Animal, page 1 of 8 © The Animal Consortium 2017
doi:10.1017/S1751731117000441

animal

Dietary verbascoside supplementation in donkeys: effects on milk fatty acid profile during lactation, and serum biochemical parameters and oxidative markers

A. G. D'Alessandro[1†], F. Vizzarri[2], M. Palazzo[2] and G. Martemucci[1]

[1]*Dipartimento di Scienze Agro-Ambientali e Territoriali, University of Bari, Via G. Amendola 165/A, 70126 Bari, Italy;* [2]*Dipartimento Agricoltura, Ambiente e Alimenti, University of Molise, Via De Sanctis snc, 86100 Campobasso, Italy*

(Received 14 June 2016; Accepted 30 January 2017)

Various uses of donkeys' milk have been recently proposed for human consumption on the basis of its nutritional characteristics. Improvements in milk fatty acid profile and animal oxidative status can be induced through dietary supplementation of phenolic compounds. The study aimed to evaluate in donkeys the effects of dietary supplementation with verbascoside (VB) on: (i) the fatty acid profile and vitamins A and E contents of milk during a whole lactation, and (ii) blood biochemical parameters and markers of oxidative status of the animals. At foaling, 12 lactating jennies were subdivided into two groups (n 6): control, without VB supplement; VB, receiving a lipid-encapsulated VB supplement. Gross composition, fatty acid profile and vitamins A and E contents in milk were assessed monthly over the 6 months of lactation. Serum total cholesterol, high-density lipoproteins cholesterol and low-density lipoproteins cholesterol, tryglicerides, non-esterified fatty acid, bilirubin, alanine aminotransferase (ALT), aspartate aminotransferase, reactive oxygen metabolites, thiobarbituric acid reactive substances (TBARs), vitamin A and vitamin E were evaluated at 8 days after foaling (D0) and then at D90, D105 and D120 of lactation. In milk, the VB supplementation decreased the saturated fatty acids ($P < 0.05$) and increased the monounsaturated fatty acids ($P < 0.05$), and vitamins A and E ($P < 0.01$) values. On the serum parameters, the VB supplementation decreased total cholesterol ($P < 0.01$), tryglicerides, bilirubin, ALT and TBARs, and increased ($P < 0.01$) vitamin E. In conclusion, the VB dietary supplementation affects the nutritional quality of donkey's milk with a benefit on the oxidative status and serum lipidic profile of the animals.

Keywords: donkey, verbascoside supplement, milk, fatty acid profile, blood biochemical parameters

Implications

In jennies, the dietary supplementation of verbascoside (VB), a natural extract from leaves of Verbenaceae, could be a strategy to modify the fat composition and vitamins A and E contents of milk, improving its nutritional quality. Moreover, the oxidative status of the animals can be positively affected by VB supplementation, resulting in a general improvement of the donkey's milk production system.

Introduction

The nutritional characteristics and the *in vitro* and *in vivo* effects of donkey's milk in humans has received scientific interest in recent years. The first potential use of donkey's milk in human feeding has concerned infants affected by cow milk protein allergy (Monti *et al.*, 2012) due to its similarity to human milk (D'Auria *et al.*, 2005), low allergenicity (Vita *et al.*, 2007) and good palatability (D'Alessandro and Martemucci, 2012). Donkey's milk has also been proposed as a functional food able to induce the release of anti-inflammatory interleukins, tumour necrosis factors-α and nitric oxide (Tafaro *et al.*, 2007).

Donkey's milk is characterized by a low milk fat content (Martemucci and D'Alessandro, 2012). Its consequent low energetic value may suggest a potential use in hypocaloric human diets (Martemucci and D'Alessandro, 2012). Moreover, the fatty acid profile shows a low content of saturated fatty acids (SFA), a high polyunsaturated fatty acids (PUFA) content, a low n-6 to n-3 fatty acid ratio and advantageous values of atherogenic and thrombogenic indices, which have been linked to potential health benefits in humans (Martemucci and D'Alessandro, 2012).

In humans, diet therapy is recognized as an important tool to reduce serum cholesterol levels (Malhotra *et al.*, 2014).

† E-mail: angelagabriella.dalessandro@uniba.it

Downloaded from https://www.cambridge.org/core. Universita Studi Molise, on 03 Jul 2017 at 14:34:37, subject to the Cambridge Core terms of use, available at https://www.cambridge.org/core/terms. https://doi.org/10.1017/S1751731117000441

The concentration of serum lipoproteins is mainly determined by the lipid present in the foods as SFA, monounsaturated fatty acids (MUFA) and PUFA, with the balance between lipid types more relevant than the total quantity of fat (Ascherio, 2002). High dietary intakes of SFA is a risk factor for the development of atherosclerosis and cardiovascular disease. In contrast, the reduction of SFA and the increase of unsaturated fatty acids improve human health (Simopoulos, 2008).

Moreover, dietary intake of fat-soluble antioxidants/vitamins (A and E) have beneficial effects on human health reducing oxidative stress, which is considered to be a risk factor for chronic conditions (Willcox *et al*., 2004). To improve the long-term health of consumers, there is considerable interest in reducing the harmful components of milk (SFA) and increasing those with beneficial effects (PUFA and PUFA/SFA ratio) by interventions in animal feeding (Martemucci and D'Alessandro, 2013). Dietary supplementation of phenolic compounds has been shown to have positive effects on the production and quality of milk, and dairy products (O'Connell and Fox, 2001) as well as on health status of the animals (Martemucci and D'Alessandro, 2013).

In a study by D'Alessandro *et al.* (2014), a short period of dietary supplementation with VB in lactating jennies resulted in influencing the serum lipidic and hepatic profiles, and the oxidative status of dams and of their suckling foals. The aim of this study was to determine whether the long-term dietary supplementation of jennies with VB affects the fatty acid composition and vitamins A and E contents of the milk during a whole lactation. The effects of the dietary supplementation on selected serum biochemical parameters and markers of oxidative status of the animals were also assessed.

Material and methods

The animals were handled following the recommendations of European Union directive 86/609/EEC and Italian law 116/92 regarding animal care.

The trial was carried out in southern Italy on a total of 12 adult lactating jennies of Martina Franca breed kept on the same farm and routinely milked twice a day (D'Alessandro and Martemucci, 2012). The trial lasted the whole lactation of 6 months. The animals were healthy and in good condition (body condition score (BCS): 3.0 to 3.5 of a scale 0 to 5; D'Alessandro and Martemucci, 2012) at the beginning and throughout the study period. The jennies were bred following a semi-extensive breeding system which is based on the use of natural scrub pasture (7 to 10 h of grazing) with supplementation of meadow hay (3 to 4 kg /day per jenny), wheat straw (2 kg/day per jenny) and concentrate (mixture of oats, corn flakes and bran; 2.0 to 2.5 kg of grain/day per jenny), and water *ad libitum*.

At foaling, the jennies were subdivided into two experimental groups (*n* 6) corresponding to control group, receiving no antioxidant supplement; and VB group, receiving antioxidant supplement containing a lipid-encapsulated VB (5 mg VB/g supplement, corresponding to 10 to 12.5 g VB/jenny). The two groups were matched for age (6 to 10 years), weight (310 ± 20 kg) and BCS (D'Alessandro and Martemucci, 2012; 3.0 to 3.5 of a scale 0 to 5).

The antioxidant supplement contained a water-soluble extract from leaves of Verbenaceae (*Lippia spp.*), titrated in phenyl propanoid glycosides expressed as verbascoside (Sintal Zootecnica, Isola Vicentina, Vicenza, Italy). The composition of the antioxidant feed supplement as phenolic compounds was as follows: gallic acid, 1.75 ± 0.07; 3,4-dihydroxybenzoic acid, 0.45 ± 0.04; methyl gallate, 1.91 ± 0.09; iso-VB, 0.43 ± 0.04; and VB, 4.47 ± 0.08 g/kg (Rastrelli L., Personal communication).

Milk sampling and analyses

Individual milk samples were collected monthly over the lactation period (6 months). Each sample was split into different aliquots for the analyses of the chemical gross composition, fatty acid profile, and vitamins A and E concentrations. The samples were then preserved (−20°C) until analysis.

The contents of fat, protein, casein and lactose of milk were analysed using an IR milk analyser (Milkoscan 6000; FOSS Italia S.r.l., Padova, Italy) previously calibrated for donkey's milk according to Federation Internationale dé Laiterie (International Dairy Federation) (2000).

The fatty acid composition was determined on milk fat according to Folch *et al.* (1957). The fatty acid composition was estimated using methyl esters prepared by direct transesterification, according to IUPAC (International Union of Pure and Applied Chemistry, 1987). The analyses were performed by a gas chromatograph Trace model 2000 series (ThermoQuest, CE, Instruments, Italy) features an open-flame ionizing detector to 270°C. The fatty acids were separated using a CP-Sil 88 fused-silica capillary column (100 m × 0.25 mm i.d. × 0.2 μm film thickness; Chrompack, Middleburg, the Netherlands). The injector temperature was 250°C and the detector temperature was 270°C. The separation was carried out at pre-programmed temperatures according to Casamassima *et al.* (2014b). The total race time was 102 min. Identification of fatty acids was performed using an external standard (Supelco TM 28 Hunger components mix; Sigma Chemical Co., St. Louis, MO, USA). Fatty acid peaks were identified by a comparative analysis with the retention times of pure standard of known concentration. Their content has been expressed as percentages of total identified fatty acids, and the atherogenicity index and the trombogenic index were calculated (Ulbricht and Southgate, 1991).

Vitamins A and E were extracted from milk samples with ethanol and chloroform (Zhao *et al*., 2004) and analysed on an HPLC system (Kontron Instruments, Milano, Italy) consisting of an autosampler (HPLC Autosampler 360; Kontron Instruments) with a 20 μl loop, two mixed pumps (HPLC Pump 422; Kontron Instruments, Milano, Italy) and a 5 μm, 250 × 4.60 mm C18 column (Phenomenex, Torrance, CA, USA). The mobile phase was 75% nitrile acid and 25% methanol (75 : 25 v/v) at a flow rate of 1.0 ml/min. A fluorimeter detector (Kontron model FMS 25) and computer with Kroma System 2000 software were used. Vitamins A and E were identified by comparing the retention time of the pure (>97%) standards (Sigma Aldrich, St. Louis, MO, USA). The concentrations were determined

Downloaded from https:/www.cambridge.org/core. Universita Studi Molise, on 03 Jul 2017 at 14:34:37, subject to the Cambridge Core terms of use, available at https:/www.cambridge.org/core/terms . https://doi.org/10.1017/S1751731117000441

using Kroma System 2000 (version 1.8.1) comparing peak area with standard reference curves.

Blood sampling and analyses

The blood samples were collected from five jennies for each of the two experimental groups 8 days after foaling (D0) and then at D90, D105 and D120 of lactation.

Blood samples were taken before the morning feeding in Vacutainer tubes (Venoject, Terumo, Europe N.V., Leuven, Belgium) with gel separator (for serum collection) or with ethylenediamine tetraacetic acid (for plasma collection). The tubes for serum collection were allowed to clot at room temperature for 3 h before centrifugation at 3000 r.p.m. for 15 min; the serum was frozen at −20°C until analysis. The tubes for plasma collection were immediately placed on ice, and within 2 h of bleeding were centrifuged at 2500 **g** for 10 min and then stored at −20°C until analysis.

The levels of total cholesterol, high-density lipoproteins cholesterol (HDL-C) and low-density lipoproteins cholesterol (LDL-C), tyglicerides, bilirubin, alanine aminotransferase (ALT) and aspartate aminotransferase were determined on serum using an automated clinical chemistry analyser (model ARCO; Biotecnica Instruments S.p.a., Rome, Italy). The non-esterified fatty acid concentrations were determined using a spectrophotometer (550 nm wavelength) with a commercially available kit (Randox Laboratories, Crumlin, UK). Plasma reactive oxygen metabolites (ROMs) were measured by the analytical method patented by Diacron (Cesarone *et al*., 1999). The results were expressed in Carr units (1 Carr unit equals 0.024 mmol/l of H_2O_2).

Thiobarbituric acid reactive substances (TBARs) in plasma were measured by the method of Esterbauer and Zollner (1989). The results are expressed as millimoles of thiobarbituric acid per litre of plasma.

Vitamins A and E were extracted from plasma samples and determined according to the procedures previously described for the analyses of vitamins A and E in milk.

Statistical analysis

All data were analysed using the GLM procedure for repeated measures (SPSS, 2010) and the animal entered the model as repeated factor. For the data on chemical composition, fatty acids profile and vitamins A and E contents of the milk analysis included the fixed effects of dietary supplement, the month of lactation, their interaction (dietary supplement × month of lactation) and an error term. The data were presented as mean values of each group and with the relative standard error; differences were tested by Scheffé test and considered significant for at least $P < 0.05$. For the blood biochemical parameters analysis included the fixed effects of dietary supplement, the day of blood sampling, their interaction (dietary supplement × day of sampling) and residual error (SPSS, 2010). Pretreatment values were used as covariate for blood parameters.

Results

Milk composition

There was no consistent difference in the content of fat, total protein, casein and lactose contents between milks from jennies supplemented with VB and that of the control group (Table 1).

The proportion of the total SFA was affected ($P < 0.05$) by the VB supplementation and lactation stage of the jennies (Table 2). The addition of VB to the diet led to lower ($P < 0.05$) values of total SFA. The SFA values in the VB group were lower ($P < 0.05$) at 4th and 6th months in comparison with those of the control group. No significant variations were observed in the control during the lactation period.

Among the individual SFA, the dietary VB supplementation affected lower concentrations of C6:0 ($P < 0.01$) and C10:0 ($P < 0.01$) acids, and greater levels for C15:0 ($P < 0.05$) and C18:0 ($P < 0.01$) acids (Table 3) in comparison with the control. Lactation stage affected the proportions of C14:0 ($P < 0.05$), C17:0 ($P < 0.01$), C18:0 ($P < 0.01$) and C20:0 ($P < 0.01$) acids (Table 3).

The total MUFA content of milk was affected by the VB supplementation ($P < 0.05$) and lactation stage ($P < 0.01$) (Table 2). In the VB group, the MUFA values at the 3rd, 4th, 5th and 6th months were greater ($P < 0.05$) in comparison with that of the 1st month.

The profile of MUFA is reported in Table 4. The main effect ($P < 0.05$) of VB supplementation was obtained for C16:1 acid, which showed higher levels ($P < 0.05$) from the 2nd to the 6th months of lactation, in comparison with the control group. The concentration of oleic acid was influenced ($P < 0.01$) by the lactation stage increasing with the increase of lactation time in both the control and VB groups (Table 4).

Table 1 *Effect of dietary verbascoside (VB) supplementation on gross composition of donkeys' milk during 6 months (M) of lactation*

	Fat (g/100 g)			Protein (g/100 g)			Casein (g/100 g)			Lactose (g/100 g)		
M of lactation	Control	VB	SEM	Control	VB	SEM	Control	VB	SEM	Control	VB	SEM
1 M	0.71	0.68		1.80	1.83		1.48	1.53		6.72	6.70	
2 M	0.63	0.60		1.76	1.79		1.44	1.48		6.70	6.68	
3 M	0.53	0.52		1.75	1.77		1.39	1.45		6.68	6.65	
4 M	0.55	0.52		1.72	1.76		1.36	1.43		6.75	6.82	
5 M	0.51	0.56		1.69	1.72		1.34	1.43		6.63	6.70	
6 M	0.50	0.55	0.178	1.60	1.65	0.23	1.30	1.40	0.18	6.60	6.64	0.20

Control = diet without VB; VB = diet supplemented with verbascoside.

Downloaded from https:/www.cambridge.org/core. Universita Studi Molise, on 03 Jul 2017 at 14:34:37, subject to the Cambridge Core terms of use, available at https:/www.cambridge.org/core/terms . https://doi.org/10.1017/S1751731117000441

Table 2 *Effect of dietary verbascoside (VB) supplementation on summarized fatty acid classes and indices of nutritional quality of fatty acids in donkey's milk during 6 months (M) of lactation*

				P-value		
Items	Control	VB	SEM	Diet	Time	Diet × time
SFA (g/100 g)						
1 M	67.96	68.40[a]	2.16			
2 M	68.39	66.47	2.17			
3 M	66.97	64.03	3.13			
4 M	65.99[c]	63.35[db]	3.49			
5 M	66.62	65.06	2.31			
6 M	67.65[c]	66.45[d]	1.63	0.028	0.049	0.301
MUFA (g/100 g)						
1 M	13.28	12.57[a]	1.23			
2 M	13.34	14.44	1.27			
3 M	13.87	15.36[b]	1.62			
4 M	14.32	15.95[b]	1.80			
5 M	14.75	15.78[b]	1.80			
6 M	15.05	15.76[b]	1.47	0.048	0.010	0.134
PUFA (g/100 g)						
1 M	18.76	19.03	2.13			
2 M	18.27	19.10	1.41			
3 M	19.17	20.63	1.97			
4 M	19.69	20.74	1.78			
5 M	18.63	19.22	1.22			
6 M	17.30	17.86	0.75	0.117	0.078	0.282
n-3 (g/100 g)						
1 M	6.56	6.67	0.13			
2 M	6.85	6.70	0.13			
3 M	6.61	6.79	0.19			
4 M	6.59	6.88	0.17			
5 M	6.67	6.97	0.19			
6 M	6.46	6.94	0.19	0.056	0.199	0.256
n-6 (g/100 g)						
1 M	12.21	12.36	0.58			
2 M	11.42	12.40	0.51			
3 M	12.59[a]	13.84[a]	0.66			
4 M	13.11[a]	13.87[a]	0.64			
5 M	11.96	12.25	0.56			
6 M	10.84[b]	10.92[b]	0.19	0.174	0.048	0.366
Atherogenic index						
1 M	1.50	1.60[a]	0.05			
2 M	1.54	1.47	0.05			
3 M	1.46	1.32	0.08			
4 M	1.40[a]	1.29[b]	0.08			
5 M	1.54	1.50	0.05			
6 M	1.63[b]	1.53	0.04	0.061	0.041	0.159
Thrombogenic index						
1 M	0.71	0.78[a]	0.03			
2 M	0.70	0.71	0.04			
3 M	0.59	0.57[b]	0.03			
4 M	0.63	0.62	0.03			
5 M	0.70	0.71	0.02			
6 M	0.80	0.76	0.02	0.059	0.049	0.455

Control = diet without VB; VB = diet supplemented with verbascoside; SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids.
[a,b,c,d]Values with different superscripts letters differ significantly at $P < 0.05$.

Table 3 *Effect of dietary verbascoside (VB) supplementation on the profile of saturated fatty acids in donkey's milk during 6 months (M) of lactation*

				P-value		
Fatty acid (g/100 g)	Control	VB	SEM	Diet	Time	Diet × time
C4:0						
1 M	0.77	0.67	0.14			
2 M	0.79	0.71	0.08			
3 M	0.93	0.76	0.12			
4 M	0.84	0.78	0.13			
5 M	0.83	0.83	0.15			
6 M	0.77	0.81	0.10	0.101	0.165	0.234
C6:0						
1 M	1.29	1.17	0.08			
2 M	1.33	1.22	0.10			
3 M	1.38	1.32	0.10			
4 M	1.39	1.39	0.05			
5 M	1.35	1.23	0.05			
6 M	1.26	1.22	0.03	0.014	0.004	0.263
C8:0						
1 M	12.73	12.33	0.99			
2 M	12.62	12.07	1.10			
3 M	12.38	12.05	10.01			
4 M	12.48	12.16	0.92			
5 M	12.01	11.84	0.98			
6 M	12.05	12.34	0.83	0.247	0.186	0.284
C10:0						
1 M	19.99	18.57	1.08			
2 M	20.42	18.66	1.19			
3 M	20.33	18.17	1.70			
4 M	20.04	17.76	1.78			
5 M	19.14	17.12	1.79			
6 M	19.03	17.78	1.31	0.001	0.153	0.232
C11:0						
1 M	0.41	0.31	0.15			
2 M	0.43	0.42	0.12			
3 M	0.39	0.31	0.15			
4 M	0.44	0.41	0.13			
5 M	0.49	0.47	0.14			
6 M	0.43	0.28	0.11	0.149	0.134	0.308
C12:0						
1 M	8.41	8.48	1.62			
2 M	8.45	8.04	1.46			
3 M	8.65	7.69	1.63			
4 M	8.44	7.35	1.71			
5 M	7.81	7.39	1.64			
6 M	6.75	6.58	0.83	0.260	0.150	0.263
C13:0						
1 M	0.13	0.12	0.04			
2 M	0.14	0.13	0.03			
3 M	0.13	0.13	0.03			
4 M	0.13	0.12	0.02			
5 M	0.13	0.11	0.04			
6 M	0.13	0.11	0.04	0.318	0.181	0.346
C14:0						
1 M	6.22	6.06	0.76			
2 M	6.38	6.41	0.81			
3 M	6.43	6.45	0.74			
4 M	6.48	6.65	0.73			
5 M	7.13	7.13	0.30			
6 M	7.22	7.07	0.43	0.557	0.037	0.397

Downloaded from https://www.cambridge.org/core. Universita Studi Molise, on 03 Jul 2017 at 14:34:37, subject to the Cambridge Core terms of use, available at https://www.cambridge.org/core/terms
. https://doi.org/10.1017/S1751731117000441

Table 3: (*Continued*)

Fatty acid (g/100 g)	Control	VB	SEM	*P*-value Diet	Time	Diet × time
C15:0						
1 M	0.37	0.36	0.09			
2 M	0.35	0.42	0.09			
3 M	0.39	0.44	0.08			
4 M	0.37	0.48	0.13			
5 M	0.40	0.44	0.08			
6 M	0.41	0.46	0.09	0.050	0.174	0.146
C16:0						
1 M	14.63	17.35	3.38			
2 M	14.65	15.23	3.83			
3 M	13.12	13.64	2.58			
4 M	12.56	12.99	3.03			
5 M	14.73	15.37	2.02			
6 M	17.06	16.53	0.90	0.345	0.087	0.306
C17:0						
1 M	0.44	0.40	0.09			
2 M	0.49	0.44	0.13			
3 M	0.47	0.47	0.40			
4 M	0.46	0.46	0.13			
5 M	0.39	0.37	0.90			
6 M	0.39	0.42	0.11	0.418	0.001	0.285
C18:0						
1 M	2.41	2.41	0.43			
2 M	2.17	2.53	0.39			
3 M	2.09	2.39	0.12			
4 M	2.22	2.64	0.46			
5 M	2.07	2.62	0.49			
6 M	2.01	2.68	0.56	0.005	0.001	0.237
C20:0						
1 M	0.17	0.17	0.06			
2 M	0.18	0.17	0.04			
3 M	0.59	0.48	0.20			
4 M	0.16	0.16	0.03			
5 M	0.14	0.15	0.04			
6 M	0.15	0.17	0.03	0.172	0.012	0.233

Control = diet without VB; VB = diet supplemented with verbascoside.

The total PUFA content of milk was not affected by the VB supplementation (Table 2). Among the PUFA, linoleic acid level was improved ($P = 0.06$) by the VB supplementation throughout the lactation period (Table 5). The lactation stage influenced ($P < 0.05$) the levels of γ-linoleic and C20:2n-6 acids.

The nutritional quality indices of milk were improved ($P = 0.06$) by the VB supplementation showing greater contents of total PUFA n-3 and lower values of atherogenic and thrombogenic indices in comparison with the control diet (Table 2). Lactation stage also affected ($P < 0.05$) total PUFA n-6 content of milk, and the atherogenic and thrombogenic indexes (Table 2).

The VB supplementation affected nutritional quality of milk increasing significantly ($P < 0.01$) the vitamins A and E contents (Table 6). In particular, greater levels of vitamin A were observed during the last 3 months of lactation (4th to 6th months), and from the 2nd to 6th months for vitamin E. Lactation stage also affected ($P < 0.05$) vitamin A ($P < 0.05$) and vitamin E ($P > 0.01$) levels.

Table 4 *Effect of dietary verbascoside (VB) supplementation on the profile of monounsaturated fatty acids in donkey's milk during 6 months (M) of lactation*

Fatty acid (g/100 g)	Control	VB	SEM	*P*-value Diet	Time	Diet × time
C14:1						
1 M	0.16	0.16	0.04			
2 M	0.16	0.14	0.04			
3 M	0.15	0.17	0.05			
4 M	0.15	0.20	0.09			
5 M	0.15	0.20	0.07			
6 M	0.15	0.15	0.03	0.257	0.128	0.316
C16:1						
1 M	2.15	2.01	0.73			
2 M	1.96^{a}	3.23^{b}	1.31			
3 M	2.22^{a}	2.90^{b}	0.75			
4 M	2.33^{a}	3.25^{b}	0.98			
5 M	2.56^{a}	3.22^{b}	0.97			
6 M	2.53^{a}	2.85^{b}	0.92	0.023	0.147	0.129
C18:1						
1 M	10.70	10.18	0.93			
2 M	10.92	10.81	1.22			
3 M	11.21	12.01	1.13			
4 M	11.58	12.19	1.31			
5 M	11.82	12.02	1.29			
6 M	12.15	12.43	0.63	0.125	0.012	0.319
C20:1 *cis*-9						
1 M	0.28	0.23	0.08			
2 M	0.30	0.25	0.06			
3 M	0.29	0.26	0.05			
4 M	0.27	0.26	0.06			
5 M	0.22	0.27	0.08			
6 M	0.22	0.26	0.06	0.164	0.151	0.281

Control = diet without VB; VB = diet supplemented with verbascoside.
[a,b]Values with different superscripts letters differ significantly at $P < 0.05$.

Blood biochemical parameters and oxidative status of jennies
The VB supplementation resulted in a decrease ($P < 0.01$) in serum levels of total cholesterol, tryglicerides, bilirubin and ALT, and although not significant, to a decrease in LDL-C levels ($P > 0.05$) (Table 7). The HDL-C, non-esterified fatty acid and aspartate aminotransferase levels were unaffected by the dietary treatment (Table 7).

Plasma concentrations of TBARs and vitamin E were higher ($P < 0.01$) in the VB group than the control, whereas no differences were observed in relation to ROMs and vitamin E levels (Table 7).

Discussion

Interventions in animal feeding are considered strategic ways to modify milk fatty acid composition and to improve the

Downloaded from https://www.cambridge.org/core. Universita Studi Molise, on 03 Jul 2017 at 14:34:37, subject to the Cambridge Core terms of use, available at https://www.cambridge.org/core/terms
. https://doi.org/10.1017/S1751731117000441

Table 5 *Effect of dietary verbascoside (VB) supplementation on the profile of polyunsaturated fatty acids in milk during 6 months (M) of lactation*

				P-value		
Fatty acid (g/100 g)	Control	VB	SEM	Diet	Time	Diet × time
C18:2n-6						
1 M	11.54	11.62	0.58			
2 M	10.77	11.74	0.51			
3 M	11.99	13.13	0.62			
4 M	12.40	13.15	0.63			
5 M	11.27	11.55	0.54			
6 M	10.18	10.31	0.19	0.059	0.183	0.238
C18:3n-3						
1 M	5.96	6.07	0.40			
2 M	6.26	6.12	0.37			
3 M	6.07	6.20	0.58			
4 M	6.00	6.28	0.56			
5 M	6.08	6.36	0.56			
6 M	5.88	6.38	0.50	0.188	0.160	0.295
C18:3n-6						
1 M	0.34[a]	0.38[a]	0.02			
2 M	0.22[b]	0.23[b]	0.01			
3 M	0.20[b]	0.25[b]	0.10			
4 M	0.37[a]	0.39[a]	0.01			
5 M	0.39[a]	0.35[a]	0.02			
6 M	0.39[a]	0.36[a]	0.01	0.075	0.033	0.199
C20:2n-6						
1 M	0.32	0.33	0.01			
2 M	0.42	0.42	0.01			
3 M	0.37	0.46	0.02			
4 M	0.34	0.33	0.01			
5 M	0.30	0.35	0.01			
6 M	0.27	0.25	0.01	0.122	0.037	0.233
C20:5n-3						
1 M	0.17	0.16	0.02			
2 M	0.19	0.17	0.03			
3 M	0.19	0.19	0.04			
4 M	0.18	0.18	0.07			
5 M	0.19	0.21	0.04			
6 M	0.21	0.18	0.02	0.197	0.112	0.133
C22:6n-3						
1 M	0.43	0.44	0.14			
2 M	0.41	0.41	0.11			
3 M	0.35	0.40	0.08			
4 M	0.41	0.42	0.06			
5 M	0.40	0.41	0.06			
6 M	0.37	0.38	0.08	0.122	0.194	0.297

Control = diet without VB; VB = diet supplemented with verbascoside.
[a,b]Values with different superscripts letters differ significantly at $P<0.05$.

nutritional and health qualities of milk (Martemucci and D'Alessandro, 2013). In this study, although the dietary VB supplementation did not influence the fat content of milk, it had a clear effect on the fatty acid profile of milk resulting in a decrease of SFA (−2.4%) and an increase of MUFA (+6.0%). The positive effects of VB supplementation on the quality of donkey milk are in agreement with the findings of Casamassima *et al.* (2014b) in ewes where VB decreased SFA and increased MUFA, PUFA and vitamins A and E contents in milk.

Table 6 *Effect of dietary verbascoside (VB) supplementation on the vitamins A and E contents in donkey's milk during 6 months (M) of lactation*

				P-value		
	Control	VB	SEM	Diet	Time	Diet × time
Vitamin A (μg/100 g)						
1 M	1.69	1.72[a]	0.07			
2 M	1.62	1.79[a]	0.06			
3 M	1.63	1.83[a]	0.08			
4 M	1.58[d]	2.07[e]	0.09			
5 M	1.70[d]	2.32[be]	0.11			
6 M	1.71[d]	2.39[be]	0.13	0.001	0.037	0.001
Vitamin E (μg/100 g)						
1 M	4.94	5.05[a]	0.05			
2 M	5.02[d]	5.49[be]	0.12			
3 M	4.99[d]	5.81[be]	0.14			
4 M	4.87[d]	6.11[ce]	0.20			
5 M	4.92[d]	6.59[ce]	0.25			
6 M	4.94[d]	6.72[ce]	0.27	0.001	0.011	0.001

Control = diet without VB; VB = diet supplemented with verbascoside.
[a,b,c,d,e]Values with different superscripts letters differ significantly at $P<0.05$.

In humans, the amount of dietary fat influences the concentration of plasma lipoproteins but the quality of lipid consumed (SFA, MUFA, PUFA) and their balance assume a major importance in terms of health (Weech *et al.*, 2014). Some speculations can be made concerning the potential implications of the changes in the fatty acid profile of donkey's milk as food for human consumption.

In general, fat of donkey milk may be considered more favourable for human health in comparison with other milks from ruminant species (cow, buffalo, ewes and goats) due to its lower percentage of SFA (Blasi *et al.*, 2008). The further reduction of SFA in favour of MUFA and PUFA, induced by the VB dietary supplementation of jennies, may be considered worthy of attention. Current recommendations include decreasing dietary intake of SFA and an increased intake of PUFA as part of a heart-healthy diet (Simopoulos, 2008; Livingstone *et al.*, 2012).

Considering the individual SFA of milk, in the present study VB administration induced a decrease of the short-chain (C4 to C8:0) and some medium (C9 to C13:0) fatty acids, and an increase of C15:0 and C18:0 acids. The exact mechanisms by which the VB acts on the lipid profile are not known. Two pathways on the biosynthesis of fatty acids at the level of mammary epithelial cells have been reported. The first is related to the primary way by which palmitic acid, stearic, oleic and all 18 carbon and longer-chain fatty acids are obtained. The source of fatty acids is through direct uptake of preformed fatty acids from blood lipoproteins derived from the intestinal absorption and mobilization of adipose tissue (Lock and Shingfield, 2004). Exogenous fatty acids are supplied to mammary epithelial cells from the

Downloaded from https:/www.cambridge.org/core. Universita Studi Molise, on 03 Jul 2017 at 14:34:37, subject to the Cambridge Core terms of use, available at https:/www.cambridge.org/core/terms. https://doi.org/10.1017/S1751731117000441

Table 7 *Effect of dietary verbascoside (VB) supplementation on the blood biochemical parameters in lactating jennies*

				P-value		
	Control	VB	SEM	Diet	Time	Diet × time
Animals (*n*)	5	5				
Total cholesterol (mg/dl)	110.56	90.69	3.60	0.001	0.547	0.001
HDL-C (mg/dl)	27.33	32.75	3.90	0.355	0.561	0.008
LDL-C (mg/dl)	78.77	66.04	4.24	0.063	0.011	0.024
Triglycerides (mg/dl) (5 months)	42.36	27.05	2.13	0.001	0.268	0.001
Non-esterified fatty acids (mg/dl)	146.45	136.70	5.51	0.246	0.015	0.140
Bilirubin (mg/dl)	0.54	0.387	0.01	0.001	0.004	0.001
ALT (UI/l)	17.80	14.76	1.05	0.008	0.175	0.130
Aspartate aminotransferase (UI/l)	160.75	138.90	13.8	0.296	0.004	0.001
ROMs (U/Carr)	169.70	160.65	7.18	0.399	0.117	0.001
TBARs (μmol/l)	0.241	0.158	0.01	0.002	0.012	0.004
Vitamin A (μmol/l)	0.518	0.555	0.05	0.584	0.739	0.423
Vitamin E (μmol/l)	4.91	7.39	0.20	0.001	0.003	0.001

Control = diet without VB; VB = diet supplemented with verbascoside; HDL-C = high-density lipoproteins cholesterol; LDL-C = low-density lipoproteins cholesterol; ALT = alanine aminotransferase; ROMs = reactive oxygen metabolites; TBARs = thiobarbituric acid reactive substances.

plasma non-esterified fatty acids pool that primarily originate by lipolytic release from adipose tissue triacylglycerol stores. In ruminants, the fatty acids in these stores are elaborated using acetate (C2) and β-hydroxybutyrate as principal precursor, whereas glucose is the principal lipogenic precursor in monogastric animals (Clegg *et al.*, 2001), such as donkeys. However, in the present study the serum levels of non-esterified fatty acids of the jennies were unaffected by the VB dietary supplementation, confirming the results of a previous study (D'Alessandro *et al.*, 2014). The second pathway for the biosynthesis of fatty acids is the so-called *de novo* synthesis by which the mammary gland produces all 4:0 to 12:0, most 14:0 and half the 16:0 fatty acids. Palmitic acid (16:0) is derived from both *de novo* synthesis and circulating blood lipids (Lock and Shingfield, 2004).

Among the total MUFA, the VB supplementation induced an increase only in C16:1 acid content, thus demonstrating a selective action on the substrate or on the activity or expression of enzyme Δ^9-desaturase, which is responsible in the udder for the desaturation of C16:0 in C16:1.

From a general nutritional point of view, the reduction in the short- and medium-chain (C4:0 to C13:0) fatty acid levels of milk induced by the VB supplementation does not seem beneficial. No nutritional relevance assume the increase of C15:0 and stearic acids in milk from the VB group. Stearic acid is considered a 'neutral' fatty acid because it has been shown to have no net impact on the plasmatic level of either LDL-C or HDL-C in humans (Bonamone and Grundy, 1988), and is not atherogenic (Mensink *et al.*, 2003). This lack of negative effect of stearic acid has been attributed to its reduced digestibility and easy desaturation into oleic acid (Bonamone and Grundy, 1988).

Among the MUFA in milk, oleic (C18:1) is the most prevalent acid in milk from donkey (Martemucci and D'Alessandro, 2012). In this study, the proportion of oleic acid was unaffected by the VB dietary supplementation and it varied in relation to the lactation stage of the jennies.

Considering the PUFA content, a slight improvement was induced by the dietary supplementation of VB on the contents of the total PUFA and linoleic (C18:2n-6) acid, and on atherogenic and thrombogenic indices, which indicate the healthfulness with respect to the milk fatty acid content.

Taking together the findings on the milk fatty acid profile, we can consider that the nutritional and health qualities of donkey milk were not completely improved by the dietary treatment with the VB. However, it is to consider that the VB supplementation induced more favourable SFA and MUFA contents and tended to improve atherogenic and thrombogenic indices.

In this study, the VB administration affected the serum parameters, confirming the results of a study carried out in ewes (Casamassima *et al.*, 2014a) and those of a recent study on lactacting jennies (D'Alessandro *et al.*, 2014). In the latter, the same effects on the serum lipid profile were observed in the suckling foals, probably induced through the mediated milk way. Therefore, in humans an indirect health effect on lipid metabolism through a mediated action of VB 'enriched' milk might be hypothesized.

Interestingly, greater levels of vitamins A and E in milk was observed in VB group in comparison with the control. This improves the quality of milk for the consumers as both of the two vitamins are involved in physiological functions, and thus play important roles on health and wellness (Jacobs *et al.*, 2002; Ross, 2004; Meydani *et al.*, 2005).

In the present study, the VB group also showed a greater blood concentration of vitamin E leading to suppose that the improvement of milk in these components is due to a greater availability for the milk synthesis at the mammary gland level. In a previous study in donkeys (D'Alessandro *et al.*, 2014) the maternal VB treatment improved also the serum levels of vitamins A and E in the suckling foals.

Evidence of VB effects on oxidative status of the jennies were observed in this study, confirming some studies on donkeys (D'Alessandro *et al.*, 2014) and ewes

Downloaded from https://www.cambridge.org/core. Universita Studi Molise, on 03 Jul 2017 at 14:34:37, subject to the Cambridge Core terms of use, available at https://www.cambridge.org/core/terms. https://doi.org/10.1017/S1751731117000441

(Casamassima *et al*., 2014a), and leading to suppose a beneficial effect on animal conditions.

Conclusions

The results provide evidence that the dietary supplementation of VB to lactating jennies changes fatty acid profile of milk, and improves its nutritional characteristics in terms of SFA, MUFA, and vitamins A and E contents. In addition, the study confirmed the VB effects on serum lipidic profile and oxidative status of the animals. Considering these novel findings, the present study suggests a potential application to modify the quality of donkey's milk. Further studies are required to assess the use of donkeys as producers of 'pharma food', as well as to validate the potential clinical utility of human consumption of donkey's milk that has been 'enriched' through the supplementation with VB in the animal diet.

Acknowledgement

The study was supported by the University of Bari.

References

Ascherio A 2002. Epidemiologic studies on dietary fats and coronary heart disease. American Journal of Medicine 113 (suppl), 9S–12S.

Blasi F, Montesano D, De Angelis M, Maurizi A, Ventura F, Cossignani L, Simonetti MS and Damiani P 2008. Results of stereospecific analysis of triacylglycerol fraction from donkey, cow, ewe, goat and buffalo milk. Journal of Food Composition and Analysis 21, 1–7.

Bonamone A and Grundy SM 1988. Effect of dietary stearic acid on plasma cholesterol and lipoprotein levels. New England Journal of Medicine 318, 1244–1248.

Casamassima D, Nardoia M, Palazzo M, Vizzarri F and Corino C 2014a. Effect of dietary extruded linseed, verbascoside and vitamin E supplements on selected serum biochemical parameters and plasma oxidative status in Lacaune ewes. Slovenian Veterinary Research 51, 89–100.

Casamassima D, Nardoia M, Palazzo M, Vizzarri F, D'Alessandro AG and Corino C 2014b. Effect of dietary extruded linseed, verbascoside and vitamin E supplements on yield and quality of milk in Lacaune ewes. Journal of Dairy Research 81, 485–493.

Cesarone MR, Belcaro G and Caratelli M 1999. A simple test to monitor oxidative stress. International Journal of Angiology 18, 127–130.

Clegg RA, Barber MC, Pooley L, Ernens I, Larondelle Y and Travers MT 2001. Milk fat synthesis and secretion: molecular and cellular aspects. Livestock Production Science 70, 3–14.

D'Alessandro AG, Casamassima D, Jirillo F and Martemucci G 2014. Effects of verbascoside administration on the blood parameters and oxidative status in jennies and their suckling foals: potential improvement of milk for human use. Endocrine, Metabolic and Immune Disorders – Drug Targets 14, 102–112.

D'Alessandro AG and Martemucci G 2012. Lactation curve and effects of milking regimen on milk yield and quality, and udder health in Martina Franca jennies (*Equus asinus*). Journal of Animal Science 90, 669–681.

D'Auria E, Agostoni C, Giovannini M, Riva E, Zetterström R, Fortin R, Greppi GF, Bonizzi L and Roncada P 2005. Proteomic evaluation of milk from different mammalian species as a substitute for breast milk. Acta Paediatrica 94, 1708–1713.

Esterbauer H and Zollner H 1989. Methods for determination of aldehydic lipid peroxidation products. Free Radical Biology and Medicine 7, 197–203.

Federation Internationale dé Laiterie (International Dairy Federation) 2000. Whole milk. Determination of milk fat, protein and lactose content, Standard 141C. FIL (IDF), Brussels, Belgium.

Folch J, Lees M and Sloane Stanley GH 1957. A simple method for isolation and purification of total lipids from animal tissues. Journal of Biological Chemistry 226, 497–509.

International Union of Pure and Applied Chemistry (IUPAC) 1987. Standard methods for the analysis of oil, fats and derivates. IUPAC Method 2.301, Report of IUPAC Working Group WG 2/87, 7th edition. Blackwell Scientific Publications, Oxford, UK.

Jacobs EJ, Henion AK, Briggs PJ, Connell CJ, McCullough ML, Jonas CR, Rodriguez C, Calle EE and Thun MJ 2002. Vitamin C and vitamin E supplement use and bladder cancer mortality in a large cohort of US men and women. American Journal of Epidemiology 156, 1002–1010.

Livingstone KM, Lovegrove JA and Givens DI 2012. The impact of substituting SFA in dairy products with MUFA or PUFA on CVD risk: evidence from human intervention studies. Nutrition Research Review 25, 193–206.

Lock AL and Shingfield KJ 2004. Optimising milk composition. In Dairying – using science to meet consumers' needs (ed. E Kebreab, J Mills and DE Beever), pp. 107–188. British Society of Animal Science and Nottingham University Press, Loughborough, Leicestershire and Nottingham, UK.

Malhotra A, Shafiq N, Arora A, Singh M, Kumar R and Malhotra S 2014. Dietary interventions (plant sterols, stanols, omega-3 fatty acids, soy protein and dietary fibers) for familial hypercholesterolaemia. Cochrane Database of Systematic Reviews 6, CD001918.

Martemucci G and D'Alessandro AG 2012. Fat content, energy value and fatty acid profile of donkey milk during lactation and implications on human nutrition. Lipids in Health and Disease 11, 113–126.

Martemucci G and D'Alessandro AG 2013. Progress in nutritional and health profile of milk and dairy products: a novel drug target. Endocrine Metabolic and Immune Disorders Drug Targets 13, 209–233.

Mensink RP, Zock PL, Kester AD and Katan MB 2003. Effects of dietary fatty acids and carbohydrates on the ratio of serum total to HDL cholesterol and on serum lipids and apolipoproteins: a meta-analysis of 60 controlled trials. The American Journal of Clinical Nutrition 77, 146–155.

Meydani SN, Han SN and Wu D 2005. Vitamin E and immune response in the aged: molecular mechanisms and clinical implications. Immunological Reviews 205, 269–284.

Monti G, Viola S, Baro C, Cresi F, Tovo PA, Moro G, Ferrero MP, Conti A and Bertino E 2012. Tolerability of donkey's milk in 92 highly-problematic cow's milk allergic children. Journal of Biological Regulators & Homeostatis Agents 26 (suppl. 3), 75–82.

O'Connell JE and Fox PF 2001. Significance and applications of phenolic compounds in the production and quality of milk and dairy products: a review. International Dairy Journal 11, 103–120.

Ross AC 2004. Introduction to vitamin A: a nutritional and life cycle perspective. In Carotenoids and retinoids: molecular aspects and health issues (ed. L Packer, U Obermueller-Jevic, K Kraemer and H Sies), pp. 23–41. American Oil Chemists Society Press, Champaign, IL, USA.

Simopoulos AP 2008. The importance of the omega-6/omega-3 fatty acid ratio in cardiovascular disease and other chronic diseases. Experimental Biology and Medicine 233, 674–688.

SPSS 2010. 18.0 Package program, user's guide. SPSS Inc., Chicago, IL, USA.

Tafaro A, Magrone T, Jirillo F, Martemucci G, D'Alessandro AG, Amati L and Jirillo E 2007. Immunological properties of donkey's milk: its potential use in the prevention of atherosclerosis. Current Pharmaceutical Design 13, 3711–3717.

Ulbricht TL and Southgate DA 1991. Coronary heart disease: seven dietary factors. Lancet 338, 985–992.

Vita D, Passalacqua G, Di Pasquale G, Caminiti L, Crisafulli G, Rulli I and Pajno GB 2007. Ass's milk in children with atopic dermatitis and cow's milk allergy: crossover comparison with goat's milk. Pediatric Allergy and Immunology 18, 594–598.

Weech M, Vafeiadou K, Hasaj M, Todd S, Yaqoob P, Jackson KG and Lovegrove JA 2014. Development of a food-exchange model to replace saturated fat with MUFAs and n-6 PUFAs in adults at moderate cardiovascular risk. Journal of Nutrition 144, 846–855.

Willcox JK, Ash SL and Catignani GL 2004. Antioxidants and prevention of chronic disease. Critical Reviews in Food Science and Nutrition 44, 275–295.

Zhao B, Tham S-Y, Lu J, Lai MH, Lee LKH and Moochhala SM 2004. Simultaneous determination of vitamins C, E and b-carotene in human plasma by high-performance liquid chromatography with photodiode-array detection. Journal of Pharmacy and Pharmaceutical Science 7, 2000–2004.

Downloaded from https://www.cambridge.org/core. Universita Studi Molise, on 03 Jul 2017 at 14:34:37, subject to the Cambridge Core terms of use, available at https://www.cambridge.org/core/terms. https://doi.org/10.1017/S1751731117000441