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Distribution of selected trace elements in the major fractions of donkey milk

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ABSTRACT

The aim of this study was to evaluate the concentrations of Zn, Cu, Mn, Se, Mo, Co, Li, B, Ti, Cr, Rb, Sr, Cd, and Pb in donkey milk and their distribution in major milk fractions (i.e., fat, casein, whey proteins, and aqueous phase). Individual milk samples were provided by 16 clinically healthy lactating donkeys. Subsequent centrifugation, ultracentrifugation, and ultrafiltration were carried out to remove fat, casein, and whey proteins to obtain skim milk, a supernatant whey fraction, and the aqueous phase of milk, respectively. Concentrations of the elements were measured in whole milk and fractions by inductively coupled plasma-mass spectrometry, and the concentrations associated with fat, casein, and whey proteins were then calculated. The effect of removal of fat, casein, and whey proteins was determined by repeated-measures ANOVA. The fat fraction of donkey milk carried a small (~4.5% to 13.5%) but significant proportion of Mo, Co, Ti, Cr, and Sr. The casein fraction in donkey milk carried almost all milk Zn, a majority of Cu and Mn, and most of Mo, Ti, and Sr. Relevant proportions, between 20% and 36%, of Se, Co, and Cr were also associated with caseins. The majority of Se, Co, Li, B, Cr, and Rb, and relevant proportions of Mn, Mo, Ti, and Sr were found in soluble form (ultracentrifuged samples) and distributed between whey proteins and the aqueous phase of milk (ultrafiltered samples). Whey proteins in donkey milk carried the majority of milk Se and Co. All Li and B was present in the aqueous phase of milk, which also contained most Rb and Cr, and 17% to 42% of Mn, Se, Mo, Co, Ti, and Sr.
Key words: dairy donkey, donkey milk, trace elements, colloidal minerals, soluble minerals

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INTRODUCTION

In the late nineteenth century, donkey milk was successfully used for feeding orphaned infants in France. The traditional use of donkey milk for the treatment of many illnesses has been reported in China, South America, and some African societies (Salimei and Fantuz, 2013, 2022; Papademas et al., 2022). In recent years, the donkey has gained interest as a species producing dairy foods for consumers sensitive to cow milk, such as infants, adults with inflammatory or allergic ailments, and healthy older people (Miraglia et al., 2020; Li et al., 2021; Papademas et al., 2022; Salimei and Fantuz, 2022). Clinical studies have shown that donkey milk can be used, when adequately supplemented, for children with IgE- and non-IgE-mediated cow milk allergy (Mansueto et al., 2013; Salimei and Fantuz, 2013; Sarti et al., 2019). A donkey milk–derived human milk fortifier has been shown to be suitable for feeding preterm and very-low-birthweight newborns, with a tendency to improve feeding tolerance and with similar auxological outcomes compared with standard bovine-derived fortifiers (Bertino et al., 2019). Donkey milk is currently marketed for human consumption in some countries in raw, pasteurized, and freeze-dried forms, and as fermented derivatives (Miraglia et al., 2020; Papademas et al., 2022).

In recent years, knowledge of donkey milk composition has greatly increased. The fat content of donkey milk (3–18 g/kg) is lower than that of human (35–40 g/kg) and cow (35–41 g/kg) milks (Fantuz et al., 2016; Papademas et al., 2022; Salimei and Fantuz, 2022). Furthermore, lactose (58–74 g/kg) and protein (13–19 g/kg) contents of donkey milk are similar to those of human milk (63–70 g of lactose/kg; 9–17 g of protein/kg) but different from those of cow milk (44–49 g of lactose/kg; 31–38 g of protein/kg). The casein:whey proteins ratio (55:45 in donkey milk) is similar to that of human milk (40:60) and markedly different from that of cow milk (80:20). Donkey milk contains 3 to 5 g/kg ash, which is slightly higher than human milk

(2–3 g/kg) but lower than cow milk (7–8 g/kg), and a high lysozyme concentration (~ 1 g/kg) compared with human (0.1 g/kg) and cow (trace) milks (Fantuz et al., 2016; Papademas et al., 2022; Salimei and Fantuz, 2022). The mineral components are less studied, and knowledge about the content of trace elements in donkey milk is sparse (Fantuz et al., 2013, 2015; Potorti et al., 2013; Bilandžić et al., 2014). Milk contains several trace elements, and some of them, including Zn, Cu, Mn, Se, and Mo, are known to be nutritionally essential, playing a primary role as cofactors of numerous enzyme activities. Other trace elements present in milk, such as Li, B, Cr, Ti, Rb, and Sr, are not considered essential but may have beneficial effects in animal and human diets (Haenlein and Anke, 2011; Nielsen, 2012).

Chemical elements occur in milk as inorganic ions or salts, or complexed with milk macromolecules (mainly proteins) or low-molecular-weight ligands such as citrate and amino acids (Vegarud et al., 2000; Gaucheron, 2013). Of the proteins in cow milk, casein has the main ability for binding chemical elements, and the distribution of minerals between the colloidal (associated with casein) and soluble forms affects the nutritional and technological properties of milk. In particular, Ca and P are of great importance for the stability of casein micelles and, consequently, for milk processing technology; the majority of Ca in cow milk is associated with the casein fraction (colloidal), directly bound to phosphoseryl residues of casein molecules, or bound to inorganic phosphorus, forming small granules called colloidal calcium phosphate (**CCP**) within the casein micelles (Dalgleish and Corredig, 2012; Gaucheron, 2013). Essential trace elements such as Zn, Cu, Mn, and Se are also distributed among milk fractions but with marked differences between cow and human milks (Fransson and Lönnerdal, 1983; Lönnerdal et al., 1985; Gaucheron, 2013), which may be related to the lower bioavailability observed for some essential trace elements in cow milk compared with human milk, using in vitro and animal models (Shen et al., 1995, 1996; Pabón and Lönnerdal, 2000). Only a few studies are available on the distribution of macrominerals in donkey milk (O'Connor and Fox, 1977; Malacarne et al., 2017; Fantuz et al., 2020), and information on trace elements is lacking.

The aim of this study, as part of a larger project on chemical elements in donkey milk, was to evaluate concentrations of Zn, Cu, Mn, Se, Mo, Co, Li, B, Ti, Cr, Rb, Sr, Cd, and Pb and their distribution in major milk fractions: fat, casein, whey proteins, and aqueous phase.

MATERIALS AND METHODS

Animals, Diet, and Sampling

Individual milk samples were obtained from 16 clinically healthy lactating donkeys (6 Amiata and 10 Ragusana breed), averaging (±SD) 7.38 (±1.54) yr old, 3.81 (±1.38) parities, and 126 (±56) d from foaling. Donkeys were reared on a private dairy farm producing donkey milk, located in a mountainous area of L'Aquila province, Italy (398 m above sea level; 42°17′11.34″ N; 13°45′43.57″ E). The research protocol was conducted in accordance with the European Commission guidelines (2010/63/EU; European Union, 2010) concerning the protection of animals used for experimental and other scientific purposes. The experimental donkeys had continuous access to alfalfa hay and water and were group-fed 1.5 kg/d of a concentrate mixture comprising corn and oats in equal parts. The donkeys were housed with their foals and were separated from them 3 h before the collection of milk samples by machine milking (Salimei et al., 2004) during the afternoon milking (1600 h). All glass and polyethylene tubes used for collection, storage, and analysis of samples were washed with a 3% HNO_3 solution (Suprapur quality, Merck).

Fractionation of Milk

The fractionation of individual milk samples was carried out as described by Fantuz et al. (2020). Briefly, whole milk samples (n = 16) were subjected to sequential centrifugation at 1,000 × *g* for 20 min at room temperature, ultracentrifugation at 100,000 × *g* for 60 min at 4°C (rotor 50 Ti, Beckman L7–55 centrifuge, Beckman Coulter), and ultrafiltration at 7,500 × *g* for 60 min at room temperature (Amicon, Ultra-4, 3-kDa cut-off; Sigma-Aldrich), to remove fat, casein, and whey proteins, and to obtain skim milk, a supernatant whey (soluble) fraction, and the aqueous phase of milk, respectively. Aliquots of whole milk, skimmed, ultracentrifuged, and ultrafiltered milk fractions were frozen at −21°C until analysis.

Analysis of Milk Fractions, Feedstuffs, and Drinking Water

All solutions were prepared using ultrapure water obtained from a Millipore Milli-Q system (resistivity 18.2 MΩ·cm). Mineralization of thawed individual whole milk samples (n = 16) and the corresponding skimmed, ultracentrifuged, and ultrafiltered fractions was carried out by placing 1.5 mL of sample in a Teflon digestion vessel, followed by the addition of 1 mL of HNO_3 (65%, Suprapur quality, Merck), 4 mL of H_2O_2, 0.5 mL of ultrapure water, and 50 μL of a solution (2 mg/L) of Be, Ru, and Au as recovery standard (Fantuz et al., 2020). The same procedure was used to mineralize 0.15 g of feedstuffs. A microwave closed-vessel system (Speedwave 4, Berghof) was used for mineralization. Mineralized solutions were transferred to a 10-mL volumetric flask and diluted with ultrapure water. Mineralized solutions were further diluted 1:10 with ultrapure water for the measurement of Zn and Rb. The concentrations of Zn, Cu, Mn, Se, Mo, Co, Li, B, Ti, Cr, Rb, Sr, Cd, and Pb in the mineralized solution and in acidified (1% HNO_3) drinking water were measured by inductively coupled plasma-MS (7500cx series, Agilent Technologies). The operating conditions were as described previously (Fantuz et al., 2020). Standard solutions (1% HNO_3) prepared with appropriate dilutions of stock standards (Fluka Analytical, Sigma-Aldrich) were used to obtain calibration curves for the investigated elements. The limits of detection (**LOD**), expressed as the concentration (μg/L) of each element in the mineralized solution, were calculated as 3 times the standard deviation of 10 repeated determinations of the blank, and were as follows: Li 0.025, B 0.867, Ti 0.502, Cr 0.133, Mn 0.147, Co 0.006, Cu 0.302, Zn 0.371, Se 0.096, Rb 0.014, Sr 0.125, Mo 0.067, Cd 0.061, and Pb 0.119. When less than 60% of the samples were below the LOD, such as for Mn (37.5% of samples <LOD) in ultracentrifuged samples, and for Mn (31.2% <LOD) and Se (50% <LOD) in ultrafiltered samples, results below the LOD were replaced by LOD/2 (EFSA, 2010). The concentration of Pb in whole milk was below the LOD in 43.7% of samples and results were also replaced by LOD/2, but this element and Cd were not further considered in the analysis of milk fractions. The accuracy of the analytical procedure was checked within each batch by analysis of blanks and certified reference material (skim milk powder ERM-BD151; European Reference Material). For the purpose of the present study, analytical results were in good agreement with certified values in reference material (certified values, mg/kg: Zn 44.9, Cu 5.0, Mn 0.29, Se 0.19, Cd 0.106, Pb 0.207; observed values, mg/kg, mean ± SD: Zn 43.38 ± 1.92, Cu 4.69 ± 0.14, Mn 0.264 ± 0.01, Se 0.195 ± 0.013, Cd 0.086 ± 0.003, Pb 0.184 ± 0.016).

The concentrations of elements associated with fat were calculated as the difference between their concentrations in corresponding individual whole and skim milks. The concentrations of elements associated with casein (colloidal form) and whey proteins were calculated as the difference between their concentration in skim milk and in ultracentrifuged samples (soluble form), and between their concentration in ultracentrifuged and ultrafiltered samples (aqueous phase), respectively. The percentage distributions of each element associated with fat, casein, whey proteins, or present in the aqueous phase were calculated and expressed with reference to their concentration in whole milk.

Table 1. Trace element concentrations in feedstuffs (mg/kg of DM; n = 1) and drinking water (μg/L; n = 1)

Element	Alfalfa hay	Corn	Oat	Water
Essential				
Zn	24.1	16.5	21.9	7.56
Cu	9.77	1.78	4.29	1.75
Mn	27.3	6.17	52.2	0.72
Se	0.05	0.04	0.14	0.22
Mo	0.51	1.70	0.63	0.68
Co	0.09	0.02	0.01	0.02
Nonessential				
Li	0.61	0.03	0.09	5.21
B	35.9	5.81	4.03	83.0
Ti	4.87	0.61	1.11	0.01
Cr	0.26	0.19	1.30	0.26
Rb	29.6	4.80	15.7	2.97
Sr	35.7	1.61	3.98	305.0
Cd	0.01	0.004	0.01	0.004
Pb	0.44	0.02	0.08	0.12

The concentrations of the investigated trace elements in feedstuffs and drinking water are given in Table 1. In the current study, milk yield per milking, milk chemical composition, including major elements concentration and distribution, and feedstuffs chemical composition were as previously published (Fantuz et al., 2020).

Statistical Analysis

The effect of milk fractionation (fat, casein, and whey proteins removal) was determined by repeated-measures ANOVA (SPSS version 25, IBM Corp.), using data on element concentrations in individual whole milk samples and in the corresponding skimmed, ultracentrifuged, and ultrafiltered fractions. The effect of breed and the covariate "days from foaling" were not significant in a preliminary analysis and excluded from the model. Least squares means were calculated using the following model:

$$y_{ij} = \mu + \alpha_i + \beta_j + \varepsilon_{ij},$$

where y_{ij} = dependent variable, μ = overall mean; α_i = milk fraction (i = 1 to 4); β_j = animal effect within milk fraction (j = 1 to 16); and ε_{ij} = error. In case of significant effects ($P < 0.05$), differences between means were analyzed by the least significant difference.

Table 2. Concentrations (μg/L) of essential trace elements in whole donkey milk and in skimmed (fat removed), ultracentrifuged (caseins removed), and ultrafiltered (aqueous phase after removal of whey proteins) milk samples (n = 16)

Element	Whole milk	Skimmed	Ultracentrifuged (soluble)	Ultrafiltered (aqueous phase)	SEM	*P*-value
Zn	2,730[a]	2,710[a]	105.4[b]	65.4[c]	43.6	<0.001
Cu	77.2[a]	76.4[a]	12.7[b]	2.91[c]	2.39	<0.001
Mn	4.71[a]	4.69[a]	0.92[b]	1.01[b]	0.21	<0.001
Se	4.13[a]	4.22[a]	2.77[b]	0.69[c]	0.16	<0.001
Mo	3.05[a]	2.63[b]	1.22[c]	0.99[d]	0.14	<0.001
Co	0.37[a]	0.34[b]	0.24[c]	0.10[d]	0.007	<0.001

[a–d]Means within a row with different superscripts differ ($P < 0.05$).

RESULTS AND DISCUSSION

Whole Milk

The concentrations of Zn, Cu, Mn, Se, Mo, Co, Li, B, Ti, Cr, Rb, and Sr, in whole milk and fractions are given in Table 2 and Table 3. The donkey milk concentrations of trace elements have not been extensively studied. Some published data on Zn concentration in donkey milk reported average values from approximately 2,200 to 2,300 μg/L (Fantuz et al., 2013; Bilandžić et al., 2014; Malacarne et al., 2019). Milk Zn concentrations of approximately 1,700 (Paksoy et al., 2018) and 2,000 μg/L (Fantuz et al., 2009) have been also reported. Our results agree with those of Potorti et al. (2013) and Martini et al. (2018), confirming that the average Zn concentration of donkey milk is generally within a range from 1,500 to 3,000 μg/L. However, Potorti et al. (2013) reported large and significant variations for Zn in donkey milk from 3 farms. Current results for Cu concentration in whole milk were slightly lower than our previous findings in donkey milk, and those for Mn, Se, Mo, Co, and Ti were similar (Fantuz et al., 2013, 2015). Compared with literature data on whole donkey milk, higher (~150 to 300 μg/L; Fantuz et al., 2009; Potorti et al., 2013; Malacarne et al., 2019) and lower (26 to 50 μg/L; Bilandžić et al., 2014; Paksoy et al., 2018) values for Cu, as well as higher values for Mn (15 to 30 μg/L) and Se (8.5 to 35 μg/L) were observed (Potorti et al., 2013; Bilandžić et al., 2014). Milk Cr was higher than published data for donkey and human milk but lower than data reported for cow milk (Björklund et al., 2012; Potorti et al., 2013; Bilandžić et al., 2015). The concentrations of Sr and Rb were, respectively, less than half and 4.2 times higher than in donkey milk samples from a dairy farm in northern Italy but consistent with values observed in milk samples from the present dairy farm located in central Italy (Fantuz et al., 2015). The concentration of Cd in whole milk was below the LOD and that of Pb was 0.78 (±0.11 SEM) μg/L, lower than previously observed in donkey milk (Potorti et al., 2013; Fantuz et al., 2015). Regarding Li and B, our data agree with that of Bilandžić et al. (2015) for Li (6.8 μg/L) and of Stergiadis et al. (2019) for B (176 μg/L) in cow milk. Compared with our results, Anderson (1992) reported higher B concentrations in cow (333 μg/L) and human (273 μg/L) milk and a lower concentration in mare milk (97 μg/L). However, in human milk, B has been observed at concentrations as low as 24 μg/L (Björklund et al., 2012), with Li ranging from 1.4 to 6.5 μg/L (Anderson, 1992). In addition to the analytical method used, a known cause of variability of trace element concentrations in donkey milk is the effect of lactation stage or season (Fantuz et al., 2013; Martini et al., 2018; Malacarne et al., 2019).

Table 3. Concentrations (μg/L) of nonessential trace elements in whole donkey milk and in skimmed (fat removed), ultracentrifuged (caseins removed), and ultrafiltered (aqueous phase after removal of whey proteins) milk samples (n = 16)

Element	Whole milk	Skimmed	Ultracentrifuged (soluble)	Ultrafiltered (aqueous phase)	SEM	*P*-value
Li	5.88[a]	5.83[a]	5.63[b]	6.16[a]	0.35	<0.01
B	209.3[a]	209.9[a]	203.8[b]	212.5[a]	5.40	<0.001
Ti	79.7[a]	74.0[b]	33.9[c]	33.2[c]	5.42	<0.001
Cr	2.17[a]	1.87[b]	1.45[c]	1.42[c]	0.07	<0.001
Rb	1,434[a]	1,397[a]	1,285[b]	1,229[c]	37.9	<0.001
Sr	370.4[a]	353.9[b]	94.8[c]	83.7[d]	9.79	<0.001

[a–d]Means within a row with different superscripts differ ($P < 0.05$).

Table 4. Distribution of essential elements in donkey milk, calculated as percentage (mean ± SEM) of that in whole milk and as concentration (μg/L; mean ± SEM) of elements associated with major fractions of donkey milk (n = 16)

Element	Associated with fat		Associated with caseins (colloidal)		Associated with whey proteins		In aqueous phase[1]	
	% of total	μg/L	% of total	μg/L	% of total	μg/L	% of total	μg/L
Zn	—	—	95.4 ± 1.70	2,604 ± 80.5	1.45 ± 0.51	39.9 ± 13.7	2.44 ± 0.26	65.4 ± 6.60
Cu	—	—	82.2 ± 1.87	63.8 ± 4.22	12.9 ± 0.71	9.75 ± 0.56	4.05 ± 0.44	2.91 ± 0.22
Mn	—	—	78.2 ± 4.13	3.76 ± 0.36	—	—	22.9 ± 2.83	1.01 ± 0.07
Se	—	—	35.8 ± 2.51	1.46 ± 0.11	50.4 ± 2.55	2.07 ± 0.16	16.8 ± 2.38	0.69 ± 0.10
Mo	13.6 ± 2.76	0.42 ± 0.09	45.8 ± 2.70	1.41 ± 0.12	7.95 ± 1.61	0.23 ± 0.05	32.7 ± 1.57	0.99 ± 0.08
Co	7.17 ± 1.72	0.03 ± 0.005	28.5 ± 1.43	0.10 ± 0.005	37.2 ± 2.38	0.14 ± 0.01	27.2 ± 1.32	0.10 ± 0.005

[1]Present in ultrafiltered milk samples.

Elements Associated with Fat

The effect of the complete milk fractionation was significant ($P < 0.01$) for all investigated elements (Table 2 and Table 3). The effect of fat removal was significant ($P < 0.05$) only for Mo, Co, Ti, Cr, and Sr (Table 2 and Table 3), indicating that the fat fraction of donkey milk carries a small but significant proportion of the mentioned elements, and no or very small amounts of Zn, Cu, Mn, Se, Li, B, and Rb (Table 4 and Table 5). Our results are in agreement with those on cow milk, in which fat is reported to carry a small percentage of total Zn (<2%), Cu (2%), Mn (1%), and Se (1%), but they differ from data on human milk, where between 15% and 18% of Zn, Cu, and Mn is associated with the fat fraction (Fransson and Lönnerdal, 1983; Lönnerdal et al., 1985; Debski et al., 1987; Van Dael et al., 1991; Xu et al., 2021). The essentiality of Mo is due to its requirement for the activity of a few Mo-containing enzymes, including xanthine oxidoreductase (**XOR**) in its 2 forms: xanthine dehydrogenase (**XDH**; EC 1.17.1.4) and xanthine oxidase (**XO**; EC 1.17.3.2); the association of XOR (XDH/XO) with milk fat globule membrane is well known in cow milk (Harrison, 2006; Silanikove and Shapiro, 2007). The presence of a small but significant proportion of Mo associated with donkey milk fat can be explained by the presence of XOR in this fraction, in agreement with findings from Li et al. (2019), who identified, by quantitative proteomic analysis, XDH as the major protein in donkey milk fat globule membrane. Unlike other essential trace elements, Co does not affect any enzymatic activity and its known biological role is as an essential constituent of cobalamin (vitamin B_{12}). Specific high-affinity cobalamin-binding proteins (i.e., haptocorrin and transcobalamin) exist in milk, with differences between species and between animal genetics (Fedosov et al., 1996, 2019). In donkey milk, transcobalamin has been identified as a protein expressed in the milk fat globule membrane (Li et al., 2019), which may explain the amount of Co associated with fat in the current study.

Elements Associated with Casein

The removal of casein from skimmed samples significantly ($P < 0.05$) reduced the concentrations of all elements in ultracentrifuged samples (Table 2 and Table 3). The casein fraction in donkey milk carries almost all milk Zn, the large majority of Cu and Mn, and the majority of Mo, Ti, and Sr. Relevant proportions, between 20% and 36%, of Se, Co, and Cr are also associated with caseins. Approximately 8% of milk Rb is associated with casein (Table 4 and Table 5). Compared with our results, the proportion of elements associated

Table 5. Distribution of nonessential trace elements in donkey milk, calculated as percentage (mean ± SEM) of that in whole milk and as concentration (μg/L; mean ± SEM) of elements associated with major fractions of donkey milk (n = 16)

Element	Associated with fat		Associated with caseins (colloidal)		Associated with whey proteins		In aqueous phase[1]	
	% of total	μg/L	% of total	μg/L	% of total	μg/L	% of total	μg/L
Li	—	—	—	—	—	—	104.8 ± 2.57	6.16 ± 0.36
B	—	—	—	—	—	—	102.0 ± 1.32	212.6 ± 4.45
Ti	7.27 ± 1.38	5.75 ± 1.15	50.0 ± 1.91	40.0 ± 2.06	—	—	41.8 ± 0.91	33.2 ± 0.76
Cr	10.4 ± 4.12	0.25 ± 0.09	20.2 ± 3.82	0.42 ± 0.08	—	—	68.5 ± 2.67	1.42 ± 0.04
Rb	—	—	7.94 ± 2.20	111.5 ± 31.3	4.04 ± 0.39	56.1 ± 4.57	87.2 ± 2.50	1,229 ± 22.1
Sr	4.48 ± 0.48	16.5 ± 2.05	69.8 ± 0.66	259.1 ± 12.4	3.04 ± 0.27	11.0 ± 0.87	22.7 ± 0.47	83.7 ± 3.55

[1]Present in ultrafiltered milk samples.

with casein (colloidal) in cow milk are reported to be similar for Zn (85–95% of total) and Mn (67–71%), but lower for Cu (45%; Fransson and Lönnerdal, 1983; Singh et al., 1989; Gulati et al., 2018; Xu et al., 2021). A higher proportion of milk Se (54–71%) is reported to be associated with casein in cow milk (Van Dael et al., 1991; Gulati et al., 2018). Conversely, the proportion of colloidal Se has been found to be limited to 29 to 45% in other studies on cow milk (Debski et al., 1987; Muñiz Naveiro et al., 2005; Liu et al., 2015), similar to our results. In human milk, the proportion of elements associated with casein is limited to 8, 7, 11, and 34%, respectively, for total milk Zn, Cu, Mn, and Se (Fransson and Lönnerdal, 1983; Lönnerdal et al., 1985; Debski et al., 1987). The only available data on the distribution of Mo in milk are from Gulati et al. (2018), who observed that 31% of the Mo present in cow skim milk is associated with the casein fraction. However, considering that a proportion of Mo is likely associated with the fat fraction via active XOR (Silanikove and Shapiro, 2007), this proportion would be reduced on a whole-milk basis, indicating that donkey casein binds a higher proportion of milk Mo compared with cow milk. Indeed, only 3.3% of XO activity is reported to be associated with casein in cow milk (Silanikove and Shapiro, 2007). The association of approximately 30% of total Co with casein in donkey milk can be due to the capacity of donkey casein in directly binding cobalamin by coordination bonds with histidine residues of caseins, as reported for cow milk when the amount of transcobalamin is not sufficient to bind all endogenous cobalamin (Fedosov et al., 2018, 2019).

In addition to the proportion of each element in milk fractions, milks from several species show marked differences in milk components (Salimei and Fantuz 2013; Fantuz et al., 2016), which results in variation in the amount of elements associated with each fraction. Based on the casein content in donkey milk (Fantuz et al., 2020), current results indicate that each gram of donkey casein carries approximately 420, 10, 0.60, 0.25, and 0.25 μg of Zn, Cu, Mn, Se, and Mo, respectively. Based on data on cow milk (Gulati et al., 2018), each gram of cow casein carries approximately one-third as much Zn and one-tenth as much Cu compared with current results. In contrast, twice as much Mn, Se and Mo was associated with each gram of cow casein. In cow milk, CCP is the major ligand for Zn. Singh et al. (1989) observed that Zn within the casein micelle is bound in 2 different forms: 63% of Zn in cow skim milk being tightly associated with CCP and 32% directly and loosely bound to casein phosphoserine residues. The casein fraction in donkey milk is reported to carry higher amounts of Ca and P, likely because of higher content of CCP in casein micelles compared with cow casein (Malacarne et al., 2017; Fantuz et al., 2020); this fact can explain the higher amount of Zn associated with the casein fraction in the current study compared with literature data on cow milk (Gulati et al., 2018). Furthermore, the fact that casein micelles are the main milk macromolecules able to bind bivalent cations such as Ca and Zn may help explain the high or relatively high proportions associated with casein also observed for Sr, and for Cu and Mn, at least in their bivalent status.

Elements in Soluble Form

The majority of Se, Co, Li, B, Cr, and Rb, and relevant proportions of Mn, Mo, Ti, and Sr were found in soluble form in the supernatant of ultracentrifuged samples (Table 2 and Table 3). Elements present in the ultracentrifuged samples (soluble) can be both partially associated with whey proteins dispersed in solution and partially present in true solution in the aqueous phase (ultrafiltered samples) (Gaucheron, 2005; Fox et al., 2015).

Elements Associated with Whey Proteins

The subsequent removal of whey proteins (>3 kDa) from the ultracentrifuged samples significantly ($P < 0.05$) reduced the concentrations of Zn, Cu, Se, Mo, Rb, and Sr, but not those of Mn, Ti, and Cr in the aqueous phase of milk (Table 2 and Table 3). Whey proteins in donkey milk carry the majority of milk Se and Co, 12.5% of Cu, approximately 8% of Mo, and <5% of Zn, Rb, and Sr (Table 4 and Table 5). Some major whey proteins in cow milk have been reported to bind trace elements. Lactoferrin binds Zn, Cu, Mn, and Co, in addition to Fe (Steijns and van Hooijdonk, 2000), β-LG binds Zn, Cu, and Mn, and both serum albumin and α-LA bind Cu (Vegarud et al., 2000; Hoac et al., 2007). In human milk, serum albumin binds Zn and Cu, and lactoferrin is the main ligand of soluble Mn (Fransson and Lönnerdal, 1983; Lönnerdal et al., 1985; Pabón and Lönnerdal, 2000). However, notwithstanding the presence of such proteins in donkey milk (Salimei and Fantuz, 2013; Fantuz et al., 2020), which can explain our results on Zn and Cu, we did not observe Mn to be associated with the whey protein fraction. The association of Cu with whey proteins observed in our study could be due to the presence in this fraction of Cu-containing enzymes, such as ceruloplasmin in its free status or bound to lactoferrin (Hoac et al., 2007). The majority of Co was associated with whey proteins in our study, probably because of the presence of soluble transcobalamin- or haptocorrin-cobalamin complexes (Fedosov et al., 2019) in this fraction. The low amount and proportion of Mo associated with whey proteins was likely due to the presence of XOR. In our study, approximately 40% of total Mo was in soluble form, consistent with the proportion of XO activity associated with the soluble fraction in cow milk (Silanikove and Shapiro, 2007); however, we observed that only about 8% of Mo was associated with whey proteins. The essentiality of Se is due to its requirement for the synthesis of 25 selenoproteins in humans in the form of the AA selenocysteine. In such proteins, selenocysteine is part of the catalytic group within the active site and is directly involved in oxidoreductase functions of most selenoproteins, including glutathione peroxidase (EC 1.11.1.9). In addition, Se, in the form of selenomethionine and selenocysteine, can be nonspecifically incorporated in other proteins typically rich in methionine and cysteine, whose S is replaced by Se. The presence of Se externally and specifically bound to the aminoacidic chain of some proteins as cofactor has been also demonstrated (Papp et al., 2007; Labunskyy et al., 2014). The majority of milk Se was found in soluble form in our study, similar to the findings of Debski et al. (1987) and Muñiz Naveiro et al. (2005) in cow and human milks. However, other studies reported that only a minority of milk Se is in soluble form in cow milk (Van Dael et al., 1991; Gulati et al., 2018). Furthermore, similar to previous observations of S distribution (Fantuz et al., 2020), most milk Se was associated with whey proteins in the current study. Although reports have indicated that cow and human milks contain glutathione peroxidase (Debski et al., 1987; Michalke, 2006) binding 15% to 30% of total Se in human milk (Milner et al., 1987), other studies did not detect the presence of the enzyme in the soluble fraction (Hoac et al., 2007) or demonstrate the lack of its activity in cow milk (Stagsted, 2006). Moreover, most of the soluble milk Se is associated with β-LG and α-LA in cow milk, because of the nonspecific incorporation of Se as selenomethionine into these 2 major whey proteins (Hoac et al., 2007).

Elements in the Aqueous Phase

Except for a small but significant ($P < 0.05$) reduction in ultracentrifuged milk fractions, it appeared that all Li and B was present in the aqueous phase of milk (Table 3, Table 5). The aqueous phase also contained the large majority of Rb; the majority of Cr; 17 to 42% of Mn, Se, Mo, Co, Ti, and Sr; and <5% of milk Zn and Cu (Table 4 and Table 5). The distribution of Li and Rb was consistent with that of other alkaline elements such as Na and K in milk of different species, including donkey (Fantuz et al., 2020), where they are present mostly free in the aqueous phase or weakly associated with ions of opposite charge (Gaucheron, 2013). The distribution of all milk B in the aqueous phase may be related to its presence as borate and boric acid, the main forms of B in aqueous solution (Kochkodan et al., 2015). The low proportion of milk Zn in the aqueous phase observed in our study probably reflects its association with low-molecular-weight ligands such as citrate, as occurs in cow milk (Fransson and Lönnerdal, 1983). Zinc citrate is the main form of this element in human milk, and it has been suggested that citrate also binds Cu and Mn (Fransson and Lönnerdal, 1983; Michalke, 2006; Hoac et al., 2007). Approximately one-fourth of milk Co was present in the aqueous phase in our study, not bound to transcobalamin or haptocorrin, which, due to their high molecular mass, may have been removed by ultrafiltration. Fedosov et al. (2018) reported that free cobalamin is absent in milk, thus the presence in donkey milk of Co bound to low-molecular-weight ligands or in inorganic form can be speculated. The same argument can apply to the 33% of milk Mo present in the aqueous phase, which is not associated with XOR due to the expected removal of this protein by ultrafiltration in our study. We observed that 25% of soluble Se, slightly less than that reported for cow milk, was present in the aqueous phase of donkey milk, likely related to the presence of selenomethionine in this fraction (Hoac et al., 2007). The presence of Mn in the aqueous phase of donkey milk could be due to the presence of a Mn-amino acid complex, possibly involving histidine, as suggested for cow and human milks (Lönnerdal et al., 1985).

CONCLUSIONS

Our results provide further data on trace element contents in donkey milk and add new knowledge on the distribution of trace elements in different milk fractions. The fat fraction of donkey milk carried low but significant amounts of milk Mo, Co, Ti, Cr, and Sr. The majority of essential trace elements were mainly associated with milk proteins but in different proportions and amounts compared with published data on cow and human milk. Almost all milk Zn, the majority of Cu, Mn, and Mo, and more than one-third of Se were associated with the casein fraction, whereas the majority of Se and Co was associated with whey proteins. Current results indicate that, compared with those in cow milk, casein micelles in donkey milk have higher affinity for Zn and Cu. Among the nonessential trace elements, the majority of milk Ti and Sr was associated with casein, whereas all Li and B and most of Cr and Rb were present in the aqueous phase. These data are important in animal and human nutrition and represent a basis for further studies about the chemical form and bioavailability of trace elements in donkey milk.

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