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

21

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42

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

44 **Abstract**

45 Donkey milk is considered a functional food for sensitive consumers, such as
46 children allergic to cow milk. No information is available regarding the effect of the
47 feeding system on the composition of donkey milk according to the feeding strategies
48 adopted on commercial farms. The study was aimed at evaluating the effect of the
49 feeding system and stage of lactation on the donkey milk gross composition, fat
50 soluble vitamins (retinol, α -tocopherol) and fatty acid (FA). Individual milk was
51 sampled from lactating jennies (n=53) on six farms located in North West Italy. The
52 performance of lactating jennies, the herd characteristics, milking management and
53 feeding strategies were recorded at each milk sampling. A greater effect of the
54 farming system and a limited effect of the lactation stage on the milk composition
55 were observed. The gross composition of the milk, and the fat-soluble vitamin
56 content differed according to the feeding system. A higher milk fat content
57 corresponded to a higher fresh herbage proportion in the diet. The highest
58 polyunsaturated FA (PUFA) content in the milk was observed for the animals fed on
59 only forage diets. The animals that were fed just pasture produced the milk with the
60 highest concentration of C18:1c9, C18:3n-3, n-3 FA, PUFA, retinol and α -tocopherol,
61 and the lowest concentrations of the FA less favorable for human health. The farms

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

67

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

70

71 **Implications**

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

80

81 **Introduction**

82 Donkey milk consumption is widespread in the Mediterranean area and, the EU
83 production is estimated to be about 300 tons per year (Eurolactis, 2016, personal
84 communication). The dairy donkey farms in the EU are mainly located in Italy, France,
85 Spain and Belgium (Salimei and Fantuz, 2012). Clinical studies have indicated that
86 donkey milk can be used successfully as an alternative to the available
87 hypoallergenic formulas for infants suffering from cow milk protein allergy (Monti *et*
88 *al.*, 2007). It has also recently been demonstrated *in vivo* that dietary
89 supplementation with donkey and human milk is associated with a decrease in
90 inflammatory status, and this decrease is in turn associated with an improvement in
91 the lipid and glucose metabolism, compared to a diet with a cow milk
92 supplementation (Trinchese *et al.*, 2015). The composition of donkey and human milk
93 are similar, in terms of average total solid, crude protein, lactose and ash content.
94 However, the fat content of donkey milk is lower than the fat content of human milk,
95 as it is in the 0.3 to 1.2 g/100 mL range. This difference is associated with a low
96 energy content (Salimei *et al.*, 2004; Medhammar *et al.*, 2012), which represents the
97 main limit to its use in the nutrition of children allergic to cow milk protein, during the
98 first year of life. However, the lipid fraction of donkey milk has shown a more
99 favorable fatty acid (FA) composition than that of the milk fat of ruminants, as it is
100 richer in polyunsaturated FA (PUFA) (Medhammar *et al.*, 2012). More in detail,
101 donkey milk fat has shown higher C18:3n-3 and n-3 FA concentration, and a lower
102 saturated FA (SFA) content than cow milk, as well as a lower n-6 to n-3 FA ratio
103 (Medhammar *et al.*, 201). On the other hand, equid milk appears to have a lower fat-
104 soluble vitamin content, that is, of α -tocopherol and retinol, than ruminant milk (Gentili
105 *et al.*, 2013; Álvarez *et al.*, 2015).

106 The variables that are significantly associated with changes in donkey milk
107 composition are (1) the lactation stage; (2) daily rhythms; and (3) the interval
108 between mechanical milkings (Salimei and Fantuz, 2012). However, feeding is also
109 believed to play a relevant role in milk yield and composition, since nutrient
110 absorption in equines precedes the ceco-colic fermentations of feeds (Doreau *et al.*,
111 2002). The feeding composition has been shown to be the main factor that affects
112 the FA composition of milk in ruminants (Shingfield *et al.*, 2013; Coppa *et al.*, 2015a).
113 In particular, pasture feeding increases in milk the concentrations of FA that are more
114 favorable for human nutrition, such as C18:3n-3, n-3 FA and conjugated linoleic acids
115 (CLA), and decreases the n6 to n3 ratio and the concentrations of FA less favorable
116 for human nutrition, such as C14:0, C16:0, and SFA (Coppa *et al.*, 2012; Farruggia *et*
117 *al.*, 2014). However, the effect of the feeding system on donkey milk composition has
118 only been studied so far in experimental conditions for a restricted group of FA
119 (Chiofalo *et al.*, 2005), and no information is available regarding the effect of the
120 feeding system on the FA composition of donkey milk according to the feeding
121 strategies adopted on commercial farms. Furthermore, changes in donkey milk fat-
122 soluble vitamins, as a result of the feeding system, have never been investigated.

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

126

127 **Materials and methods**

128 *Milk Sampling and Survey*

129 Individual milks were sampled (0.5 L) from 53 lactating jennies reared on six
130 commercial farms located in the Piedmont Region, in North West Italy, during Spring
131 2014. The performance of the lactating jennies and herd characteristics (number of
132 jennies, breed, DIM, milk yield, body condition scores (BCS), milking management,
133 feeding strategies, forage type and conservation methods adopted were recorded at
134 each milk sampling and characterized through a detailed on farm survey. The BCS
135 were determined as described by Burden (2012), and body weight according to
136 Pearson and Ouassat (2000). The farm characteristics, herd composition and diets of
137 the jennies are reported in Table 1. The milk samples were immediately refrigerated,
138 stored at -20°C and lyophilized within 72 h. The lyophilized samples were then stored
139 at -20°C.

140

141 *Milk Gross Composition Analyses*

142 The donkey milk samples were analyzed for fat, proteins, lactose and total solids
143 contents. The fat content and protein content were assessed as described by
144 Cavallarin *et al.*, (2015). The lactose content was determined by means of
145 spectrophotometric absorbance at 340 nm (Cary 60 UV-Vis, Agilent Technologies,
146 Santa Clara, CA), according to the AOAC 984.15 Official Method (2005).

147

148 *Milk Fat-soluble Vitamin Analysis*

149 The retinol and α -tocopherol in the milk samples were extracted according to the
150 Kuhl *et al.* (2012), with some adaptations. The retinol and α -tocopherol
151 concentrations were quantified according to Prola *et al.* (2013), by means of a HPLC
152 system (Dionex, Sunnyvale, CA, USA). The analytical column was an XTerra RP18
153 column (250-mm \times 4.6-mm, 5 μ m particles) (Waters, Milford, MA).

154 A calibration curve was obtained with two determinations of six concentration
155 levels of α -tocopherol and retinol standard solutions (Sigma-Aldrich, St. Louis, MO)
156 between 0.7 and 100 μ g/mL. The linearity was excellent ($R^2 = 0.999$). Recovery
157 experiments were performed by spiking blank donkey milk samples with retinol and
158 with α -tocopherol. The recoveries of the method were good, ranging from 91.1% to
159 96.8% (Table 3).

160

161 *Milk Fatty Acid Analysis*

162 Milk samples were analyzed for FA composition by gas chromatography (GC), as
163 described by Coppa *et al.* (2015b). The method was adapted to donkey milk,
164 because of the lower lipid content and its larger variation in donkey milk than in cow
165 milk. The lipids in 0.7 g of the lyophilized milk samples were methylated directly using
166 4 mL of 0.5 M sodium methanolate plus 1.5 mL of hexane for 15 min at 50°C, and
167 this was followed, after cooling, by the addition of 2 mL of 12 M HCl at 50°C for 15
168 min. Six mL of 6% K_2CO_3 water solution was added after cooling. The FA methyl
169 esters were separated as a supernatant after centrifugation and injected into a GC
170 equipped with a flame ionization detector, separating and identifying the FA methyl
171 esters as described by Coppa *et al.* (2015b), with the sole adaptation of the split ratio

172 to the lower fat content of donkey milk: a volume of 1 μ L was injected into the column
173 at a split ratio ranging from 2.5:1 to 100:1, according to the fat content of the sample.

174 *Statistics*

175 Statistical analyses were performed using the SPSS for Windows software package
176 (version 17.0; SPSS Inc., Chicago, IL). The milk composition data were processed
177 using the general linear model (GLM) of ANOVA, in which the farm was the fixed
178 factor and the lactation stage (DIM) was the covariate. The Bonferroni test was used
179 as the *post-hoc* test. A principal components analysis (PCA) was performed on the
180 main FA.

181 **Results**

182 *Milk Gross Composition and Fat-Soluble Vitamin Content*

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

190 The retinol content was within the 0.89 to 4.66 μ g/100 mL range, and α -tocopherol
191 was within the 2.14 to 38.40 μ g/100 mL range. A farm effect was seen for both
192 vitamins, with the highest levels being found in the milk on Farm 3 (Table 2).

193 *Milk Fatty Acid Composition*

194 The FA composition of the donkey milk differed significantly over the farms (Table 5,
195 and supplementary Table 1, for the detailed FA profile). The milk from Farm 3 showed
196 the highest concentrations of C18:1c9, total C18:1cis isomers, C22:5n-3,
197 CLAc9t11 and total CLA, and the lowest concentrations of C8:0, C12:0, C14:0, total
198 *de novo* synthesis FA, and even chain-saturated FA (ECSFA). The highest
199 concentration of C18:3n-3, PUFA, and n-3 FA and the lowest value of the
200 Atherogenicity and Thrombogenicity indexes were observed in the milk from Farms
201 3, 4 and 5. The odd chain-FA (OCFA) and branched chain-FA (BCFA) concentrations
202 were the highest in the milk from Farm 2 and the lowest in the milk from Farms 3 and
203 6, with intermediate values in the milk from Farms 4 and 5 for BCFA. The
204 OCFA/BCFA ratio showed the lowest value in the milk from Farm 2 and the highest
205 in the milk from Farms 3 and 4.

206 Only a few FA were affected to a great extent by DIM. An increase in the
207 concentrations of C14:1c9, C15:0, isoC16:0, C17:0, C18:1t11, C18:2c9t12, C18:2n-
208 6, C18:3n-3, C22:0, C20:3n-3+C22:1c13, OCFA, PUFA, total C18:1trans isomers
209 and n-3 FA increased with increasing DIM, whereas the concentrations of C8:0,
210 C20:4n-6, then-6/n-3 ratio and the Trombogenicity Index decreased with increasing
211 DIM. However, Fischer's F for those FA that showed a significant effect of both DIM
212 and farm were far higher for the farm effect than for the DIM (Table 4).

213 The results of the PCA performed on the main FA concentrations are given in Fig.
214 1. The PCA separated samples according to the farm in which milk was produced on
215 both the first principal component (PC1) and the second PC (PC2) (Figure 1). The
216 milk samples from Farm 3 were clearly separated from those of the other farms on
217 PC1, whereas the samples from Farm 4 was separated for Farm 3 and from Farms 1,

218 2 and 6 on both PC1 and PC2. The samples from Farm 5 were in an intermediate
219 position between those from Farm 4 and from Farms 1, 2, and 6, which were not
220 separated by the PCA (Figure 1). The first principal component (PC 1, 46.4% of
221 variance) was positively and closely correlated to ECSFA, the total *de novo* synthesis
222 FA, the Atherogeicity index and the Trombogenicity index (correlation coefficients >
223 0.88), while PUFA, n-3FA and total CLA were negatively correlated to PC1
224 (correlation coefficients < -0.76). PC2 (33.6% of variance) was highly positively
225 correlated with C16:0, C18:1c9 and MUFA (correlation coefficients > 0.80) and
226 negatively correlated with n-3 FA, total *de novo* synthesis FA and PUFA (correlation
227 coefficients < -0.53). The n-6/n-3 ration and the OCFA/BCFA texture also made
228 significant and positive contribution to PC2 and negative contribution to PC1,
229 respectively (correlation coefficients > 0.51 and < -0.46).

230 **Discussion**

231 *Effect of Lactation Stage on Donkey Milk Gross Composition*

232 The mean protein content of milk observed in the present study is in accordance with
233 previous data reported for donkey milk in Italy (e.g. Salimei *et al.*, 2004; Cavallarini *et al.*,
234 2015). The decrease in the protein content of the donkey milk during lactation is
235 in agreement with the findings of Salimei *et al.* (2004), Giosuè *et al.* (2008), Salimei
236 and Fantuz (2012) who reported overall values ranging from a maximum of 2.1 g/100
237 mL, at the beginning of lactation, to a minimum of 1.6 g/100 mL in late lactation.

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

239 The effect of lactation on the FA composition of donkey milk was studied by
240 Martemucci and D'Alessandro (2012), Gubić *et al.* (2015) and Martini *et al.* (2015).
241 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

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

270 *Effect of Feeding System on the Fat-soluble vitamin content of the milk*

271 Little is known about the fat-soluble vitamin content in donkey milk. Gentili *et al.*
272 (2013) and Clayer *et al.* (2014) reported the average contents of α -tocopherol and
273 retinol in donkey milk, and compared them with milk from other species. However,
274 the variations in fat-soluble vitamins in donkey milk fed different diets have never
275 been studied before. Álvarez *et al.* (2015) reported a concentration of retinol in milk
276 from mares fed at pasture that was double that reported by other authors for mares
277 fed hay (Khul *et al.*, 2012). Similarly, the amount of α -tocopherol and retinol in cow
278 milk was shown to double approximately when cows were fed at pasture instead of
279 conserved forages (Nozière *et al.*, 2006). These provitamin carotenoids originate
280 from β -carotene through enzymatic oxidative. As β -carotene is highly sensitive to
281 ultraviolet light, it is degraded into forages during herbage wilting in the field, and this
282 results in the hay having lower β -carotene contents than the fresh herbage (Nozière
283 *et al.*, 2006). Thus, the higher concentrations of α -tocopherol and retinol in the milk
284 from Farm 3 than in milk from the other farms are coherent with the high proportion of
285 fresh herbage in the donkey diet.

286 *Effect of Feeding System on the Fatty Acid Composition of the Milk*

287 The present results are the first evidence of the effect of feeding system on the
288 detailed milk FA profile of donkey milk on commercial farms, as the only study
289 available in literature, in which the FA composition of donkeys fed different diets was
290 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 Δ^9 -
322 desaturase 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

341 and BCFA in the milk of ruminants are mainly derived from the lipid membrane of
342 ruminal bacteria (Vlaemink *et al.*, 2006). Their concentration in cow milk varies
343 according to the shift in ruminal population due to the changes in ruminal substrate,
344 as a function of the different diets (Vlaemink *et al.*, 2006). In particular, forage-based
345 diets favor the cellulolytic bacteria population in rumen, and determine an increase in
346 BCFA in milk (Vlaemink *et al.*, 2006; Coppa *et al.*, 2015a). On the other hand, the
347 substitution of hay or pasture feeding with corn silage or cereal based-concentrates,
348 which are rich in starch, favors the ruminal population of amylolytic bacteria, with a
349 resultant increase in the milk concentration of OCFA and of the OCFA/BCFA ratio. In
350 addition, the concentration of BCFA in cow milk has also been negatively related to
351 the diet protein and total FA contents (Vlaemink *et al.*, 2006), that arise from legume
352 and oilseed supplementations. The main cellulolytic bacteria in cow rumen are
353 *Ruminococcus flavescentis*, *R. albus*, *Fibrobacter succinogenes* and *Butyrivibrio* sp.
354 These bacteria are also the main cellulolytic bacteria in the equine gastrointestinal
355 compartments (Sadet-Bourgeteau and Julliand, 2012; Costa *et al.*, 2015). Similarly,
356 *Megasphaera elsdenii* and *Streptococcus bovis*, which are among the main amylolytic
357 bacteria of cow rumen, are also important components of the gastrointestinal flora of
358 equines (Sadet-Bourgeteau and Julliand, 2012; Costa *et al.*, 2015). *Streptococcus*
359 *bovis* also plays a proteolytic role in cow rumen (Vlaemink *et al.*, 2006). The changes
360 in microbiota population in the gastrointestinal compartments of equine fed grass or
361 concentrate diets (Daly *et al.*, 2012) are also in line with the findings observed for the
362 ruminal population in cows (Vlaemink *et al.*, 2006), which would therefore suggest a
363 similar regulation mechanism of the microbiota in cow rumen and equine
364 gastrointestinal compartments. Even though the *de novo* synthesis of small amounts
365 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

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387

388 **References**

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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%	Pasture 0%			Pasture 40%	Pasture 0%
	Hay 90%	Hay 90%	Pasture 100%	Pasture 50%	Hay 50%	Hay 100%
	Cereal mix A ² 10%	Cereal mix B ² 10%		Hay 50%	Cereal mix A ² 10%	

¹Counted during the visit.

²Cereal 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	
	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	*

¹DIM = 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 ($\mu\text{g}/100\text{ mL}$)	Recovery \pm SD ¹ (%)	RSD ² (%)
Retinol	2.70	91.6 \pm 1.67	1.82
	43.0	96.8 \pm 8.51	8.80
	107	91.1 \pm 4.95	5.43
	Mean of means	93.2 \pm 3.16	3.39
α -Tocopherol	5.40	105 \pm 4.25	4.02
	54.0	86.5 \pm 3.04	3.52
	86.0	79.9 \pm 2.55	3.19
	Mean of means	90.7 \pm 13.4	14.8

¹SD= Standard Deviation (no = 3 replicates)

²RSD = relative standard deviation

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	DIM ¹ Coefficient	Fisher's F ¹	
		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

¹DIM = 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								NS	**
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	*	***

[†]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; \sum cis18:1= sum of cis isomers of C18:1; \sum trans18:1= sum of isomers of C18:1; \sum n-6 = sum of n-6 FA; \sum n-3 = sum of n-3 FA; \sum *de novo synthesis* FA = sum of even-chain SFA from C4:0 to C14:0; \sum 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 variable¹ 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; \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.