Effect of farming system on donkey milk composition

Effect of farming system on donkey milk composition.

Valle E, Pozzo L, Giribaldi M, Bergero D, Gennero MS, Dezzutto D, McLean A, Borreani G, Coppa M, Cavallarin L.

J Sci Food Agric. 2017 Nov 13. doi: 10.1002/jsfa.8777.

Feeding system and lactation stage affect the donkey milk fatty acid composition and fat-soluble vitamin composition

E. Valle[1], L. Pozzo[2,3], M. Giribaldi[3,4], F. Raspa[1], M.S. Gennero[5], D. Dezzutto[5], A. McLean[6,a], G. Borreani[7], M. Coppa [7], L.Cavallarin[3].

[1] Dipartimento di Scienze Veterinarie, Università di Torino, Largo P. Braccini 2, 10095 Grugliasco (Turin), Italy

[2] Istituto di Biologia e Biotecnologia Agraria, Consiglio Nazionale delle Ricerche, Via Moruzzi 1, 56124 Pisa, Italy

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

[4] Unità di ricerca per i processi dell'industria agroalimentare (IAA), Consiglio per la Ricerca in Agricoltura e l'Analisi dell'Economia Agraria (CREA), Via Venezian 26, Milan, Italy.

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

[6] Department of Animal Science, North Carolina State University, Raleigh 27695.

[7] *Dipartimento di Scienze Agrarie, Forestali e Alimentari, Università di Torino, Largo P. Braccini 2, 10095 Grugliasco (Turin), Italy.*

[a] *Present address:UC Davis Animal Science Horse Barn, University of California at Davis, Davis 95616.*

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

Short title: The feeding system affects the composition of donkey milk

Abstract

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 the feeding system on the composition of donkey milk according to the feeding strategies adopted on commercial farms. The study was aimed at evaluating the effect of the feeding system and stage of lactation on the donkey milk gross composition, fat soluble vitamins (retinol, α-tocopherol) and fatty acid (FA). Individual milk was sampled from lactating jennies (n=53) on six farms located in North West Italy. The performance of lactating jennies, the herd characteristics, milking management and feeding strategies were recorded at each milk sampling. A greater effect of the farming system and a limited effect of the lactation stage on the milk composition were observed. The gross composition of the milk, and the fat-soluble vitamin content differed according to the feeding system. A higher milk fat content corresponded to a higher fresh herbage proportion in the diet. The highest polyunsaturated FA (PUFA) content in the milk was observed for the animals fed on only forage diets. The animals that were fed just pasture produced the milk with the highest concentration of C18:1c9, C18:3n-3, n-3 FA,PUFA, retinol and α-tocopherol, and the lowest concentrations of the FA less favorable for human health. The farms

that fed intermediate fresh herbage proportions in the diets showed intermediate concentrations of C18:3n-3 in the milk. Pasture feeding has been shown to improve the fat content and fat-soluble vitamin concentration of donkey milk and to move the FA composition to a more favorable profile for human nutrition, as already observed for ruminants.

Keywords: *Equus asinus,* Donkey milk, Lactation stage, Feeding system, Fatty acids.

Implications

The present study has evaluated the effect of the feeding system and stage of lactation on the composition of donkey milk, considering data collected during a survey on dairy donkey farms in North West Italy. The results have shown that it is possible to move donkey milk composition to a more favorable profile for human nutrition, by means of feeding pasture to the lactating donkeys. These findings will be useful for dairy donkey breeders for improving the quality of donkey milk that is considered a functional food for sensitive consumers, such as children allergic to cow milk.

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).The dairy donkey farms in the EU are mainly located in Italy, France, Spain and Belgium (Salimei and Fantuz, 2012). 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 (Monti *et al.*, 2007). It has also 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 (Trinchese *et al.*, 2015). 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 0.3 to 1.2 g/100 mL range. This difference is associated with a low energy content (Salimei *et al.*, 2004; Medhammar *et al.*, 2012), which represents the main limit to its use in the nutrition of 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) (Medhammar *et al.*, 2012). More in detail, donkey milk fat has shown higher C18:3n-3 and n-3 FA concentration, and a lower saturated FA (SFA) content than cow milk, as well as a lower n-6 to n-3 FA ratio (Medhammar *et al.,* 201). On the other hand, equid milk appears to have a lower fat-soluble vitamin content, that is, of α-tocopherol and retinol, than ruminant milk (Gentili *et al*., 2013; Álvarez *et al.,* 2015).

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 (Salimei and Fantuz, 2012). 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 (Doreau *et al.,* 2002). The feeding composition has been shown to be the main factor that affects the FA composition of milk in ruminants (Shingfield *et al*., 2013; Coppa *et al*., 2015a). In particular, pasture feeding increases in 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), and decreases the n6 to n3 ratio and the concentrations of FA less favorable for human nutrition, such as C14:0, C16:0, and SFA (Coppa *et al.,*2012; Farruggia *et al.,* 2014).However, the effect of the feeding system on donkey milk composition has only been studied so far in experimental conditions for a restricted group of FA (Chiofalo *et al.,* 2005), and no information is available regarding the effect of the feeding system on the FA composition of donkey milk according to the feeding strategies adopted on commercial farms. Furthermore, changes in donkey milk fat-soluble vitamins, as a result of the feeding system, have never been investigated.

The aim of this study was to evaluate the effect of the feeding system and lactation stage on the milk composition of dairy asses, on the basis of observational data collected during a survey on six commercial farms located in North-West Italy.

Materials and methods

Milk Sampling and Survey

Individual milks were sampled (0.5 L) from 53 lactating jennies reared on six commercial farms located in the Piedmont Region, in North West Italy, during Spring 2014. The performance of the lactating jennies and herd characteristics (number of jennies, breed, DIM, milk yield, body condition scores (BCS), milking management, 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 Burden (2012), and body weight according to Pearson and Ouassat (2000). 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 contents. The fat content and protein content were assessed as described by Cavallarin *et al*., (2015). 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

The retinol and α-tocopherol in the milk samples were extracted according to the Kuhl *et al.* (2012), with some adaptations. The retinol and α-tocopherol concentrations were quantified according to Prola *et al.* (2013), by means of a HPLC system (Dionex, Sunnyvale, CA, USA). The analytical column was an XTerra RP18 column (250-mm × 4.6-mm, 5 µm 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 0.7 and 100 µg/mL. 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% (Table 3).

Milk Fatty Acid Analysis

Milk samples were analyzed for FA composition by gas chromatography (GC), as described by Coppa *et al.* (2015b). 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, and 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% K_2CO_3 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, (2015b), with the sole adaptation of the split ratio

to the lower fat content of donkey milk: a volume of 1 µL 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.

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. The Bonferroni test was used as the *post-hoc* test. A principal components analysis (PCA) was performed on the main FA.

Results

Milk Gross Composition and Fat-Soluble Vitamin Content

The fat-soluble vitamin content of the donkey milk differed significantly for all the parameters over the different farms (Table 2), except for the lactose concentration. The highest protein content was found in the milk collected on Farm 5, while the highest fat content was found in the 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 0.89 to 4.66µg/100 mL range, and α-tocopherol was within the 2.14 to 38.40µg/100 mL 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 5, and supplementary Table 1, for the detailed FA profile).The milk from Farm 3 showed the highest concentrations of C18:1c9, total C18:1cis isomers, C22:5n-3, CLAc9t11and total CLA, and the lowest concentrations of C8:0, C12:0, C14:0, total *de novo* synthesis FA, and even chain-saturated FA (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 odd chain-FA (OCFA) and branched chain-FA (BCFA) concentrations were the 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 Atherogeicity 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).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 (e.g. Salimei *et al.*, 2004; Cavallarin *et al.,* 2015). The decrease in the protein content of the donkey milk during lactation is in agreement with the findings of Salimei *et al.* (2004), Giosuè *et al.* (2008), Salimei and Fantuz (2012) 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.

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 (2012), Gubić *et al.* (2015) and Martini *et al.* (2015). These authors highlighted an increase in concentration of long chain FA and a

242 decrease in concentrations of short chain FA from *de novo* synthesis in the
243 mammary gland, with the development of the lactation stage. These results are in
244 agreement with the significant increase observed for several long-chain FA during
245 lactation in the present study, even if the differences found in literature in donkey milk
246 FA composition during lactation were larger than those observed in the present
247 study. However, the aforementioned studies followed the evolution of the FA
248 composition of milk collected from individual animals throughout the entire lactation
249 period in controlled condition and with a constant diet (Martemucci and D'Alessandro,
250 2012; Martini *et al.,* 2015). On the other hand, the effect of animal related factors,
251 such as breed and lactation stage, are known to have a negligible effect on the FA
252 composition of milk in dairy cows on farms, compared to animal diet (Coppa *et al.,*
253 2015a). The results of the present study have shown a greater effect on milk FA of
254 the farming system, with a limited effect of the lactation stage, which is pointed out by
255 the higher ANOVA Fisher's F coefficients for the Farm effect than for DIM.

256 *Effect of Feeding System on the Gross Composition of Milk*

257 To the best of our knowledge, the effect of the feeding system on donkey milk quality
258 has never been studied before. The higher content of fat in the milk collected on
259 Farm 3 and 4 corresponded to a higher pasture proportion in the diet than on the
260 other farms. In addition, the hay sampled on Farm 4 in two different periods (data not
261 shown) resulted to be of high quality, in terms of protein and ADF content. This
262 indicates that forage quality plays an important role in the fat concentration of donkey
263 milk.

264 It is well known that, in ruminants, genetics may also accounts for the difference
265 between the protein and fat contents of milk (Shingfield *et al.,* 2013). No evidence is
266 available in this regard for equine species. It can be speculated that the higher

content of milk protein from Farm 2 and 5 might depends on the fact that a homogenous breed is reared on these farms (Martina Franca and Ragusana, respectively), unlike the other farms, where crossbreeds animal are reared.

Effect of Feeding 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.* (2013) and Clayes *et al.* (2014) 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 fed different diets have never been studied before. Álvarez *et al.* (2015) reported a concentration of retinol in milk from mares fed at pasture that was double that reported by other authors for mares fed hay (Khul *et al*., 2012). Similarly, the amount of α-tocopherol and retinol in cow milk was shown to double approximately when cows were fed at pasture instead of conserved forages (Nozière *et al.,* 2006). These provitamin carotenoids originate from β-carotene through enzymatic oxidative. 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 (Nozière *et al.,* 2006). 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 Feeding System on the Fatty Acid Composition of the Milk

The present results are the first evidence of the effect of feeding system on the detailed milk FA profile of donkey milk on commercial farms, as the only study available in literature, in which the FA composition of donkeys fed different diets was compared in controlled conditions, was focused on a few groups of FA (Chiofalo *et*

291 *al.*, 2005). Our study points out an important influence of animal diets on the FA
292 profile of donkey milk. The milk collected in Farm 3 showed the highest concentration
293 of the FA that are favorable for human nutrition, such as C18:1c9, C18:3n-3, n-3 FA
294 and PUFA, and the lowest concentration of the FA less favorable for human health,
295 such as ECSFA, and *de novo* synthesis FA (Salimei and Santuz, 2012, Claeys *et al.,*
296 2014). The key factor that can explain the FA pattern of the milk from Farm 3 is
297 related to the donkey diets, which were exclusively constituted by fresh forage from
298 pastures. The higher concentration of C18:3n-3, compared to that in the milk from the
299 other farms, could be derived from a direct transfer of this FA from the ingested
300 pasture (Chiofalo *et al.,* 2005), as C18:3n-3 is the most abundant FA in fresh
301 herbage (Coppa *et al.,* 2015b). A higher transfer of C18:3n-3 in the milk of equids
302 than that of ruminants is allowed by the lack of biohydrogenation (Claeys *et al.,*
303 2014), which conversely occurs for most of the ingested long-chain PUFA in
304 ruminants (Shingfield *et al*., 2013).The C18:3n-3 has been shown to be a valuable
305 indicator of pasture feeding for dairy cows (Farruggia *et al*., 2014; Hurtaud *et al.,*
306 2014), and its concentration has been shown to increase with increasing fresh
307 herbage proportions in cow diets (Coppa *et al*., 2012). The increase in the C18:3n-3
308 concentration in donkey milk, with increasing proportions of fresh herbage in the diet,
309 is also consistent with the intermediate concentration of this FA in the milk from
310 Farms 4 and 5, which had 50 and 40% of fresh herbage in the diets, respectively.
311 The higher C18:3n-3, C22:3n-3 and PUFA proportions in the donkey milk for Farm
312 3, due to the full grazing diet, could also have partially inhibited the *de novo* synthesis
313 process in the mammary gland (Shingfield *et al.,* 2013; Claeys *et al.,* 2014), thus
314 resulting in lower concentrations in the milk of C8:0, C10:0, C12:0, C14:0, total *de*
315 *novo synthesis* FA, and ECSFA. A lower concentration of SFA in the milk from

316 donkeys fed fresh herbage than those from donkeys fed hay was also observed by
317 Chiofalo *et al.* (2005), as observed for ruminants (Shingfield *et al.,* 2013).
318 Small concentrations of CLAc9t11 have been observed in horse milk, but have
319 never been detected in donkey milk before (Devle *et al.,* 2012; Medhammar *et al.,*
320 2012). However, the same authors reported concentrations of C18:1t11 in donkey
321 milk. This FA is known to be the substrate for CLAc9t11 desaturation by to Δ9-
322 desautrase activity in the mammary gland in ruminants (Shingfield *et al.,* 2013), and
323 to be responsible for the desaturation in the mammary gland of donkeys (Martemucci
324 and D'Alessandro 2012), thus suggesting a possible similar origin in donkey milk.
325 The CLAc9t11 in ruminants can also originate from dietary C18:2n-6
326 biohydrogenation by *Butyrivibrio* sp. bacteria, as well as C18:1t11 from C18:3n-3
327 (Kemp and Lander, 1984). *Butyrivibrio* sp. bacteria were also identified as main
328 components of equine gastrointestinal compartments (Daly *et al.,* 2012; Sadet-
329 Bourgeteau and Julliand, 2012). This would seem to suggest that a small part of
330 ingested C18:3n-3 may have been biohydrogenated, by these bacteria to C18:1t11,
331 which could have been desaturated to CLAc9t11 in the mammary gland. This
332 hypothesis also seems to be supported by the higher concentrations of both
333 C18:1t11 and CLAc9t11 in the milk of Farm 3, in which the donkeys were fed at
334 pasture. In fact,C18:1t11 and CLAc9t11 have been identified as indicators of pasture
335 proportion in cow diets for ruminants (Hurtaud *et al*., 2014;Coppa *et al.,*2012 and
336 2015b).
337 The variations in OCFA and BCFA in donkey milk, according to the feeding
338 system, are more difficult to interpret, as little is known about the mechanism that
339 determines their concentration. Only Devle *et al*. (2012) and Medhammar *et al.*(2012)
340 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 (Vlaemnik *et al.,* 2006). Their concentration in cow milk varies according to the shift in ruminal population due to the changes in ruminal substrate, as a function of the different diets (Vlaemink *et al*., 2006). In particular, forage-based diets favor the cellulolytic bacteria population in rumen, and determine an increase in BCFA in milk (Vlaemink *et al*., 2006: Coppa *et al.,* 2015a). On the other hand, the substitution of hay or pasture feeding with corn silage or cereal based-concentrates, which are rich in starch, favors the ruminal population of amylolytic bacteria, with a resultant increase in the milk concentration of OCFA and of the OCFA/BCFA ratio. In addition, the concentration of BCFA in cow milk has also been negatively related to the diet protein and total FA contents (Vlaemink *et al.,* 2006), that arise from legume and oilseed supplementations. The main cellulosolytic bacteria in cow rumen are *Ruminococcus flavescens*, *R. albus*, *Fibrobacter succinogenes* and *Butyrivibrio* sp. These bacteria are also the main cellulolytic bacteria in the equine gastrointestinal compartments (Sadet-Bourgeteau and Julliand, 2012; Costa *et al,* 2015). Similarly, *Megaspheraelsdenii* and *Streptococcus bovis,* which are among the main amylolitic bacteria of cow rumen, are also important components of the gastrointestinal flora of equines (Sadet-Bourgeteau and Julliand, 2012; Costa *et al*., 2015). *Streptococcus bovis* also plays a proteolytic role in cow rumen (Vlaemink *et al.*, 2006). The changes in microbiota population in the gastrointestinal compartments of equine fed grass or concentrate diets (Daly *et al.,* 2012) are also in line with the findings observed for the ruminal population in cows (Vlaemink *et al*., 2006), 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 (Vlaemink *et al*., 2006), the

366 results on the milk OCFA and BCFA concentrations in donkey milk also seem to
367 support the hypothesis of their bacterial origin in equids. In fact, the OCFA
368 concentrations were the highest in the farms in which the donkeys were
369 supplemented with cereal-based concentrates. The OCFA/BCFA ratio showed the
370 lowest values in the milk from Farms 3 and 4, in which the donkeys were fed at
371 pasture (with a higher protein content and lower fiber content than hay) and with
372 pasture and hay, respectively, without any cereal-based concentrate. The
373 concentrate supplementation on Farm 5, which had a slightly lower fresh herbage
374 proportion than Farm 4, could have reduced the effect of the diet on the OCFA/BCFA
375 ratio. Farm 2 showed the lowest OCFA/BCFA ratio, which could be explained by the
376 presence of oilseeds in the concentrate composition.
377 The present research has highlighted the effect of feeding system on the composition
378 of donkey milk, which has here been shown to be more relevant than the effect of
379 lactation stage. Pasture feeding has been shown to improve the milk fat content and
380 fat-soluble vitamin concentration of donkeys and to move the FA composition of the
381 milk to a more favorable profile for human nutrition, as already observed for
382 ruminants.
383

384 **Acknowledgements**

385 Dr. Amy McLean was recipient of a Short-Mobility Program Fellowship of CNR
386 (Contract Ref. N. AMMCNT-CNR 35203/2014).

387

388 **References**

389 Álvarez R, Meléndez-Martínez AJ, Vicario IM, and Alcalde MJ 2015. Carotenoids and fat-
390 soluble vitamins in horse tissues: a comparison with cattle. Animal 9, 1230-1238.

391 Association of Official Analytical Chemists (AOAC) 2005. Official methods of analysis, 18th
392 ed. AOAC International, Gaithersburg, MD, USA.
393 Burden F 2012. Practical feeding and condition scoring for donkeys and mules. Equine
394 Veterinary Education 24, 589-596.
395 Cavallarin L, Giribaldi M, Soto-Del Rio MD, Valle E, Barbarino G, Gennero MS and Civera T
396 2015. A survey on the milk chemical and microbiological quality in dairy donkey farms
397 located in NorthWestern Italy. Food Control 50,230-235.
398 Chiofalo BM, Polidori P, Costa R and Salimei E 2005. Fresh forage in dairy ass's ration:
399 effect on milk fatty acid composition and flavours. Italian Journal Animal Science 4,
400 433-435.
401 Claeys W L, Verraes C, Cardoen S, De Block J, Huyghebaert A, Raes K, Dewettinck K and
402 Herman L 2014. Consumption of raw or heated milk from different species: An
403 evaluation of the nutritional and potential health benefits. Food Control 42, 188-201.
404 Coppa M, Chassaing C, Ferlay A, Agabriel C, Laurent C, Borreani G, Barcarolo R, Baars T,
405 Kusche D, Harstad OM, Verbiþ J, Golecký J, Delavaud C, Chilliard Y and Martin B
406 2015a. Potential of milk fatty acid composition to predict diet composition and
407 authenticate feeding systems and altitude origin of European bulk milk. Journal of
408 Dairy Science 98, 1539–1551.
409 Coppa M, Farruggia A, Ravaglia P, Pomiès D, Borreani G, Le Morvan A, and Ferlay A
410 2015b. Frequent moving of grazing dairy cows to new paddocks increases the
411 variability of milk fatty acid composition. Animal 9, 604–613.
412 Coppa M, Gorlier A, Lonati M, Martin B, Russo EM and Lombardi G 2012. The management
413 of the transition from hay- to pasture-based diets affects milk fatty acid kinetics. Journal
414 of Dairy Science Technology 92, 279–295.
415 Costa MC, Silva G, Ramos RV, Staempfli HR, Arroyo LG, Kim P and Weese JS 2015.
416 Characterization and comparison of the bacterial microbiota indifferent gastrointestinal
417 tract compartments in horses. Veterinary Journal 205, 74–80.

418 Daly K, Proudman CJ, Duncan SH, Flint HJ, Dyer J and Shirazi-Beechey SP 2012.
419 Alterations in microbiota and fermentation products in equine large intestine in
420 response to dietary variation and intestinal disease. British Journal of Nutrition 107,
421 989-995.
422 Devle H, Vetti I, Naess-Andresen CF, Rukke EO, Vegarud G and Ekeberg D 2012. A
423 comparative study of fatty acid profiles in ruminant and non-ruminant milk. European
424 Journal of Lipid Science Technology 114, 1036–1043.
425 Doreau M, Gaillard JL, Chobert JM, Léonil J, Egito AS and Haertlé T 2002. Composition of
426 mare and donkey milk fatty acids and proteins and consequences on milk utilization. In
427 Proceedings of the 4th Meeting New findings in equine practice, 11-13 July 2002,
428 Campobasso, Italy, pp. 51-71.
429 Farruggia A, Pomiès D, Coppa M, Ferlay A, Verdier-Metz I, Le Morvan A, Bethier A,
430 Pompanon F, Troquier O and Martin B 2014. Animal performances, pasture
431 biodiversity and dairy product quality: how it works in contrasted mountain grazing
432 systems. Agriculture, Ecosystems and Environment 185, 231–244.
433 Gentili A, Caretti F, Bellante S, Ventura S, Canepari S and Curini R 2013. Comprehensive
434 profiling of carotenoids and fat-soluble vitamins in milk from different animal species by
435 LC-DAD-MS/MS hyphenation. Journal of Agricultural and Food Chemistry 61,
436 1628−1639.
437 Giosuè C, Alabiso M, Russo G, Alicata ML and Torrisi C 2008. Jennet milk production during
438 the lactation in a Sicilian farming system. Animal 2, 1491-1495.
439 Gubić J, Milovanović I, Iličić M, Tomić J, Torbica A, Šarić L and Ilić N 2015. Comparison of
440 the protein and fatty acid fraction of Balkan donkey and human milk. Mljekarstvo 65,
441 168-176.
442 Hurtaud C, Dutreuil M, Coppa M, Agabriel C and Martin B 2014. Characterization of milk
443 from feeding systems based on herbage or corn silage with or without flaxseed and
444 authentication through fatty acid profile. Dairy Science and Technology 94, 103–123.

445 Kemp P and Lander DJ 1984. Hydrogenation *in vitro* of α-linolenic acid to stearic acid by
446 mixed cultures of pure strains of rumen bacteria. Journal of General Microbiology 130,
447 527–533.
448 Kuhl J, Aurich JE, Wulf M, Hurtienne A, Schweigert FJ and Aurich C 2012. Effects of oral
449 supplementation with β-carotene on concentrations of β-carotene, vitamin A and α-
450 tocopherol in plasma, colostrum and milk of mares and plasma of their foals and on
451 fertility in mares. Journal of Animal Physiology and Animal Nutrition 96, 376-384.
452 Martini M, Altomonte I, Manicac E and Salari F 2015. Changes in donkey milk lipids in
453 relation to season and lactation. Journal of Food Composition and Analysis 41, 30–34.
454 Martemucci D and D'Alessandro AG 2012. Fat content, energy value and fatty acid profile of
455 donkey milk during lactation and implications for human nutrition. Lipids Health Disease
456 11, 113-126.
457 Medhammar E, Wijesinha-Bettoni R, Stadlmayr B, Nilsson E, Charrondiere UR and
458 Burlingame B 2012. Composition of milk from minor dairy animals and buffalo breeds:
459 a biodiversity perspective. Journal of the Science of Food and Agriculture 92, 445–474.
460 Monti G, Bertino E, Muratore MC, Coscia A, Cresi F, Silvestro L, Fabris C, Fortunato D,
461 Giuffrida MG and Conti A 2007. Efficacy of donkey's milk in treating highly problematic
462 cow's milk allergic children: an *in vivo* and in vitro study. Pediatric Allergy and
463 Immunology 18, 258-264.
464 Nozière P, Graulet B, LucasA , Martin B, Grolier P and Doreau M 2006. Carotenoids for
465 ruminants: from forages to dairy products. Animal Feed Science and Technology 131,
466 418–450.
467 Pearson AR and Ouassat M 2000. A guide to live weight estimation and body condition
468 scoring of donkeys. Centre for Tropical Veterinary Medicine, Edinburgh, UK.
469 Prola L, Nery J, Lauwaerts A, Bianchi C, Sterpone L, De Marco M, Pozzo L and Schiavone A
470 2013. Effects of *N*,*N*-dimethylglycine sodium salt on apparent digestibility, vitamin E
471 absorption, and serum proteins in broiler chickens fed a high- or low-fat diet. Poultry
472 Science 92, 1221-1226.

473 Sadet-Bourgeteau S and Julliand V 2012. La diversité de l'écosystème microbien du tractus
474 digestif équin. INRA Productions Animales 25, 407-418.
475 Salimei E and Fantuz F 2012. Equid milk for human consumption. International Dairy Journal
476 24, 130-142.
477 Salimei E, Fantuz F, Coppola R, Chiofalo B, Polidori P and Varisco G 2004. Composition and
478 characteristics of ass's milk. Animal Research 53, 67-78.
479 Shingfield K J, Bonnet M and Scollan ND 2013. Recent developments in altering the fatty
480 acid composition of ruminant-derived foods. Animal 7, 132–162.
481 Trinchese G, Cavaliere G, Canani RB, Matamoros S, Bergamo P, De Filippo C, Aceto S,
482 Gaita M, Cerino P, Negri R, Greco L, Cani PD and Mollica MP 2015. Human, donkey
483 and cow milk differently affects energy efficiency and inflammatory state by modulating
484 mitochondrial function and gut microbiota. Journal of Nutritional Biochemistry 26,1136-
485 1146.
486 Vlaeminck B, Fievez V,Cabrita ARJ, Fonseca AJM and Dewhurst RJ 2006. Factors affecting
487 odd- and branched-chain fatty acids in milk: A review. Animal Feed Science and
488 Technology 131, 389–417.
489

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		40	80	32
Milking jennies[1] (n.)	6	12	9	6	10	10
BW 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
Milking system	Automatic in milking room	Automatic in milking room	Hand milking	Automatic in cowshed	Automatic in milking room	Automatic in milking room
Milk yield (L/animal×d)	0.5	0.7	0.8	1.1	2.0	1.0
Feeding	Pasture 0% Hay 90% Cereal mix A[2]10%	Pasture 0% Hay 90% Cereal mix B[2]10%	Pasture 100%	Pasture 50% Hay 50%	Pasture 40% Hay 50% Cereal mix A[2]10%	Pasture 0% Hay 100%

[1]Counted during the visit.

[2]Cereal 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 vitamin contents of the donkey milk on the studied farms*

Milk constituents	Farm						SEM	Effect and significance	
	1	2	3	4	5	6		DIM	Farm
Fat (g/100 g milk)	0.13 [b]	0.17 [b]	0.36 [ab]	0.65 [a]	0.25 [b]	0.26 [b]	0.03	NS	**
Protein (g/100 g milk)	1.76 [b]	1.96 [a]	1.84 [b]	1.65 [b]	2.03 [a]	1.93 [b]	0.04	***	***
Lactose (g/100 g milk)	7.90	7.49	6.87	6.39	7.60	6.68	0.23	NS	NS
Retinol (µg/100 mL)	0.91 [b]	1.36 [ab]	3.04 [a]	2.78 [ab]	2.84 [ab]	1.82 [ab]	0.21	NS	**
α-Tocopherol (µg/100 mL)	3.13 [b]	5.80 [b]	25.79 [a]	19.31 [ab]	8.57 [b]	5.58 [b]	2.23	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 *Recoveries of the method used for the determination of retinol and α-tocopherol in donkey milk*

	Spiking level (µg/100 mL)	Recovery ± SD[1] (%)	RSD[2] (%)
Retinol	2.70	91.6 ± 1.67	1.82
	43.0	96.8 ± 8.51	8.80
	107	91.1 ± 4.95	5.43
	Mean of means	93.2 ± 3.16	3.39
α-Tocopherol	5.40	105 ± 4.25	4.02
	54.0	86.5 ± 3.04	3.52
	86.0	79.9 ±2.55	3.19
	Mean of means	90.7 ± 13.4	14.8

[1]SD= Standard Deviation (no = 3 replicates)

[2]RSD = relative standard deviation

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

Item	DIM[1]	Fisher's F[1]	
	Coefficient	DIM[1]	Farm
Protein (g/100 g milk)	-0.002069	6.55	22.48
Fatty acid (g/100g FA)			
C8:0	-0.006593	5.08	8.35
C14:1c9	0.000402	5.44	13.84
C15:0	0.000228	3.84	10.40
isoC16:0	0.000223	2.47	6.40
C17:0	0.000724	3.27	6.42
C18:1t11	0.000463	14.54	29.95
C18:2c9t12	0.000059	3.90	6.60
C18:3n-6	0.000114	8.50	11.10
C18:3n-3	0.026447	8.49	18.34
C22:0	0.000041	4.19	9.65
C20:3n-3+C22:1c13	0.000372	6.65	11.20
C20:4n-6	-0.000058	3.70	9.94
OCFA	0.001575	2.92	6.62
PUFA	0.02557	8.82	14.83
∑ n-3	0.028655	8.33	18.51
∑ n-6/∑ n-3	-0.000655	6.76	10.70
Trombogenicity Index	-0.000431	6.89	20.28

[1]DIM = days in milk; NS = not significant; FA = fatty acids; OCFA = odd chain-FA; PUFA = polyunsaturated FA; ∑ n-6 = sum of n-6 FA; ∑ n-3 = sum of n-3 FA.
† = $P < 0.1$.

Table 5 *Fatty acid composition of the donkey milk on the studied farms*

Fatty acids (g/100 g FA)	Farm						SEM	Effect and significance[1]	
	1	2	3	4	5	6		DIM	Farm
C4:0	0.60	0.57	0.31	0.53	0.85	1.02	0.09	NS	NS
C6:0	0.38 ab	0.50 a	0.33 b	0.32 b	0.41 ab	0.33 ab	0.02	NS	**
C8:0	4.53 ab	5.25 a	3.54 b	4.04 ab	4.48 ab	4.31 ab	0.18	**	**
C10:0	9.71 ab	9.42 ab	6.44 b	8.44 ab	9.45 ab	10.01 a	0.37	NS	*
C10:1c9	1.57 ab	1.26 ab	0.75 c	1.68 a	1.04 bc	1.26 ab	0.06	NS	***
C12:0	9.20 a	7.76 a	4.88 b	8.74 a	8.30 a	9.28 a	0.35	NS	**
C12:1c9	0.18 a	0.12 b	0.07 c	0.21 a	0.11 bc	0.14 ab	0.01	NS	***
isoC14:0	0.12 a	0.11 ab	0.07 bc	0.07 bc	0.12 a	0.06 c	0.01	NS	**
C14:0	7.52 a	6.60 a	4.10 b	7.52 a	6.61 a	7.44 a	0.25	NS	***
isoC15:0	0.11 ab	0.13 a	0.06 c	0.08 bc	0.08 bc	0.08 bc	0.01	NS	***
anteisoC15:0	0.10 b	0.13 a	0.06 c	0.05 c	0.08 bc	0.06 c	0.01	NS	***
C14:1c9	0.40 ab	0.29 bc	0.19 c	0.48 a	0.26 bc	0.40 ab	0.02	*	***
C15:0	0.44 a	0.38 b	0.29 cd	0.33 bc	0.34 bc	0.25 d	0.01	†	***
isoC16:0	0.23 a	0.24 a	0.15 b	0.15 b	0.16 ab	0.16 ab	0.01	*	***
C16:0	20.72	20.50	18.99	19.00	18.76	19.25	0.29	NS	NS
C16:1c9	3.78	3.32	3.79	3.28	2.45	4.47	0.20	NS	NS
anteisoC17:0	0.22 b	0.27 a	0.17 bc	0.19 bc	0.21 b	0.15 c	0.01	NS	***
C17:0	0.35 ab	0.47 a	0.22 ab	0.22 ab	0.30 ab	0.19 b	0.02	*	*
C17:1c9	0.43 a	0.41 a	0.35 ab	0.43 a	0.28 b	0.30 b	0.01	NS	**
C18:0	1.55 bc	1.86 ab	1.90 ab	1.01 c	2.02 a	1.49 bc	0.06	NS	***
C18:1t11	0.10 b	0.09 b	0.30 a	0.14 b	0.21 ab	0.11 b	0.02	**	***
C18:1c9	17.19 ab	17.64 ab	20.74 a	12.59 b	14.70 b	16.84 ab	0.67	NS	*
C18:1c11	1.28	1.31	1.38	0.89	1.02	1.45	0.05	NS	†
C18:2c9t12	0.031 ab	0.027 b	0.047 a	0.027 b	0.034 ab	0.026 ab	0.002	*	***
C18:2n-6	6.03 b	6.87 b	8.77 a	5.43 b	9.05 a	5.48 b	0.30	NS	***
C18:3n-6	0.061 b	0.071 b	0.097 ab	0.111 a	0.076 bc	0.076 bc	0.003	**	***
C18:3n-3	9.68 b	10.69 b	17.97 a	20.54 a	14.70 ab	11.99 b	0.70	***	***
C20:1c11	0.22 ab	0.23 ab	0.25 a	0.16 b	0.18 ab	0.20 ab	0.01	NS	*
CLAc9t11	0.06 b	0.06 b	0.09 a	0.07 b	0.07 b	0.07 b	0.00	NS	**
C20:2n-6	0.16 bc	0.15 bc	0.18 ab	0.11 c	0.21 a	0.11 c	0.01	NS	***
C22:0	0.022 a	0.018 ab	0.023 a	0.016 ab	0.020 a	0.011 b	0.001	**	**
C20:3n-3+C22:1c13	0.29 b	0.32 b	0.46 a	0.47 a	0.38 ab	0.33 b	0.01	*	***
C20:4n-6	0.043 bc	0.031 c	0.066 a	0.031 c	0.064 a	0.054 ab	0.003	†	***
C24:0	0.015 b	0.018 b	0.039 a	0.010 b	0.028 ab	0.020 b	0.002	NS	***
C22:5n-3	0.077 ab	0.047 b	0.113 a	0.076 ab	0.097 ab	0.077 ab	0.006	NS	**
ECSFA	54.31 a	52.60 a	40.62 b	49.65 ab	51.00 a	53.20 ab	1.03	NS	***
OCFA	1.41 a	1.53 a	1.09 b	1.24 ab	1.20 ab	1.00 b	0.05	*	*
BCFA	0.93 ab	1.10 a	0.63 c	0.66 bc	0.77 b	0.63 c	0.04	NS	***
MUFA	26.16	25.66	29.10	20.76	21.25	26.10	0.89	NS	NS
PUFA	16.71 b	18.56 b	28.11 a	27.14 a	24.98 a	18.45 b	0.89	**	***
∑ cis18:1	18.56 ab	19.06 ab	22.25 a	13.57 b	15.81 b	18.40 ab	0.72	NS	*
∑ trans18:1	0.18 b	0.19 b	0.46 a	0.22 b	0.38 ab	0.20 b	0.02	**	***
∑ n-6	6.36 b	7.19 b	9.17 a	5.71 b	9.47 a	5.78 b	0.31	NS	***
∑ n-3	10.09 b	11.11 b	18.61 a	21.17 a	15.24 ab	12.45 b	0.71	***	***
∑ 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	*
∑*de novo* synthesis FA	31.94 a	30.12 a	19.60 b	29.58 a	30.10 a	32.39 ab	1.08	NS	**
∑ CLA	0.12 b	0.12 b	0.17 a	0.13 b	0.13 b	0.11 b	0.00	NS	***

Atherogenicity Index	1.83	a	1.50	ab	0.75	d	1.11	c	1.11	c	1.61	ab	0.06	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

† $P< 0.1$; * $P< 0.05$; ** $P< 0.01$; *** $P< 0.001$.

Figure captions

Figure 1. *Principal component analysis performed on the main FA of the milk: plot of the variable*[1] *distribution and of the sample distribution.*

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