3.8	ab	2.0	b	0.2	**	***
∑ n-6	63.6	b	71.9	b	91.7	a	57.1	b	94.7	a	57.8	b	3.1	NS	***
∑ n-3	100.9	b	111.1	b	186.1	a	211.7	a	152.4	ab	124.5	b	7.1	***	***
∑ n-6/∑ n-3	0.64	a	0.65	a	0.51	ab	0.27	b	0.65	a	0.47	ab	0.02	*	***
OCFA/BCFA	1.58	ab	1.39	b	1.73	a	1.89	a	1.59	ab	1.65	ab	0.04	NS	*
∑ <i>de novo</i> synthesis FA	319.4	a	301.2	a	196.0	b	295.8	a	301.0	a	323.9	ab	10.8	NS	**
∑ CLA	1.2	b	1.2	b	1.7	a	1.3	b	1.3	b	1.1	b	0.01	NS	***
Atherogenicity Index	1.83	a	1.50	ab	0.75	d	1.11	c	1.11	c	16.1	ab	0.6	NS	***
Trombogenicity Index	0.62	a	0.57	a	0.34	c	0.35	c	0.45	bc	0.51	ab	0.02	*	***

¹ DIM = days in milk; NS = not significant ; FA = fatty acids; ECSFA = even chain-saturated FA; OCFA = odd chain-FA; BCFA=

branched chain-FA; MUFA = mono-unsaturated FA; PUFA = polyunsaturated FA; CLA = conjugated linoleic acid; ∑ cis18:1= sum of cis isomers of C18:1; ∑ trans18:1= sum of isomers of C18:1; ∑ n-6 = sum of n-6 FA; ∑ n-3 = sum of n-3 FA; ∑ *de novo synthesis* FA = sum of even-chain SFA from C4:0 to C14:0; ∑ CLA = sum of CLA isomers.

^{a-d} Values within a row with different superscripts differ significantly at: † $p < 0.1$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Table 4 Fischer's F for farm effect and days in milk (covariate factor) from the analysis of the variance and significant regressive coefficients of the covariate factor

Item ¹	DIM ¹ Coefficient	Fisher's F	
		DIM ¹	Farm
Protein (g/ kg ⁻¹ milk)	-0.0002069	6.55	22.48
Fatty acid (g kg ⁻¹ FA)			
C8:0	-0.0006593	5.08	8.35
C14:1c9	0.0000402	5.44	13.84
C15:0	0.0000228	3.84	10.40
isoC16:0	0.0000223	2.47	6.40
C17:0	0.0000724	3.27	6.42
C18:1t11	0.0000463	14.54	29.95
C18:2c9t12	0.0000059	3.90	6.60
C18:3n-6	0.0000114	8.50	11.10
C18:3n-3	0.0026447	8.49	18.34
C22:0	0.0000041	4.19	9.65
C20:3n-3+C22:1c13	0.0000372	6.65	11.20
C20:4n-6	-0.0000058	3.70	9.94
OCFA	0.0001575	2.92	6.62
PUFA	0.002557	8.82	14.83
∑ n-3	0.0028655	8.33	18.51
∑ n-6/∑ n-3	-0.0000655	6.76	10.70
Trombogenicity Index	-0.0000431	6.89	20.28

¹ DIM = days in milk; FA = fatty acids; OCFA = odd chain-FA; PUFA = polyunsaturated FA; ∑ n-6 = sum of n-6 FA; ∑ n-3 = sum of n-3 FA.

Figure 1. Principal component analysis performed on the main fatty acids of the milk: plot of the variable distribution and of the sample distribution.

¹ ECSFA = even chain-saturated FA; OCFA/BCFA = odd chain-FA to branched chain-FA ratio; MUFA = monounsaturated FA; PUFA = polyunsaturated FA; $\sum$ n-3 = sum of n-3 FA; $\sum$ n-6/ $\sum$ n-3 = sum of n-6 FA to sum of n-3 FA ratio; $\sum$ *de novo synthesis* FA = sum of even-chain SFA from C4:0 to C14:0; $\sum$ CLA = sum of CLA isomers; AI: Atherogenicity Index; TI: Trombogenicity Index.

