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Cocoa husks fed to lactating dairy ewes affect milk fatty acid profile and oxidative status of blood and milk

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ABSTRACT

The objective of this study was to evaluate the effect of cocoa husks (CH) containing theobromine on milk fatty acids (FA) and on milk and blood oxidative status in dairy ewes. The experiment was carried out with 24 dairy ewes and it lasted 8 weeks with 3 weeks of adaptation period and 5 weeks of experimental period. Animals were divided in three homogeneous groups and all ewes were fed with the same basal diet, composed of a total mix ration (TMR). The first group (CON) was fed with the TMR and a supplementation of 100 g/d per head of soybean hulls, while the second (CH50) and the third groups (CH100) were fed with the TMR and a supplementation of 50 and 100 g/d per head of CH, respectively. Blood samples were analyzed for the activities of superoxide dismutase (SOD), glutathione transferase (GSTS), glutathione reductase (GR) and glutathione peroxidase (GPx), while milk samples were analyzed for SOD, GR and lactoperoxidase (LPO). Total antioxidant capacity and oxidative stress biomarkers in blood and milk samples were determined. Orthogonal polynomial contrasts (linear and quadratic) were used to investigate the effect of the diet on the variables. The C16:0 and MCFA (medium-chain fatty acids) decreased, whereas C18:0 and LCFA (long-chain fatty acids) increased quadratically with the dose of CH. The supplementation of CH decreased linearly the OBCFA (branched-chain fatty acid). Regarding antioxidant results, mean protein carbonyls (PC) decreased linearly and SOD activity tended to increase linearly in blood with increasing CH dose. Meanwhile, antioxidant analysis of milk samples showed that CH supplementation increased quadratically the LPO and tended to decrease quadratically the malondialdehyde level (MDA). In conclusion, the addition of CH as supplement to the diet of ewes could modify the milk FA profile and could improve antioxidant capacity of blood and milk.

1. Introduction

Agro-industrial by-products could reduce feed–food competition, feeding cost and environmental impact both for livestock and food industries, without detrimental effects on performances or quality of products (Mirzaei-Aghsaghali and Maheri-Sis, N., 2008). Recently, the effects of using these by-products in livestock diets on milk production and rumen parameters has been largely studied (Nudda et al., 2017; Buffa et al., 2020a). Some of them are rich in beneficial physiologically active compounds, such as polyphenols and tannins, that have received notable attention for their role in preserving animal and human health (Liu et al., 2013; López-Andrés et al., 2013; Schroeter et al., 2006). Despite enzymatic antioxidants such as catalase, superoxide dismutase (SOD), glutathione peroxidase (GPx), occur both in blood and milk, recent studies in dairy ewes (Buffa et al., 2020b) and cows (Safari et al., 2018) evidenced that the administration of feeds and by-products rich in natural antioxidant compounds enhances the antioxidant defense mechanism in blood and milk. The bioactive components help in reducing lipid peroxidation, especially when animals were fed diets that increase the polyunsaturated fatty acids content in milk, making it more vulnerable to oxidation during processing and storage (Girard et al., 2016). However, in comparison with PUFA, the oleic acid that is one of the major native fatty acids (FA) in ruminant milk, has been reported to exert antioxidant properties in cellular models, animals (Cho et al., 2010) and humans (Bonanome et al., 1992).

Cocoa husks (CH), a by-product derived from processing of cocoa (*Theobroma cacao L.*), with 700 thousands of tons produced per year, represent a disposal problem for chocolate industry worldwide,

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relegating the most convenient residual use to energy production (Barišić et al., 2020). However, CH are a precious source of nutraceutical compounds which can represent a resource for animal health and be conveyed to human nutrition through their use as animal feed: actually, they contain large amount of theobromine, caffeine and phenolic compounds, including epicatechin, catechin, and other flavonoids (Hernández-Hernández et al., 2019; Lu et al., 2018) which have antioxidant (Lecumberri et al., 2007; Okiyama et al., 2017; Wu et al., 2019), antimutagenic (Osowski et al., 2010) and antitumoral (Delgado et al., 2009; Haza and Morales, 2011) properties.

The effects of CH or their bioactive compounds, specifically theobromine, on ruminant oxidative status have not been yet investigated. Studies on humans cells evidenced a strong positive correlation of theobromine with antioxidants assays, as 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) and ferric reducing antioxidant power assay (FRAP) (Hu et al., 2016), probably because of quenching effect of theobromine on the production of hydroxyl radicals, as well as on oxidative DNA breakage by free radicals (Azam et al., 2003). Theobromine has been found also to mitigate IL-1β-induced oxidative stress and inflammatory response and degradation of type II collagen in human chondrocytes (Gu et al., 2020). The main limit to the use of this by-product is related to the theobromine concentration, which could be toxic for animals when present in large amount (Adamafio, 2013). The European Union in 2002 established that the concentration of theobromine in a complete feedstuff for animal should be below 300 mg/kg (Directive, 2002/32/EC).

The aim of this study was to evaluate the use of CH as feeding supplement given to dairy ewes. We hypothesized that this byproduct could improve milk and blood oxidation stability. Instead, polyphenols contain in CH could modify rumen biohydrogenation with a consequent alteration of milk fatty acids profile.

2. Materials and methods

2.1. Animals and diets

The experiment was approved by the Ethics Committee of the University of Sassari (no. 54584/2018) and the animals were managed according to the European Union Guidelines on Animal Care (European Union, 2010). Twenty-four Sarda ewes in middle lactation were allotted in three experimental groups as detailed in the previous paper (Carta et al., 2020). Briefly, animals received 2.580 kg/d per head of a total mix ration (TMR) and 100 g/d of soybean hulls (SH) as control group (CON group), or 50 g/d (CH50 group) and 100 g/d (CH100 group) of cocoa husks. The animal groups were homogeneous for (mean ± SD) daily milk yield (1.8 ± 0.04 kg/d per head), body weight (BW = 42.5 ± 1.1 kg), body condition score BCS (2.7 ± 0.01), days in milk (DIM = 120 ± 15 d), and parity (3.1 ± 1.2). Animals were machine milked twice daily at 0800 and 1600. The in vivo experiment lasted 8 weeks including 3 weeks of adaptation to the experimental condition and 5 weeks of experimental period. The animals were allocated in twelve pens (two ewes in each pen) for a total of 4 experimental units per group. The TMR was offered four times a day, while the SH and CH were administrated individually in the morning milking mixed with 70 g of beet pulp.

2.2. Sampling and analysis

2.2.1. Feed sampling and analysis

Samples of TMR and by-products were collected at beginning and end of the trial and were analyzed to determine the chemical composition. The samples were analyzed for dry matter content (DM) using an oven-drying at 105 °C for 24 h. NDF, ADF and ADL were determined following the method of Mertens (2002), the AOAC method 973.18 ("AOAC, Official Method of Analysis," 1990;) and the Robertson and Van Soest (1981) method, respectively. Crude protein (CP) and ether extract (EE) were analyzed following the Kjeldahl method (AOAC, 2000; method 988.05) and the Soxhlet method (AOAC, 2005; method 920.39), respectively. Non-fiber carbohydrates (NFC) were calculated according to Weiss (1999): NFC (% of DM) = 100- (NDF + CP + ash + EE). By-products and samples of the three diets were analyzed for FA following Correddu et al. (2016), with the gas chromatograph Agilent and a CP-Sil88-fused silica capillary column SP™-2560 (100 m ×0.25 mm ID, 0.20-μm film, Supelco, Bellefonte, PA, USA). CH and SH were also analyzed for protein fraction following the method of Licitra et al. (1996): A (NPN), B1 (buffer-soluble true protein), B2 (buffer-insoluble protein – neutral detergent soluble protein), B3 (neutral detergent insoluble protein – acid detergent insoluble protein) and C (acid detergent insoluble protein).

2.2.2. Milk and blood samples

During the 5 weeks of experimental period individual milk and blood samples were collected. Milk samples were collected weekly and the samples from the last day of 2th, 3th, 4th and 5thweek of experimental period were stored at −20 °C for FA analysis. Samples milk for antioxidant analysis were collected at the end of the 2th and at the 5th week and degreased for the determination of SOD, glutathione reductase (GR), lactoperoxidase (LPO) and protein carbonyl (PC) and stored at −80 °C. Samples milk for FRAP and ABTS assays were processed for extracted antioxidant compounds, following Alyaqoubi et al. (2014). An aliquot of whole milk was stored at −20 °C for malondialdehyde assay (MDA).

Individual blood samples, collected at 2th and at 5th week and were taken from jugular vein into a heparinized vacuum tube and then centrifugated at 3000 x g, 4 °C, 10 min to separate plasma from corpusculate fraction and stored at −80 °C for antioxidant analysis.

2.2.3. Fatty acids analysis

Milk samples were analyzed for fatty acid methyl ester (FAME). The milk fat was extract by direct extract as described by Feng et al. (2004), with some modification. Milk samples were centrifuged at 12,000 rpm, 7 °C and 15 min and then an aliquot of fat located in the top of the centrifuge tube (11−14 mg) was taken and placed in a vial. After, the fat was methylated using 500 μl of sodium methoxide (0.5 N) and the solution was mixed with a vortex for 2 min. Subsequently, 1 mL of methylated, an internal standard composed by C 5:0 and C 13:0 (0.4 mg/mL in hexane), was added to each sample. Samples were mixed by vortex for 1 min and incubated for 10 min and then the upper part was transfer in a new vial for GC analysis.

FA were determined using a 7890A GC System (Agilent Technologies, Santa Clara, CA, USA), equipped with 7693 Autosampler (Agilent Technologies, Santa Clara, CA, USA) and a flame ionization detector (FID). Helium was used as gas carrier (1 ml/ min flow rate) with a pressure of 28 psi. The initial temperature of the instrument was 45 °C, maintained for 4 min, and then the temperature was increased by 13 °C/min until to reach 175 °C. This temperature was held for 27 min and after was increase by 4 °C/min until 215 °C. The temperature of the injector and the detector was 250 °C. Split ratio was 1:80. OpenLAB CDS GC ChemStation Upgrade software data system (Revision C.01.04, Agilent Technologies Inc., Santa Clara, CA, USA) was used to determine the area of the FAME. The peaks were identified by comparing their retention time with those of methyl standard, as detailed in Nudda et al. (2005).

The FA were referred as g/100 g of FAME and the groups were calculated as following: SFA, saturated fatty acid (sum of individual saturated fatty acids), UFA, unsaturated fatty acid (sum of individual unsaturated fatty acids), MUFA, monounsaturated fatty acids (sum of individual monounsaturated fatty acids), PUFA, polyunsaturated fatty acids (sum of individual polyunsaturated fatty acids), TFA, trans fatty acids (sum of individual trans fatty acids), BCFA, branched-chain fatty acids (sum of individual branched-chain fatty acids), OBCFA, odd- and branched-chain fatty acids (sum of individual odd- and branched-chain fatty acids), SCFA, short-chain fatty acids (sum of individual fatty acids

from C4:0 to C10:0), MCFA, medium-chain fatty acids (sum of individual fatty acids from C11:0 to C17:0), LCFA, long-chain fatty acids (sum of individual fatty acids from C18:0 to C22:6 (docosahexaenoic acid, DHA), PUFA 3-n (sum of individual n-3 fatty acids), PUFA 6-n (sum of individual n-6 fatty acids), total CLA, conjugated linoleic acids (sum of individual conjugated linoleic acids). The nutritional properties were valuated as the ratio between n-6 and n-3 and three indices, that are the atherogenic index (AI) and trombogenic index (TI), calculated according to Ulbricht and Southgate (1991) except for the substitution of C18:0 with C12:0, as suggested by Nudda et al. (2013): AI = [12:0 + (4 × 14:0) + 16:0] / [(PUFA) + (MUFA)]; TI = (14:0 + 16:0) / [(0.5 × MUFA) + (0.5 × n-6) + (3 × n-3) + (n-3: n-6)]; the hypocholesterolemic to hypercholesterolemic ratio (h:H), calculated according to Fernández et al. (2007) as following: [(sum of 18:1cis-9, 18:1cis-11, 18:2 n-6, 18:3 n-6, 18:3 n-3, 20:3 n-6, 20:4 n-6, 20:5 n-3, 22:4 n-6, 22:5 n-3 and 22:6 n-3)/(14:0 + 16:0)].

The Δ9-desaturase indices (DI) were calculated as following according to Schennink et al. (2008) to evaluate the effect of the different diets on the capacity of desaturating SFA to Δ9- UFA: C10 index = [C10:1/(C10:0 + C10:1)] × 100; C14 index = [C14:1 cis-9/(C14:0 + C14:1 cis-9)] × 100; C16 index = [C16:1 cis-9/(C16:0 + C16:1 cis-9)] × 100; C18 index = [C18:1 cis-9/(C18:0 + C18:1 cis-9)] × 100; CLA index = [CLA cis-9,trans-11/(C18:1 trans-11 + CLA cis-9,trans-11)] × 100; total index = [(C10:1 + C14:1 cis-9 + C16:1 cis-9 + C18:1 cis-9 + CLA cis-9,trans-11)/(C10:0 + C14:0 + C16:0 + C18:0 + C18:1 trans-11+ C10:1 + C14:1 cis-9 + C16:1 cis-9 + C18:1 cis-9 + CLA cis-9,trans-11)] × 100.

2.2.4. Antioxidant capacity analysis

The antioxidant capacity in milk and blood was evaluated using different assays. In blood, the activity of SOD, GR, glutathione transferase (GST) and GPx were determined while in milk SOD, GR, LPO activities were assessed. Also, in milk and blood the total antioxidant capacity was evaluated by FRAP, ABTS and the oxidative stress biomarkers were determined by MDA and PCs assays.

Total antioxidant capacity, enzymatic activities and oxidative stress biomarkers were measured spectrophotometrically as described by Tsiplakou et al. (2017). More specifically, ferric reducing ability of plasma (FRAP) assay was used to measure total antioxidant capacity according to the method described by Benzie and Strain (1996). Ferric tripyridyltriazine (Fe^{III}-TPTZ) complex is reduced to ferrous tripyridyltriazine (Fe^{II}-TPTZ) form and develops an intense blue color, with maximum absorption at 593 nm. The 2,2′-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) radical scavenging assay was based on the published methods (Li et al., 2011; Pellegrini et al., 2003). This method is based on the ability of an antioxidant to discolorate the ABTS cation radical, which is previously formed through the reaction between ABTS and potassium persulfate. The activity of GSTS was measured by observing the conjugation of 1-chloro, 2,4-dinitrobenzene (CDNB) with reduced glutathione at 340 nm (Labrou et al., 2001). LPO activity was performed according to the methods of Keesey (1987) by measuring the oxidation of ABTS present in hydrogen peroxide at 340 nm. GPx activity was measured by observing the decrease in NADPH absorbance measured at 340 nm during the oxidation of NADPH to NADP+, according to Paglia and Valentine (1967).

SOD activity was assayed using the method of McCord and Fridovich (1969) by measuring the inhibition of cytochrome c oxidation at 550 nm. GR activity was measured according to the method of Mavis and Stellwagen (1968) by measuring the reduction in oxidized glutathione at 340 nm. MDA was determined according to the method of Nielsen et al. (1997) with some modifications. This assay measures MDA, which reacts with thiobarbituric acid (TBA) forming a pink chromogen (TBARS), which is measured at 532 nm. The PC content was determined according to the method of Patsoukis et al. (2004) by measuring the conjunction of 2,4-dinitrophenylhydrazine (DNPH) on protein carbonyls at 375 nm.

2.3. Statistical analysis

All data were analyzed as a completely randomized design with repeated measures using the PROC MIXED procedure of SAS version 9.2 (SAS Institute, (2008)). The model included the fixed effect of diet, sampling and sampling x diet and pen as random effect. The pens, that contained 2 animals each one, were considered as experimental units. Therefore, 4 experimental units were considered for each group. Orthogonal polynomial contrasts (linear and quadratic) were included in the model in order to evaluate the effect of the diet on the variables. Significant differences are declared at $P < 0.05$. Correlations between antioxidant parameters on blood and milk were assessed by Pearson's correlation test using PROC CORR procedure of SAS.

3. Results

3.1. Diet and chemical composition

The chemical composition of the diets is reported in Table 1. The protein concentration in CH was 16.7 % on DM, 34.4 % of which represented C fraction. Neutral detergent fiber was 46 % DM and ether extract was 5.6 % DM, where oleic acid was predominant (34.2 % of total fatty acids), followed by stearic (25.8 %) and palmitic acid (24.2 %). The CH contained 3.36 g of gallic acid equivalent (GAE)/100 g of DM of total polyphenols, and 6,850 mg/kg of DM of theobromine (Carta et al., 2020).

3.2. Fatty acids profile

Both doses of CH influenced partially the milk fatty acid composition, as showed in Table 2: the quadratic effect was the most observed, as the FAs affected by the experimental factor were changed by the low but not by the high dose of CH. A quadratic effect was found for the C16:0 content ($P = 0.03$), being lowest in CH50 group and of C18:0 ($P < 0.01$) that was highest in CH50, whereas C18:1 cis-9 increased linearly ($P = 0.02$) with dose of CH.

Quadratic effect was found also for the content of some minor C18:1 trans isomers, as trans-4 ($P < 0.001$) and C18:1 trans-13 + trans-14 ($P = 0.01$). The VA and RA contents were not affected by the inclusion of CH in the diet.

Sampling time influenced almost all FA, in particular some of SFA, such as C14:0 and C16:0, that increased over the time, whereas some PUFA, such as C18:2n6, C18:3n3, CLAcis-9, trans-11 and C18:1trans-11, decreased during the experiment ($P < 0.05$).

Regarding the FA groups, a linear effect was observed on OBCFA ($P = 0.03$). The C15:0 concentration was not changed, whereas that of C17:0 was lowered ($P = 0.006$) by the inclusion of CH in the diet. The CH supplementation quadratically reduced the MCFA ($P = 0.002$) and increased the LCFA ($P = 0.01$).

Desaturase index of C14, C18 and CLA decrease quadratically ($P < 0.05$) and C16 index tended to decrease quadratically ($P = 0.10$), being the lowest values in CH50 group.

The AI and TI nutritional indexes of milk fat were not affected by CH supplementation, whereas h:H tended to increase with CH supplementation.

3.3. Antioxidant status in blood plasma

The results of the effect of CH on plasma antioxidant status are presented in Table 3. The inclusion of CH in ewes' diets decreased linearly PC ($P < 0.001$), but it did not affect MDA content in blood plasma. Meanwhile, in blood plasma an increase in SOD activity of ewes supplement with CH (Table 3) was found. The inclusion of CH did not modify the total antioxidant capacity (measured by FRAP and ABTS).

Sampling time influenced some oxidation parameters ($P < 0.05$), as FRAP, PC, GPx and SOD activities that increased over the time, whereas

Table 1
Ingredients and chemical composition of offered diets.

Item	Diet[a]		
	CON	CH50	CH100
Ingredient (kg/d per animal, as fed)			
TMR[b]	2.58	2.58	2.58
By-product mix			
Beet pulp[c]	0.07	0.07	0.07
Cocoa husks[d]	–	0.05	0.10
Soybean hulls[d]	0.10	–	–
Total DM supplied	2.42	2.38	2.42
Chemical composition (% of DM unless otherwise noted)			
DM (%)	88.08	88.06	88.09
NDF	40.20	39.29	39.42
NFC	35.74	36.23	35.99
ADL	4.30	4.67	4.95
CP	16.99	17.23	17.22
Ash	6.09	6.18	6.23
Ether extract	0.98	1.07	1.15
Theobromine (mg/kg DM)	–	130	252
Total polyphenol (g GAE/100 of DM)	1.06	1.11	1.17
ME, Mcal/kg of DM[e]	2.77	2.76	2.74
ME supplied, Mcal/d	5.93	5.89	6.05
Major FA (g / 100 g of total FA)			
C12:0	0.13	0.10	0.18
C14	0.41	0.40	0.42
C16:0	17.0	17.5	17.8
C16:1c7	0.21	0.19	0.19
C16:1c9	0.22	0.23	0.22
C18:0	3.97	5.65	6.43
C18:1c9	22.5	22.8	23.1
C18:1c11	1.13	1.10	1.05
C18:2n6	43.5	41.6	40.7
C18:3n3	5.44	5.25	4.93
C20:0	0.98	0.69	0.66
C22:0	0.68	0.68	0.66
C24:0	0.77	0.77	0.76

[a] Diet: CON = diet containing 100 g/d per head of soybean hulls; CH50 = diet containing 50 g/d per head of cocoa husks; CH100 = diet containing 100 g/d per head of cocoa husks.

[b] TMR (total mix ration) composition: beet pulp = 37.98 %; soybean meal = 14.34 %; flaked corn = 14.11 %; dehydrated alfalfa = 8.53 %; barley = 5.81 %; rise = 4.65 %; straw = 4.57 %; hay = 3.33; m qualiter = 5.89 %; steecker 150 = 0.78 %. Chemical composition: DM = 87.45; NDF = 38.95 % of DM; NFC = 36.47 % of DM; ADL = 4.31 % of DM; CP = 17.42 % of DM; Ash = 6.17 % of DM; Ether extract = 0.99 % of DM.

[c] Beet pulp, chemical composition: DM = 90.46 %; NDF = 46.89 % of DM; NFC = 36.97 % of DM; ADL = 7.14 % of DM; CP = 10.64 % of DM; Ash = 4.88 % of DM; Ether extract = 0.66 % of DM.

[d] Fatty acid profile of cocoa husk, CH (g/100 g of total FA): C16:0 = 24.24 %; C16:1 cis-9 = 0.79; C18:0 = 25.84 %; C18:1 cis-9 = 34.25 %; C18:1 cis-11 = 1.64 %; C18:2n6 = 8.44 %; C18:3n3 = 0.68 %; SFA = 53.59 %; UFA = 46.41 %. Fatty acid profile of soybean hulls, SH (g/100 g of total FA): C16:0 = 16.72 %; C16:1 cis-9 = 0.50 %; C18:0 = 6.94 %; C18:1 cis-9 = 15.02 %; C18:1 cis-11 = 3.39 %; C18:2n6 = 40.84 %; C18:3n3 = 10.20 %; SFA = 28.95 %; UFA = 71.05 %. Nitrogen fraction of CH (% CP): A = 22.27; B1 = 3.10; B2 = 25.59; B3 = 4.67; C = 34.37. Nitrogen fraction of SH (% CP): A = 0.86; B1 = 11.51; B2 = 42.22; B3 = 29.83; C = 15.58.

[e] Calculated using the Small Ruminant Nutrition Model (Tedeschi et al., 2010).

no effects on ABTS, MDA, GR and GSTS parameters ($P > 0.05$) were observed.

3.4. Antioxidant capacity of milk

The CH effects on antioxidant capacity of milk are reported in Table 4. The supplementation of CH tended to reduce quadratically the MDA content. The PCs content was not affected by the CH supplementation. The LPO activity in the milk responded quadratically to the CH supplementation with the highest value achieved by the CH50 group.

Table 2
Fatty acid profile and nutritional indices of fatty acids of milk of ewes fed with 100 g of soybean hulls (CON), 50 g of cocoa husks replacing 50 g of soybean hulls (CH50) and 100 g of cocoa husks replacing 100 g of soybean hulls (CH100).

Fatty acid (g/100 g of FAME)[a]	Diet[b]			SEM	P-value	
	CON	CH50	CH100		Linear	Quadratic
Groups						
SFA	73.42	72.69	72.96	0.262	0.70	0.56
UFA	26.56	27.30	27.03	0.262	0.70	0.56
MUFA	20.52	21.57	21.23	0.184	0.44	0.37
PUFA	6.04	5.73	5.80	0.104	0.58	0.78
TFA	5.82	5.82	5.25	0.163	0.41	0.51
BCFA	2.13[a,b]	2.26[a]	1.98[b]	0.028	0.15	0.03
OCFA	2.93	2.69	2.67	0.030	0.10	0.22
OBCFA	5.06[a]	4.93[a,b]	4.68[b]	0.044	0.03	0.83
SCFA	14.93	15.87	15.10	0.131	0.78	0.13
MCFA	58.37[a]	54.42[b]	56.62[a,b]	0.325	0.11	<0.001
LCFA	26.69[b]	29.70[a]	28.28[a,b]	0.293	0.13	0.01
PUFA n-3	0.48	0.41	0.42	0.011	0.27	0.37
PUFA n-6	3.98	3.63	3.74	0.083	0.53	0.52
n-6:n3	8.33[b]	8.94[a]	8.94[a]	0.056	0.02	0.17
Total CLA	0.86	0.98	0.95	0.037	0.63	0.43
FA						
C4:0	2.37	2.52	2.46	0.023	0.37	0.34
C6:0	1.85	2.04	1.94	0.025	0.40	0.11
C7:0	0.05	0.05	0.05	0.004	0.18	0.30
C8:0	1.98	2.22	2.06	0.025	0.43	0.03
C9:0	0.11	0.10	0.10	0.005	0.63	0.46
C10:0	8.52	8.88	8.45	0.085	0.84	0.23
C10:1	0.04	0.04	0.04	0.002	0.99	0.35
C11:0	0.52	0.46	0.49	0.008	0.31	0.12
C12:0	6.21	5.96	5.90	0.084	0.42	0.88
iso C13:0	0.01	0.01	0.01	0.000	0.82	0.68
anteiso C13:0	0.11	0.09	0.10	0.003	0.21	0.08
iso C14:0	0.11[b]	0.14[a]	0.13[a]	0.005	0.03	0.22
C14:0	13.59	12.99	13.10	0.105	0.26	0.38
C14:1cis-9	0.52[a]	0.36[b]	0.45[a,b]	0.015	0.27	0.03
iso C15:0	0.14	0.16	0.14	0.004	0.94	0.17
anteiso C15:0	0.47	0.52	0.42	0.011	0.26	0.12
C15:0	1.28	1.17	1.19	0.015	0.26	0.36
C15:1	0.11	0.12	0.11	0.004	0.84	0.48
iso C16:0	0.33	0.38	0.35	0.008	0.61	0.10
C16:0	30.34[a]	28.01[b]	30.04[a]	0.276	0.79	0.03
C16:1 trans-6 + trans-7	0.08	0.10	0.08	0.002	0.93	0.06
C16:1 trans-9	0.10	0.13	0.12	0.006	0.43	0.22
C16:1 trans-10	0.01[b]	0.02[b]	0.02[a,b]	0.000	0.41	0.01
C16:1 cis-7	0.23	0.25	0.23	0.003	0.68	0.08
C16:1 cis- 9	1.76[a]	1.23[b]	1.57[a,b]	0.052	0.38	0.03
C16:1 cis-10	0.02	0.02	0.02	0.001	0.64	0.31
iso C17:0	0.36	0.35	0.33	0.007	0.39	0.79
anteiso C17:0	0.52	0.54	0.45	0.011	0.10	0.27
C17:0	0.90[a]	0.82[b]	0.81[b]	0.009	<0.001	0.18
C17:1 cis-9	0.30	0.25	0.25	0.006	0.19	0.30
C18:0 (SA)	3.12[b]	4.69[a]	3.97[a,b]	0.121	0.05	<0.001
C18:1 trans-4	0.017[b]	0.023[a]	0.018[b]	0.001	0.47	<0.001
C18:1 trans-6+ trans-8	0.34	0.39	0.32	0.010	0.71	0.08
C18:1 trans-9	0.27	0.31	0.27	0.007	0.99	0.10
C18:1 trans-10	2.60	1.91	1.81	0.148	0.26	0.64
C18:1 trans-11 (VA)	0.87	1.23	1.08	0.056	0.43	0.15
C18:1 trans-13 + trans-14	0.41[b]	0.49[a]	0.41[b]	0.010	0.97	0.01
C18:1 cis-9	11.04[b]	12.75[a]	12.64[a]	0.180	0.02	0.17
C18:1 cis-11	0.52	0.50	0.50	0.010	0.59	0.83
C18:1 cis-12	0.18	0.23	0.19	0.008	0.78	0.06
C18:1 cis- 13	0.06	0.07	0.06	0.001	0.56	0.08
C18:1 trans-16 + cis-14	0.14	0.19	0.16	0.006	0.42	0.05
C18:2 trans-9, trans-12	0.03	0.02	0.03	0.001	0.26	0.29
C18:2 trans-8, cis-13	0.09	0.10	0.09	0.003	0.71	0.36

(continued on next page)

Table 2 (*continued*)

Fatty acid (g/100 g of FAME)[a]	Diet[b]				P-value	
	CON	CH50	CH100	SEM	Linear	Quadratic
C18:2 cis-9, trans-12	0.08	0.09	0.08	0.002	0.61	0.07
C18:2 trans-9, cis-12	0.03	0.03	0.03	0.001	0.23	0.81
C18:2n-6 (LA)	3.52	3.18	3.29	0.079	0.53	0.50
C18:3n-6	0.11	0.11	0.11	0.003	0.84	0.79
C18:3n-3 (LNA)	0.32	0.27	0.28	0.009	0.30	0.42
CLA cis-9, trans-11 (RA)	0.74	0.86	0.84	0.037	0.61	0.46
C18:4n-3	0.01	0.01	0.01	0.000	0.67	0.11
C20:0	0.13[b]	0.16[a]	0.15[a]	0.002	<0.001	<0.001
CLA trans-9, cis-11 +C21:0	0.056[b]	0.064[a]	0.057[b]	0.001	0.86	0.01
CLA trans-10, cis-12	0.01	0.01	0.01	0.000	0.89	0.25
CLA trans-11, trans-13	0.02	0.02	0.02	0.001	0.31	0.75
CLA trans-9, trans-11	0.02	0.02	0.02	0.001	0.48	0.98
C20:2n-9	0.02	0.02	0.02	0.000	0.72	0.26
C20:2n-6	0.04	0.04	0.04	0.001	0.34	0.67
C20:3n-9	0.03	0.03	0.03	0.001	0.96	0.50
C20:3n-6	0.04	0.04	0.04	0.001	0.52	0.41
C20:4n-6	0.19	0.18	0.19	0.003	0.86	0.49
C20:3n-3	0.01	0.01	0.01	0.000	0.98	0.73
C22:0	0.09[b]	0.10[a]	0.08[b]	0.001	0.75	<0.001
C20:4n-3	0.19	0.18	0.19	0.003	0.86	0.49
C22:1n-9	0.022[a,b]	0.025[a]	0.021[b]	0.000	0.36	0.03
C22:5n-3 (EPA)	0.035[a]	0.030[b]	0.031[b]	0.001	0.04	0.04
C22:2n-6	0.03[a,b]	0.04[a]	0.03[b]	0.001	0.30	0.03
C22:4n-6	0.03	0.03	0.03	0.001	0.47	0.96
C24:0	0.032[a,b]	0.035[a]	0.030[b]	0.001	0.18	0.02
C22:5n-3 (DPA)	0.07	0.06	0.06	0.001	0.22	0.21
C22:6n-3 (DHA)	0.03	0.02	0.03	0.001	0.46	0.30
Δ9- desaturase indices						
C10 index	0.48	0.49	0.49	0.021	0.80	0.87
C14 index	3.63[a]	2.69[b]	3.30[a,b]	0.092	0.39	0.03
C16 index	5.45	4.19	4.93	0.140	0.45	0.10
C18 index	78.36[a]	73.26[b]	76.50[a,b]	0.427	0.29	0.01
CLA cis-9, trans-11 index	47.06[a]	41.44[b]	45.92[a,b]	0.558	0.66	0.03
AI	3.47	3.16	3.34	0.051	0.54	0.19
TI	3.23	2.95	3.18	0.049	0.82	0.17
h:H	0.36	0.42	0.40	0.006	0.10	0.09

[a] SA = stearic acid; VA = vaccenic acid; LA = linoleic acid; LNA = linolenic acid; RA = rumenic acid; EPA = eicosapentaenoic acid; DPA = docosapentaenoic acid; DHA = docosahexaenoic acid; SFA = sum of the individual saturated fatty acids; UFA = sum of the individual unsaturated fatty acids; MUFA = sum of the individual monounsaturated fatty acids; PUFA = sum of the individual polyunsaturated fatty acids; TFA = trans fatty acids, sum of the individual trans fatty acids, except CLA isomers; BCFA = branched-chain fatty acids, sum of iso- and anteiso-FA; OBCFA = odd- and branched-chain fatty acids, sum of odd-, iso-, and anteiso-FA; SCFA = short-chain fatty acids, sum of the individual fatty acids from C4:0 to C10:0; MCFA = medium-chain fatty acids, sum of the individual fatty acids from C11:0 to C17:0; LCFA = long-chain fatty acids, sum of the individual fatty acids from C18:0 to DHA; PUFA n-3 and PUFA n-6 = sum of individual n-3 and n-6 fatty acids, respectively; CLA = sum of individual conjugated linoleic acids.TI = thrombogenic index; AI = atherogenic index; h:H = hypocholesterolemic to hypercholesterolemic ratio.

[b] Diet: CON = diet containing 100 g/d per head of soybean hulls; CH50 = diet containing 50 g/d per head of cocoa husks; CH100 = diet containing 100 g/d per head of cocoa husks.

Table 3
Total antioxidant capacity, enzyme activities, malondialdehyde, and protein carbonyls content from blood plasma of ewes fed with 100 g of soybean hulls (CON), 50 g of cocoa husks replacing 50 g of soybean hulls (CH50), and 100 g of cocoa husks replacing 100 g of soybean hulls (CH100).

	Diet[a]				*P*-value	
	CON	CH50	CH100	SEM	Linear	Quadratic
FRAP[b], μmol ascorbic acid/mL	0.82	0.85	0.94	0.02	0.14	0.55
ABTS[c], % inhibition	40.88	40.63	41.07	0.51	0.80	0.82
GSTS[d], units/mL	0.17	0.19	0.12	0.04	0.41	0.46
GR[e], units/mL	0.03	0.03	0.03	0.00	0.46	0.64
GPx[f], units/mL	0.07	0.07	0.08	0.00	0.61	0.90
SOD[g], units/mL	19.00	19.43	22.98	1.33	0.07	0.35
MDA[h], μM	0.53	0.44	0.43	0.02	0.99	0.33
PC[i], nmol/mL	2.89[a]	2.67[a]	2.23[b]	0.10	<0.001	0.19

[a] Diet: CON = diet containing 100 g/d per head of soybean hulls; CH50 = diet containing 50 g/d per head of cocoa husks; CH100 = diet containing 100 g/d per head of cocoa husks.
[b] FRAP: Ferric Reducing Ability of Plasma.
[c] ABTS: 2,2′-azino-di(3-ethylbenzthiazoline-6-sulforic acid).
[d] GSTS: Glutathione transferase.
[e] GR: Glutathione reductase.
[f] GPx: Glutathione peroxidase.
[g] SOD: Superoxide Dismutase.
[h] MDA: Malondialdehyde.
[i] PC: Protein carbonyls.

Table 4
Total antioxidant capacity, enzyme activities, malondialdehyde, and protein carbonyls content from milk of ewes fed with 100 g of soybean hulls (CON), 50 g of cocoa husks replacing 50 g of soybean hulls (CH50) and 100 g of cocoa husks replacing 100 g of soybean hulls (CH100).

	Diet[a]				*P*-value	
	CON	CH50	CH100	SEM	Linear	Quadratic
FRAP[b], μmol ascorbic acid/mL	1.80	1.67	1.55	0.07	0.59	0.98
ABTS[c], % inhibition	48.41	46.30	41.64	1.15	0.22	0.70
LPO[d], units/mL	0.45[ab]	0.81[a]	0.36[b]	0.14	0.66	0.02
GR[e], units/mL	0.24	0.17	0.22	0.03	0.77	0.12
SOD[f], units/mL	89.77	89.49	81.09	12.35	0.63	0.79
MDA[g], μM	0.49	0.41	0.49	0.02	0.11	0.07
PC[h], nmol/mL	4.36	3.87	3.99	0.10	0.64	0.16

[a] Diet: CON = diet containing 100 g/d per head of soybean hulls; CH50 = diet containing 50 g/d per head of cocoa husks; CH100 = diet containing 100 g/d per head of cocoa husks.
[b] FRAP: Ferric Reducing Ability of Plasma.
[c] ABTS: 2,2′-azino-di(3-ethylbenzthiazoline-6-sulforic acid).
[d] LPO: Lactoperoxidase.
[e] GR: Glutathione reductase.
[f] SOD: Superoxide Dismutase.
[g] MDA: Malondialdehyde.
[h] PC: Protein carbonyls.

MDA and LPO activity were also affected by the sampling time: MDA increased over the time, meanwhile LPO decreased ($P < 0.05$).

4. Discussion

4.1. Fatty acids profile

Diet plays an important role in the modification of ruminal biohydrogenation and fatty acid profile (Correddu et al., 2016). Moreover, FA profile can be also improved through genetic selection (Cesarani et al., 2019). CH contains more C18:0 and C18:1 cis-9 than SH (Table 1) and this could explain partially the quadratical effect found in milk of animal fed with CH. In fact, a passage of both FA to the mammary gland

is plausible. However, the lack of change in C18:0 content with the highest dose of CH could be partly related to higher rumen biohydrogenation of C18:1 cis9 to C18:0 and partly to the chain elongation of palmitate (C16:0) to stearate (C18:0) catalyzed by fatty acid elongase 6 (ELOVL6) that has been found active also in mammary gland (Shi et al., 2017).

Regarding FA groups, the linear reduction of OBCFA with increasing amounts of CH suggests a direct effect of this by-product on composition or richness of rumen microbial population. In ruminants the OCFA and BCFA are mostly derived from ruminal bacteria cell wall composition and therefore are considered a biomarker of rumen microbial fermentation and microbial de-novo lipogenesis (Toral et al., 2020; Prado et al., 2019; Vlaeminck et al., 2006). The C15:0 and C17:0, despite their negligible amount in milk, could be considered valuable markers of dairy fat intake because humans are not able to synthesize these FA (Albani et al., 2017; Brevik et al., 2005); in addition the interest on these FA is recently increased among scientists because of their inverse association with cardiovascular diseases (Jenkins et al., 2015; Khaw et al., 2012), incidence of type 2 diabetes (Hodge et al., 2007; Krachler et al., 2008), and tumoral cell proliferation (Cai et al., 2013).

Usually, high PUFA content in milk fat might partly explain its decreased desaturase activity index, because they reduce the genetic expression of Δ9 desaturase (Sessler and Ntambi, 1998). This is not the cause of our study as no differences in PUFA among groups have been observed. Therefore, although the concentrations of C18:1 cis-9 was the highest in CH50, the desaturase index related to these FA did not follow the same pattern, suggesting that the increase in these FA was related not to an increasing activity of Δ9 -desaturase but, more likely, to the increase in the concentration of their substrates C18:0, as explained before.

The AI and TI nutritional indexes of milk fat have not been affected by CH supplementation, in agreement with previous observation on dairy ewes' milk by using other by-products (Buffa et al., 2020b).

4.2. Antioxidant status in blood plasma

MDA and PC are the most frequently used biomarkers of lipids (Lykkesfeldt, 2007) and proteins (Weber et al., 2015) damage respectively, while the last one is considered as the most sensitive in oxidation (Cheah et al., 2008). The consumption of dark chocolate was reported to reduce protein oxidation in skeletal muscles of bicycle-treated humans (Taub et al., 2016), which is in accordance with our findings. A significant decline in PCs content of skeletal muscles of heart failure and type-2 diabetes patients was found, after three months of consuming 18 g of cocoa powder daily, which contained 100 mg of epicatechin (Ramirez-Sanchez et al., 2013). Moreover, a reduction in protein oxidation in the liver of rats treated with cocoa products has been also observed (Cordero-Herrera et al., 2015; Granado-Serrano et al., 2009). The protective role of cocoa against proteins oxidation might be due to its high polyphenols content (Felice et al., 2020). It has been shown that phenols possess high binding affinity with plasma proteins, and form complexes (López-Yerena et al., 2020; Pandey and Rizvi, 2009), which might be also biologically active (D'Archivio et al., 2007; Dufour et al., 2007). Further to polyphenols, the high MUFA (mainly oleic acid) content in CH (Table 1) might have also an involvement with the decline in PCs content in blood plasma of ewes fed the highest CH dose (Table 3). It has been reported that oleic acid in comparison with PUFA, inhibit lipid peroxidation in humans (Bonanome et al., 1992) and did not affected PC in bovine serum in vitro (Refsgaard et al., 2000).

However, in blood plasma, the increase in SOD activity of ewes supplement with CH (Table 3) could protect proteins and lipids from oxidation. In agreement with our results, the dietary supplementation of rats with cocoa extracts for 4 weeks enhanced SOD activity in their blood plasma (Jalil et al., 2008). Moreover, cocoa consumption increases significantly SOD activity in skeletal muscles of diabetic rats (Nikolić et al., 2016). Additionally, the higher (20 %) compared with the lower (10 %) inclusion level of cocoa powder in diets of oxidative stressed rats, had a better impact on lowering the MDA content and on increasing the SOD activity (Tamrin et al., 2017). The antioxidant capacity measured by ABTS and FRAP were not influenced by CH, in contrast with observation in human, blood plasma, where has been reported a dose response increases of antioxidant capacity after acute consumption of procyanidin-rich chocolate (Wang et al., 2020). Furthermore, the consumption of flavanol-rich cocoa enhances the blood plasma antioxidant capacity in hypercholesterolemic patients (Adamson et al., 1999; Rein et al., 2000). An improvement in blood plasma antioxidant status of healthy smokers-humans by dark chocolate (74 % cocoa) administration has been also reported (Hermann et al., 2006).

4.3. Antioxidant capacity of milk

The MDA and PCs in milk were not affected by CH supplementation, in contrast with previous observation in dairy ewes, in which a significant decline in PCs content in milk was found when were fed by products (grape pomace, tomato pomace, myrtle berries) rich in polyphenols (Buffa et al., 2020b). Polyphenols are very effective in forming complexes with milk proteins, and in particular caseins, which protect them from oxidation (Semo et al., 2007; Shukla et al., 2009; Xiao et al., 2011). LPO, increased only in CH50 group. It is an important enzyme since catalyzes the oxidation of numerous organic and inorganic substrates (Sarikaya et al., 2015), and has a crucial role in the mammary gland and in the defense of newborn intestine from bacteria. Moreover, the LPO system has pivotal role in the preservation of pasteurized and raw milk as well as of dairy products (Barrett et al., 1999; Martinez et al., 1988). On the other hand, the highest supplementation level of CH caused a sharp decline on LPO activity in ewes' milk. Although, some in vitro studies have shown that phenolic compounds (taxifolin, olivetol, cynarine and phloretin) inhibit LPO activity in bovine milk (Koksal et al., 2020) some others, indicate that none of all phenols have the same inhibitory power (Sarikaya et al., 2015). Indeed, epicatechin (the main polyphenol in CH) compared with caffeic acid, ferulic acid and quercetin have an average inhibition effect on LPO activity in bovine milk in vitro (Sarikaya et al., 2015). Thus, it can be assumed that the inhibitory effect of CH on LPO activity only in ewes fed the highest dose of CH, is closely relate with the supplemented dose of CH (dose response) in animals' diets. The negative impact of CH on LPO activity in milk with the highest inclusion level of CH was further confirmed not only by the sharpest numerical reduction of its total antioxidant capacity (measured by both FRAP and TBARS assays) but also by its MDA content, which was similar with the SBH one (Table 4). Thus, these results show that the inclusion level of CH in ewes' diets affects differently the oxidative capacity of milk.

4.4. Correlation between milk and blood antioxidant parameters

Pearson correlations between milk and blood parameters are reported in Table 5. No correlation between the same parameter measured on plasma and on milk was found. Probably, they respond differently to the treatment because the antioxidant effect of polyphenols in vivo is rather multifarious (Correddu et al., 2020). In fact, the antioxidant framework is a dynamic and complex system which converts oxidative products into others metabolites by specific enzymatic and non-enzymatic antioxidant compounds. This suggests that the antioxidant physiology in the systemic and the mammary gland compartments seemed to behave independently, and therefore both measurements are needed to understand the effect of antioxidant compounds supplemented to animals.

5. Conclusion

The incorporation of 100 g of cocoa hulls in ewes' diets, could be a good nutritional strategy to reduce the oxidative stress in animals'

Table 5
Pearson correlation between milk and blood antioxidant parameters of dairy sheep. Bold values represent significant correlation ($P < 0.05$).

	ABTS milk	FRAP milk	MDA milk	PC milk	LPO milk	GR milk	SOD milk
ABTS blood	0.185	−0.135	0.144	0.121	−0.098	0.098	0.008
	0.208	0.361	0.328	0.413	0.508	0.507	0.956
FRAP blood	−0.181	−0.008	**0.309**	−0.134	−0.019	0.02	−0.256
	0.225	0.957	**0.034**	0.369	0.901	0.894	0.086
MDA blood	0.09	−0.013	0.175	0.158	−0.22	0.083	−0.237
	0.57	0.936	0.269	0.319	0.161	0.601	0.135
PC blood	0.052	0.185	0.245	−0.227	−0.054	0.174	−0.095
	0.732	0.225	0.105	0.133	0.727	0.253	0.539
GPx blood	−0.262	0.17	0.249	−0.071	0.03	0.006	0.019
	0.093	0.283	0.112	0.654	0.853	0.969	0.907
SOD blood	−0.113	−0.082	0.215	−0.126	−0.19	**0.372**	−0.202
	0.445	0.578	0.142	0.394	0.197	**0.009**	0.174
GSTS blood	0.103	**−0.289**	0.104	−0.009	0.032	−0.175	−0.186
	0.487	**0.046**	0.482	0.952	0.829	0.235	0.212
GR blood	−0.233	−0.1	−0.215	**−0.294**	−0.071	−0.118	−0.037
	0.112	0.499	0.142	**0.042**	0.634	0.424	0.806

organism, as indicated by the PCs content in their blood plasma. The inclusion of CH at 5% is also favorable for the oxidative stability of ewes' milk as shown from the MDA values and LPO activity on it. Highest level of CH enhances oxidative stress in milk, and more research is needed to define the highest optimum inclusion level of them in ewes' diets. Moreover, the influence of CH on milk fatty acid composition is related to the dose, but we conclude that incorporation of 50 and 100 g of this byproduct in sheep diet does not cause a worsening of fatty acid profile. Finally, the lack of relationship of the same parameters in milk and blood, suggest that the measuring the blood antioxidant status is not indicative of the antioxidant spectrum of milk and vice versa.

Authors contributions

S. Carta: investigation, formal analysis, writing - original draft; E. Tsiplakou: writing- review and editing; C. Mitsiopoulou: formal analysis, investigation, G. Pulina: conceptualization, validation, writing-review; A. Nudda: methodology, conceptualization, validation, supervision, writing- review. All the authors have read and approved the final version of this manuscript.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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