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Short communication: Technological and seasonal variations of vitamin D and other nutritional components in donkey milk

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ABSTRACT

Vitamin D is an essential nutrient that plays a crucial role in calcium homeostasis and bone metabolism and also acts as a hormone. Although several studies on the content of vitamin D in bovine milk have been conducted, little information is available regarding donkey milk. In the context of the nutritional assessment of donkey milk, the aim of this study was to assess the vitamin D content in donkey milk and its chemical profile, with particular reference to seasonal and technological modifications after pasteurization. The study was conducted on a dairy farm that produces donkey milk for human consumption located in central Italy. At sampling time, an aliquot of total bulk milk production was sampled before and after pasteurization (63°C for 30 min without homogenization) with a total of 20 raw and 20 pasteurized milk samples. The samples were collected for 10 mo, every 15 d, from May to February 2017. All the samples were analyzed for the chemical composition and vitamin D_2 and D_3 content by HPLC after saponification. The donkey milk analyzed showed a higher average vitamin D content (raw milk: vitamin D_2 = 1.68, vitamin D_3 = 0.60 μg/100 mL; pasteurized milk: vitamin D_2 = 1.38, vitamin D_3 = 0.30 μg/100 mL) than reported for bovine and human milk. The results of the effect of pasteurization on milk did not highlight significant differences in the total content of vitamin D. However, vitamin D_3 has a poor thermal stability, which led to a significant reduction in content in pasteurized milk compared with raw milk. The total vitamin D content of donkey milk did not show significant variations between seasons; however, a higher concentration of vitamin D_3 was found in spring and summer. In conclusion, raw and pasteurized donkey milk showed a high content of vitamin D, which could be useful in meeting the deficiencies of this vitamin in humans. Further investigations are needed to improve the vitamin D content in donkey milk by increasing its endogenous synthesis or its transfer in milk and to clarify other variability factors.
Key words: donkey milk, vitamin D, seasonal variability, pasteurization

Short Communication

Vitamin D plays a key role in calcium homeostasis and bone metabolism and acts as a hormone (Müller et al., 2011). Vitamin D_2 (ergocalciferol) is derived from the UV radiation of ergosterol (in particular UV-B radiation), which is a vitamin D precursor naturally found in plants, fungi, and invertebrates. Vitamin D_3 (cholecalciferol) is derived by sunlight exposure from 7-dehydrocholesterol, which is a precursor of cholesterol and also acts as a provitamin D_3 (Schmid and Walther, 2013).

The major source of vitamin D for children and adults is exposure to natural sunlight that is required for UV-B–induced vitamin D production in the skin. Vitamin D that comes from the skin or diet is biologically inert and requires its first hydroxylation in the liver to 25(OH)D_3. The latter requires a further hydroxylation in the kidneys to form the biologically active form of vitamin D1,25$(OH)_2$D.

An oral intake of vitamin D may be an important source in winter, when the UV-B–related synthesis is limited and for people who are not exposed to sunlight (Gill et al., 2016). Vitamin D deficiency is well known, and the concentration in blood serum of the hydroxylated form of calciferol [25(OH)D_3] is recognized as a sensitive accurate indicator of the functional status of vitamin D (Heaney, 2004). The Institute of Medicine (IOM, 2011) defined a vitamin D deficiency as a content of 25(OH)D_3 less than 20 ng/mL in serum. In addition, the widespread risk of deficiency and insufficiency worldwide has been reported (Holick et al., 2011), due to the current mostly indoor life style. In Italy, vitamin D deficiency and insufficiency were detected in 49.9 and 32.3% of adolescents, respectively, and 8.9% of Italian

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adolescents were severely deficient (Vierucci et al., 2014).

Vitamin D dietary intake is also of great importance, and animal foodstuffs (e.g., fish, meat, offal, eggs, and dairy) are the main sources for naturally occurring cholecalciferol (vitamin D_3). However, it is difficult to cover the requirements of vitamin D solely by foodstuffs (Schmid and Walther, 2013). Milk mainly contains 2 forms of vitamin D (D_3 and D_2).

Research has highlighted the variability factors of vitamin D in milk by analyzing endogenous, exogenous, and technological factors, such as season, age of the animal, treatment, and conservation (Jakobsen and Saxholt, 2009; Weir et al., 2017). However, most of the studies focus on bovine milk, whereas little information regarding donkey milk is available (Gentili et al., 2013; Bulgari et al., 2015). The properties of donkey milk have been known since ancient times, and in the last few decades there has been renewed interest from the scientific community due to donkey milk's use as a therapeutic product for children with bovine milk protein allergy (Martini et al., 2017).

Despite having a low bacterial count, the thermal treatment of donkey raw milk is recommended to avoid the risk of food-borne diseases (Martini et al., 2016). Pasteurization is known to eliminate pathogenic microorganisms that could be present in milk and guarantees its preservation. Furthermore, the literature on the effects of pasteurization on nutritional characteristics of donkey milk is not yet exhaustive. In addition, unlike cow milk that is mostly standardized, donkey milk has a certain variability in terms of its components.

Nutritional characteristics of donkey milk are especially interesting, as this milk is targeted for consumption by children for its similarity to human milk. In addition, consumption in the elderly has been proposed given that donkey milk has good calcium content, is low in fat, and easily digestible. Children and the elderly are at particular risk for developing nutritional deficiencies; therefore, they require careful nutrition management, from a quantitative and qualitative point of view, to avoid undernourishment and malnutrition. In the context of the nutritional assessment of donkey milk, the aim of our paper was to carry out an evaluation of the vitamin D content in donkey milk and its chemical profile, with particular reference to seasonal and technological modifications after pasteurization.

The study was conducted on dairy farm that produces donkey milk for human consumption. The farm, located in central Italy, raised about 160 Amiata donkeys, reared outdoors, in a semi-intensive system. The animals were fed about 2.5 kg/d per head of concentrate for dairy donkeys (Progeo S.C.A., Masone, Italy) and polyphite hay ad libitum. The jennies were routinely machine milked twice a day.

At sampling time, an aliquot of total daily bulk milk production was sampled before and after pasteurization (63°C for 30 min without homogenization). The samples were collected for 10 mo, every 15 d, from May to February 2017 (20 raw and 20 pasteurized milk samples), for a total of 40 samples.

All the samples were analyzed for DM, fat, and lactose content by infrared analysis (Milkoscan, Italian Foss Electric, Padova, Italy) as well as protein, casein, and ash (AOAC International, 2006). Individual mineral content (Ca, P, Mg, K, Na, and Zn; mg/L) was determined by atomic absorption spectroscopy and UV-visible spectroscopy according to Horwitz (2000) and Murthy and Rhea (1967). Milk fat extraction was performed following the Röse-Gottlieb method (933.05, AOAC International, 1995), and FAME were prepared using methanolic sodium methoxide according to Christie (1982). A Perkin Elmer Clarus 480 (Perkin Elmer, Norwolk, CT) equipped with a flame ionization detector and a capillary column (60 m × 0.25 mm; film thickness 0.25 mm; ThermoScientific TR-FAME 60 m × 0.25 mm ID; film thickness 0.25 μm, Thermo Fisher Scientific, Waltham, MA) were used.

The helium carrier gas flow rate was 1 mL/min. The oven temperature program level 1 was 50°C held for 5 min; level 2 was 50 to 140°C at 3°C/min, then held for 2 min; and level 3 was 140 to 240°C at 1°C/min, then held for 10 min. The injector and detector temperatures were set at 270 and 300°C, respectively. The peak areas of individual fatty acids (**FA**) were identified using an FA standard injection (Food Industry FAME Mix – Restek Corporation, Bellefonte, PA) and quantified as the percentage of total FA. In addition, nonadecanoic acid methyl ester (C19:0, Restek Corporation) was also used as an internal standard.

Milk fatty acids were grouped as SFA, MUFA, PUFA, and short-chain (**SCFA**), medium-chain (**MCFA**), and long-chain fatty acids (**LCFA**). The SFA included ΣC4:0, C6:0, C8:0, C10:0, C11:0, C12:0, C13:0, C14:0, C15:0, C16:0, C17:0, C18:0, C20:0, C21:0, C22:0, C23:0, and C24:0. The MUFA included ΣC14:1, C15:1, C16:1, C17:1, C18:1 *trans*-9, C18:1 *trans*-11, C18:1 *cis*-9, C20:1, C22:1, and C24:1. Thee PUFA included ΣC18:2 tranis-9,12, C18:2 *cis*-9,12, C18:3 n6, C18:3 n3, C20:2, C20:3 n6, C20:4, C20:3 n3, C20:5, C22:2, C22:5, and C22:6. The SCFA included the sum of FA from 4 to 10 C, MCFA included the sum of FA from 11 to 17 C, and LCFA included the sum of FA from 18 to 24 C.

For the determination of vitamin D, 75 mL of donkey milk were saponified by adding KOH pellets directly to the milk according to Perales et al. (2005). As a

Table 1. Effects of pasteurization (63°C for 30 min) on nutritional characteristics and vitamin D content of donkey milk (n = 40)[1]

Item	Raw milk	SE	Pasteurized milk	SE
Fat, %	0.15	0.021	0.15	0.022
Protein, %	1.64	0.054	1.58	0.057
DM, %	9.30	0.070	9.28	0.074
Ash, %	0.37	0.008	0.36	0.008
Ca, mg/L	647.25	49.969	674.69	47.314
P, mg/L	375.11	21.682	390.70	20.530
Mg, mg/L	101.31	10.740	101.72	10.167
K, mg/L	652.88	29.193	626.75	27.642
Na, mg/L	202.25	34.677	176.69	32.835
Zn, mg/L	2.88	0.560	3.28	0.530
SCFA, %	11.79	0.472	11.96	0.499
MCFA, %	42.60	0.877	41.61	0.927
LCFA, %	45.61	1.049	46.43	1.109
SFA, %	51.70	1.135	50.75	1.200
MUFA, %	28.56	0.773	29.29	0.818
PUFA, %	19.74	0.729	19.97	0.771
UFA/SFA	0.94	0.035	0.98	0.037
n-3/n-6	0.69	0.082	0.73	0.087
Total vitamin D, μg/100 mL	2.23	0.240	1.68	0.253
Vitamin D_2, μg/100 mL	1.64	0.211	1.38	0.223
Vitamin D_3, μg/100 mL	0.60[a]	0.127	0.30[b]	0.134

[a,b]Means within a row with different superscripts differ ($P \leq 0.05$).

[1]SCFA = short-chain fatty acids; MCFA = medium-chain fatty acids; LCFA = long-chain fatty acids.

hot temperature may lead to the isomerization of D vitamins, saponification occurred at 40°C for 32 min. Ethanol and double-distilled water were then added to the sample to remove the polar compounds and to avoid foaming. Afterward, the solution was transferred to a 500-mL separatory funnel, and an initial extraction of the unsaponifiable fraction was performed using 75 mL of hexane. The aqueous phase was thus drained and collected to repeat 2 extractions by adding 75 mL of hexane each time. Each time, the organic phase was collected in a rotary evaporator flask. Finally, the organic phase was evaporated to dryness on a rotary evaporator and the extract was resuspended in 500 μL of acetonitrile and filtered through a 0.45-μm diameter syringe filter. One hundred microliters of the extract were injected into an HPLC and isocratically eluted using as a mobile phase acetonitrile-methanol 97:3 at a flow of 1 mL/min, as described by Hagar et al. (1994). A Kinetex core-shell column (Phenomenex Inc., Torrance, CA) was used as the stationary phase and the UV detector was set at 254 nm. Vitamins D_2 and D_3 in the milk samples were quantified by comparison with a calibration curve obtained with the injection of the pure standards (Sigma-Aldrich, St. Louis).

All the results were analyzed by ANOVA, where season (autumn-winter or spring-summer) and thermal treatment (raw or pasteurized milk) were the fixed effects. Significant differences were considered at $P \leq 0.05$. The statistical analysis was carried out using JMP (2007) software.

The donkey milk analyzed showed a higher average vitamin D content (raw milk: vitamin D_2 = 1.68, vitamin D_3 = 0.60 μg/100 mL; pasteurized milk: vitamin D_2 = 1.38, vitamin D_3 = 0.30 μg/100 mL) than reported for bovine and human milk (Zhang et al., 2012; Schmid and Walther 2013). Our results shows that the main vitamin D form present in donkey milk was vitamin D_2.

The results related to the effect of heat treatment on milk (Table 1) did not highlight significant differences in the chemical composition either in relation to the total content of vitamin D or in the main constituents of milk and FA classes. Our results therefore indicate that pasteurization treatment at 63°C does not involve substantial changes to the quality of the milk, similar to results from other authors (Addo and Ferragut 2015; Martini et al., 2016).

As reported by Tsai et al. (2017), vitamin D_3 has a poor thermal stability, which entails a significant reduction ($P \leq 0.05$) of about 50% of vitamin D_3 in pasteurized milk compared with raw milk (Table 1). Despite the strong decrease in the vitamin D_3 content, the total vitamin D amount was only partially affected. In fact, the total vitamin D decreased but not significantly. This can be attributed to the large contribution of vitamin D_2, which has been described as heat-stable both after pasteurization at 63°C for 30 min and after sterilization (Kaushik et al., 2014).

Seasonal variations in donkey milk quality were found (Table 2). In particular, DM content and several mineral salts, such as phosphorus and zinc, were signifi-

Table 2. Effects of the season on the nutritional characteristics and vitamin D content of donkey milk (n = 40)[1]

Item	Autumn/winter	SE	Spring/summer	SE
Fat, %	0.14	0.020	0.16	0.018
Protein, %	1.57	0.048	1.64	0.042
DM, %	9.09[b]	0.079	9.46[a]	0.070
Ash, %	0.37	0.008	0.36	0.007
Ca, mg/L	603.36	53.706	699.38	44.015
P, mg/L	354.02[b]	23.304	402.17[a]	19.098
Mg, mg/L	90.71	11.541	108.75	9.458
K, mg/L	675.24	31.376	616.20	25.714
Na, mg/L	164.22	37.270	208.81	30.545
Zn, mg/L	2.16[b]	0.601	3.69[a]	0.493
SCFA, %	12.20	0.422	11.60	0.374
MCFA, %	41.63	0.805	42.52	0.712
LCFA, %	46.16	0.960	45.88	0.849
SFA, %	50.77	1.032	51.61	0.913
MUFA, %	30.57[A]	0.734	27.60[B]	0.650
PUFA, %	18.66[b]	0.680	20.78[a]	0.601
UFA/SFA	0.98	0.038	0.94	0.033
n-3/n-6	0.54[B]	0.083	0.84[A]	0.074
Total vitamin D, μg/100 mL	1.65	0.250	2.21	0.240
Vitamin D_2, μg/100 mL	1.49	0.224	1.53	0.210
Vitamin D_3, μg/100 mL	0.16[B]	0.133	0.68[A]	0.127

[a,b]Means within a row with different superscripts differ ($P \leq 0.05$).

[A,B]Means within a row with different superscripts differ ($P \leq 0.01$).

[1]SCFA = short-chain fatty acids; MCFA = medium-chain fatty acids; LCFA = long-chain fatty acids.

cantly ($P \leq 0.05$) higher in the spring and summer season. These findings are supported by a previous study by Martini et al. (2014), in which seasonal changes in milk quality were found and were suggested as being the result of the better adaptability of the Amiatina donkey to a warm and temperate climate.

In addition, significant differences in the FA classes were recorded, in particular regarding UFA. In fact, in the spring and summer season, we found a significantly lower content of MUFA ($P \leq 0.01$) and higher content of PUFA ($P \leq 0.05$) and n-3/n-6 ratio ($P \leq 0.01$), and therefore a greater incidence of n-3 on the total PUFA.

In vitro studies have found that the vitamin D-binding protein, the main carrier of vitamin D in blood and milk, competitively binds some MUFA (Williams et al., 1988; Calvo and Ena, 1989), such as C18:1 *cis*-9, which is one of the main MUFA in donkey milk (Martini et al., 2015). Although other factors may be involved in the decrease in the synthesis of MUFA in summer milk, competition for the carrier protein due to the increase in vitamin D secretion may to some extent contribute to the decrease in this family of FA in milk. However, this hypothesis needs further investigation.

In donkey milk, the total vitamin D content did not undergo significant variations; however, a higher ($P \leq 0.01$) concentration of vitamin D_3 was found in spring and summer than in autumn and winter, in particular the vitamin D_3 content was 4 times higher than in the autumn and winter. This is probably due to the different sun exposure of the animals facilitated by the outdoor farming system. Seasonal variations in vitamin D content of milk have been well documented in cows, with higher concentrations in the summer months than in the winter (Jakobsen and Saxholt, 2009; Weir et al., 2017)

In conclusion, raw and pasteurized donkey milk showed a high content of vitamin D. Although donkey milk is a niche product, the interest of our results derives from its use in consumers at risk of nutritional deficiencies. In fact, for these categories of people, donkey milk could be helpful (together with appropriate integrations) in meeting deficiencies of vitamin D.

Seasonal variations in vitamin D content as well as of other milk components were also highlighted, in particular the spring and summer season tends to increase the vitamin D_3 concentration in milk. Pasteurization affected the vitamin D_3 content, which is more thermolabile than D_2, but it did not influence the total vitamin D uptake. Further investigations are needed to improve the vitamin D content in donkey milk through increasing its endogenous synthesis or its transfer in milk and to clarify other variability factors.

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