Inclusion of cocoa by-product in the diet of dairy sheep: Effect on the fatty acid profile of ruminal content and on the composition of milk and cheese

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ABSTRACT

In this study, we hypothesized that dietary cocoa bean shell (CBS) as a partial replacer of human edible cereal grains in the diet of lactating ewes may affect performance and milk and cheese composition. Twenty Comisana lactating ewes allotted into control (CTRL; $n = 10$) or cocoa (CBS; $n = 10$) group received alfalfa hay *ad libitum* and 800 g of conventional (CTRL) or experimental (CBS) concentrate containing 11.7% CBS to partially replace corn and barley of the CTRL concentrate. Milk yield and composition did not differ between groups, and only urea concentration was lower in CBS milk. Dietary CBS increased cheese fat and reduced protein percentage in CBS group. Fatty acid composition of rumen content partially reflected that of the ingested diet, with total saturated fatty acids (SFA), total monounsaturated fatty acids (MUFA), 16:0, 18:0 and 18:1c9 greater in the CBS group. Moreover, all the identified *trans*- and *cis*-18:1 isomers were greater in CBS rumen content. Milk and cheese showed a similar fatty acid composition. Total MUFAs were greater in milk and cheese of CBS, mainly due to the proportion of 18:1c9, and conversely, total polyunsaturated fatty acids (PUFA), PUFAn-6 and PUFAn-6-to-PUFAn-3 ratio was greater in CTRL group. Concluding, the inclusion of CBS in the diet of lactating ewes within the limit imposed by the current legislation did not cause detrimental effects on animal performance and milk composition. Interestingly, dietary CBS reduced milk urea concentration probably due to the phenols contained in CBS concentrate. However, our results support that biohydrogenation was weakly impaired by dietary CBS. Finally, CBS negatively affected cheese nutritional characteristics due to lower protein and greater fat content, but improved fat health indexes in milk and cheese.

Implications

Cocoa bean shell, a by-product of chocolate industry, can be used as an alternative feed to partially replace cereals traditionally included in ruminant diet. This could represent a strategy to reduce feed-to-food competition and feeding cost when pasture is scarcely available. Within the limit imposed by the regulation, dietary cocoa bean shell does not affect animal performance and major milk components in sheep, but reduces milk urea. It could determine a reduction of protein and an increase of fat percentage in cheese. Improved health indexes can be observed in milk and cheese fat from ewes fed cocoa bean shell.

Introduction

In the Mediterranean, small ruminants are principally fed on pasture, which provides bioactive compounds that contribute to improve milk and meat nutritive value, fatty acid composition and oxidative stability (Cabiddu et al., 2016). However, the climatic conditions of that region make pasture unevenly available during the year. Thus, providing alternative feeds would be important to maintain acceptable productive levels and product quality when pasture is lacking. Agro-industrial by-products are generally safe and widely accepted as animal feed and could represent an important source of nutrients for ruminants (Salami et al., 2019). Introducing nonhuman edible agro-industrial by-products as an alternative ingredient in the ruminant diet could reduce the feed-to-food competition in livestock production and the feeding

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costs (Kasapidou et al., 2015). Cocoa industry produces a vast amount of by-products across the world. Some of them are generated in the cultivation areas during the harvesting and postharvesting processes (Martínez et al., 2012). Among these, cocoa pod husk (70–75% of the fruit weight) represents the part of the fruit discarded after cocoa beans removing, whereas cocoa mucilage is a white mass which surrounds the cocoa beans (Vasquez et al., 2019). Though cocoa is not a Mediterranean cultivation, a considerable amount of cocoa by-products is present in this area due to the presence of processing industries. Specifically, this study focused on cocoa bean shell **(CBS)**, which is removed from the seeds during the winnowing process to prevent foods off-flavours and miscoloration (Okiyama et al., 2017). CBS contains 12–18% protein, 19–60% dietary fibre and 6–8% fat (Vasquez et al., 2019). Additionally, it is a natural source of bioactive compounds that show antioxidant (Okiyama et al., 2017) and antiradical (Bordiga et al., 2015) activity. Bordiga et al. (2015) reported that polyphenols, among these tannins, are mainly responsible for the bioactive properties of cocoa by-products. These compounds have the potential to affect ruminant performance and product quality by altering the rumen metabolism (Frutos et al., 2020). Therefore, CBS may represent a potential alternative ingredient in the diet for ruminants. However, the presence of theobromine in cocoa by-products must be taken into account for a safe inclusion of CBS in animal diet. Theobromine is an alkaloid that may cause a reduction of palatability and toxicity symptoms when fed at large quantities to livestock (Adamafio, 2013). For this reason, the European legislation established that the concentration of theobromine in animal feeds cannot exceed 300 mg/kg. The effect of the dietary administration of CBS on dairy ruminant performance and product quality has been scarcely investigated. To the best of our knowledge, only Carta et al. (2020) investigated the effect of dietary cocoa bean shell on milk composition and haematological parameters in dairy sheep. Considering all of the above, in this study we supposed that dietary administration of a safe dose of CBS as a partial replacer of cereals in the diet of lactating ewes may have an effect on animal performance, fatty acid composition of rumen content and milk and cheese composition.

Material and methods

Animals and diets

The trial was performed in the facility of the Department of Agriculture, Food and Environmental Science of the University of Perugia (Italy), where animals are conducted as in a real commercial dairy farm and only common agricultural practices are followed. Twenty multiparous lactating Comisana ewes (80 ± 8 days in milk), homogeneous for BW (65 ± 8 kg), were randomly allotted into control (CTRL; n = 10) or cocoa (CBS; n = 10) group and confined in multiple pens. Within each group, the animals were housed into three different sawdust bedded pens (two pens hosting three animals and one pen hosting four animals) where water was always available. The trial lasted a total of 35 days. During the first 14 days (1–14th day) animals were adapted to the experimental dietary regimen. Before the adaptation period, the ewes were kept on the same diet. During the experimental period (21 days), each animal received chopped alfalfa hay *ad libitum* (particle size > 4 cm in length) and 800 g/day/ewe of a conventional concentrate (**CTRL**) or an experimental concentrate (**CBS**) containing 116.7 g/kg DM of CBS to partially replace corn and barley of the CTRL concentrate. Theobromine content in the CBS declared by the furnisher was 7 220 mg/kg DM. The experimental concentrates were offered individually (400 g/head at each of the two daily milking at 7.30 a.m.

and 5.30 p.m.) and were completely consumed by all the animals. The concentrate ingredients were incorporated into pellets (5 mm length) using a pelleting machine (CMS-IEM – Colognola ai Colli, Verona, Italy). Diets were formulated to be isoproteic and according to the nutrient requirement of a dairy ewe weighing 68 kg and producing 1 kg of milk at 6.5% of fat (Cannas et al., 2004). Table 1 reports ingredients and chemical composition of the experimental feeds. Offered feeds and orts were daily weighed per pen while individual milk production was recorded weekly.

Feed sampling and analysis

A weekly collected subsample of each feed was stored at −30 °C until analysis. An equal amount of the above subsamples was mixed to obtain a representative feed sample destined to the chemical analyses and freeze-dried. Crude protein, ether extract, and ash were determined according to AOAC (AOAC

Table 1
Ingredients, chemical and fatty acid composition of the experimental treatments of the hay and cocoa bean shell administrated to the ewes.

Items	Hay	Cocoa bean shell	Experimental Concentrate CTRL[1]	CBS[2]
Ingredients (g/kg of DM)				
Cocoa bean shell			-	116.7
Corn			316.6	259.7
Barley			309.6	251.1
Wheat bran			148.8	147.9
Soybean meal (44% CP)			119.5	118.8
Sunflower meal			50.8	50.4
Molasses			34.2	34.0
Calcium carbonate			5.1	5.1
Sodium bicarbonate			5.1	5.1
Dicalcium phosphate			5.1	5.1
Sodium chloride			5.1	5.1
Chemical composition (g/kg of DM)				
CP	78.4	120.3	163.2	166.2
Ether extract	16.3	204.2	21.7	31.6
NDF	562.8	330.8	181.0	217.9
ADF	409.0	273.2	90.0	132.7
ADL	62.9	137.0	22.7	38.5
NFC[3]	302	350.6	602.1	553.9
Ash	58.3	46.3	50.1	57.6
Protein fraction[4] (g/100 g total protein)				
A	20.7	14.5	17.4	21.0
B1	15.4	1.6	24.4	15.4
B2	39.4	52.0	103.3	102.7
B3	15.9	8.3	11.5	14.2
C	8.64	43.9	6.6	12.9
Fatty acids (g/100 g of fatty acids)				
14:0	1.29	0.23	0.09	0.27
16:0	25.0	22.4	16.9	18.5
18:0	4.45	23.9	2.24	9.04
18:1c9	7.78	39.0	20.9	26.0
18:2c9c12	30.0	8.50	52.6	39.1
18:3c9c12c15	26.8	0.50	3.02	2.42
Total extractable phenols (g/kg DM)	8.70	43.93	1.94	5.20
Total extractable tannins (g/kg DM)	3.92	30.3	0.98	3.64

[1] CTRL = control concentrate.
[2] CBS = concentrate containing cocoa bean shell diet.
[3] NFC = Nonfibre detergent carbohydrates calculated as 1 000-(CP-(NDF + NDFIP)-Ether Extract – Ash) where NDFIP represents the protein fraction linked to NDF.
[4] Protein fractions: A = nonproteic nitrogen; 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 (Licitra et al., 1996).

International, 1995). The crude protein was partitioned into five fractions, according to the Cornell Net Carbohydrate and Protein System, as modified by Licitra et al (1996). The content of NDF, ADF and ADL was determined using heat-stable amylase and sodium sulphite according to Van Soest et al. (1991). The results were expressed inclusive of residual ash. Fatty acids of the feeds were extracted from freeze-dried sample and converted to fatty acid methyl esters (**FAMEs**) with a one-step procedure using chloroform and 2% (v/v) sulfuric acid in methanol (Shingfield et al., 2003) and nonadecanoic acid (Sigma-Aldrich, St. Louis, MO, U.S. A) as internal standard. Individual FAME separation and quantification were performed by gas-chromatography according to Campione et al. (2020) (Supplementary Material S1). Total extractable phenolic compounds and tannins in feeds were analysed according to Valenti et al. (2019) (Supplementary material S2).

Rumen sample collection and fatty acid profile

Individual rumen content was collected before morning feeding on 35th day of trial using a stomach tube connected to an electrical pump, immediately frozen at −80 °C, freeze-dried and stored at −18 °C pending analysis. Trans-esterification of rumen content lipid was performed as described by Natalello et al. (2019) (Supplementary Material S3). Individual FAMEs were separated and quantified as described for feeds (Supplementary Material S1).

Milk sampling and analysis

Milk samples were collected during the morning and evening milking at the beginning of the adaptation period (day 1) and weekly throughout the experimental period (day 21st, 28th and 35th). Individual milk samples from the morning and evening milking were proportionally gathered in a single sample according to the morning and afternoon yield and, subsequently, split into aliquots for analysis. An aliquot was analysed for fat, lactose, protein, casein, urea and somatic cell count (Milkoscan 6000 FT supplied by Foss Electric, Hillerod, Denmark). Somatic cell count data were expressed as linear score ((log$_2$ (Somatic cell count/12 500)). Another aliquot was analysed for fatty acid composition as reported by Campione et al. (2020) (Supplementary Material S1).

Cheese manufacturing and analysis

Bulk milk from each of the two feeding groups was daily collected during the experimental period and stored at −30 °C until the quantity of 15 kg was reached, each representing a batch of milk. A total of fifteen cheeses per group were obtained in five different days of cheese making across the experimental period. The cheese-making procedure is detailed in the Supplementary Material S4. The determination of moisture, lipid and protein content of the cheese samples were performed as reported by Bradley and Vanderwarn (2001), Gerber-Van Gulik method (ISO 1975) and Kjeldhal method (total nitrogen × 6.38), respectively. Cheese FAME preparation was performed according to Nudda et al. (2005) (Supplementary Material S4). Individual FAMEs of cheese were separated and quantified as described for feeds (Supplementary Material S1).

Statistical analysis

Data on DM intake, milk yield, milk chemical and fatty acid composition were analysed using the mixed model procedure to test the effect of dietary treatment, time of sampling (day 21st, 28th and 35th) as fixed factors and their interaction. The individual animal was used as random factor for the analysis of milk chemical and fatty acid composition, whereas individual pen was used as

random factor for the analysis of DM intake and milk yield. The pretreatment data of each parameter were used as a covariate. When the covariate was not significant ($P > 0.05$), it was excluded from the model. The general liner model procedure for repeated measures was used to test the effect of dietary treatment, time of cheese production (1–5) as fixed factors and their interactive effect on the chemical and fatty acid composition of cheese. Least square difference was used for the multiple comparisons of the means. Data on the fatty acid composition of rumen content were analysed by one-way ANOVA, using the dietary treatment as the fixed factor. Significance was declared when ($P \leq 0.05$). The analyses were performed using the statistical software SPSS, version 25 (SPSS Inc., Illinois).

Results

Feed composition

The protein concentration (Table 1) in CBS was 120 g/kg DM, 4.4% of which represented by unavailable nitrogen (C fraction). Neutral detergent fibre was 331 g/kg DM and ether extract was 204 g/kg DM, the latter dominated by oleic acid (39% of total fatty acids). CBS contained 43.9 g/kg DM total extractable phenols, 30.3 g of which were tannins. Consequently, the CBS concentrate was enriched with 5.20 and 3.64 g/kg DM of total phenols and tannins, respectively. The CTRL concentrate was lower in total phenols and tannins (1.94 and 0.98 mg/kg DM, respectively). The CBS concentrate showed a greater amount of NDF, ADF and ADL, C fraction of crude protein and ether extract in comparison with the CTRL. Regarding fatty acids, CBS inclusion increased the percentage of 18:0 and 18:1*c*9 in CBS concentrate, while the CTRL concentrate was richer in 18:2c9c12 and 18:3c9c12c15.

Milk yield and chemical composition of milk and cheese

Table 2 reports the effect of the dietary treatment on milk yield and milk and cheese composition. Milk yield, protein, fat percent-

Table 2
Effect of the inclusion of cocoa bean shell in the diet of lactating ewes on milk yield and milk and cheese composition.

Items	Dietary treatment[1] (D)			P-value[2]		
	CTRL	CBS	SEM	D	Time (T)	D × T
DM Intake (g/day)	2 080	2 070	22.9	0.823	0.165	0.998
Milk Yield (g/day)	628	627	78.36	0.989	0.011	0.289
FPCM[3] (g/day)	627	651	86.90	0.674	0.005	0.128
Milk composition (%)						
Fat	6.47	6.87	0.490	0.135	0.282	0.028
Protein	6.07	5.87	0.134	0.125	0.005	0.447
Lactose	4.39	4.25	0.071	0.095	0.001	0.040
Casein	4.72	4.54	0.118	0.085	0.005	0.382
Casein index[4]	77.8	77.3	0.427	0.055	0.005	0.551
Urea (mg/dl)	38.6	34.9	2.973	0.033	0.001	0.251
Linear Score[5]	4.21	3.81	1.05	0.665	0.009	0.278
Cheese composition (%)						
Moisture	35.7	34.8	0.687	0.390	<0.001	0.184
Fat	30.3	33.3	0.472	<0.001	0.009	0.012
Protein	25.8	23.7	0.414	0.003	0.260	0.360
Sodium chloride	2.24	2.22	0.069	0.907	0.016	0.387
Ash	5.89	5.96	0.099	0.751	0.198	0.838

[1] Dietary treatment consisted of hay *ad libitum* + 800 g/head/day of a control concentrate (CTRL) or a concentrate containing 11.7% cocoa bean shell (CBS).
[2] Probability of significant effect ($P \leq 0.05$).
[3] Fat and protein corrected milk.
[4] Caseins/Proteins * 100.
[5] Linear score calculated as LS = log$_2$ (SCC/12 500) where SCC is the somatic cell count.

age and linear score were not affected by the dietary treatment ($P > 0.05$). Conversely, urea concentration was significantly lower ($P = 0.033$) in the CBS milk. Regarding cheese, fat percentage was greater in the CBS group ($P < 0.001$), while protein was greater in the CTRL group ($P = 0.003$).

Fatty acid composition of rumen content

Table 3 reports the effect of dietary treatment on rumen content fatty acid composition. Feeding CBS increased the concentration of total saturated fatty acids (SFA; $P = 0.006$). Among individual SFA, 16:0 and 18:0 were greater ($P \leq 0.05$) in the rumen content of CBS ewes. In particular, 18:0 was found at double concentration in the rumen of CBS group. Similarly, total monounsaturated fatty acids (**MUFAs**) were greater ($P = 0.008$) in the CBS rumen content. As regards individual MUFA, 18:1t6 + t7 + t8, 18:1t9, 18:1t10 18:1t11, 18:1c6, 18:1c9, 18:1c11, 18:1c12 and 18:1c14 were greater ($P \leq 0.05$) in the rumen content of CBS group. Among polyunsaturated fatty acids (**PUFAs**), only 20:4n-6 was greater ($P = 0.001$) in the CTRL group. As for the individual odd and branched chain fatty acids (**OBCFAs**), 13iso was greater in the CTRL rumen content ($P = 0.021$).

Milk and cheese fatty acids

Total MUFA and OBCFA were greater ($P \leq 0.05$) in CBS milk (Table 4). Conversely, the sum of PUFA ($P < 0.001$), PUFAn-6 ($P < 0.001$), and the ratio PUFAn6-to-PUFAn3 ($P = 0.001$) were greater in the milk of CTRL group. Although total SFAs were not affected by the dietary treatment, saturated fatty acids from 10:0 to 16:0 were greater ($P \leq 0.05$) in CTRL milk, while 4:0, 18:0, 20:0 and 24:0 were greater ($P \leq 0.05$) in CBS milk. As for the individual MUFA, the inclusion of CBS in diet reduced 12:1c9, 14:1c9, 16:1c9 and 20:1c11 ($P \leq 0.05$). Regarding 18-carbon MUFA, 18:1c9 ($P < 0.001$) was greater in the CBS milk, while 18:1t10 and 18:1c12 were found at greater ($P \leq 0.05$) proportion in the CTRL milk. Among PUFA, 18:3n-6, 20:4n-6 and 20:5n-3 were greater ($P \leq 0.05$) in CTRL milk. Finally, regarding individual OBCFA, CTRL milk was richer ($P < 0.001$) in 17iso and 17anteiso than CBS.

The fatty acid composition of the cheese largely reflected that of milk (Table 5). The sum of SFA did not differ ($P > 0.05$) between groups, but of 8:0–14:0 were greater ($P < 0.001$) in the CTRL cheese, while CBS increased ($P \leq 0.05$) 4:0 and 16:0–24:0. The greater proportion of total MUFA ($P < 0.001$) in the cheese of CBS group was mainly due to 18:1c9 ($P < 0.001$), as the other MUFAs that differed ($P \leq 0.05$) between groups (12:1c9, 14:1c9; 16:1c9 and 18:1c12) were greater in the CTRL cheese. Total PUFA, PUFAn-6 and PUFAn-3 were greater ($P \leq 0.05$) in the CTRL cheese as well as 18:2c9c12 and 20:2n-6. Conversely, 18:3n-6 and 18:3c9c12c15 were greater ($P \leq 0.05$) in the CBS cheese. The dietary inclusion of CBS did not affect ($P > 0.05$) total OBCFA; however, 15:0 was greater ($P = 0.039$) in CBS cheese, and 17iso and 17anteiso were greater ($P \leq 0.05$) in CTRL cheese. Lastly, regarding indexes of health importance, the cheese from CBS group showed a lower ($P \leq 0.05$) PUFAn-6-to-PUFAn-3 ratio, atherogenic and thrombogenic indexes.

Discussion

We investigated for the first time the effect of the dietary administration of cocoa bean shell on animal performance, fatty acid profile of rumen content and milk and cheese composition in lactating ewes. Specifically, 11.7% of cocoa bean shell was included in a pelleted feed given to lactating ewes, representing

Table 3
Effect of the inclusion of cocoa bean shell in the diet of lactating ewes on rumen content fatty acids (g/kg DM).

Items	Dietary treatment[1]			P-value[2]
	CTRL	CBS	SEM	
12:0	0.107	0.112	0.033	0.810
13:0iso	0.008	0.013	0.003	0.021
14:0	0.371	0.354	0.083	0.766
14iso	0.105	0.105	0.017	0.996
14:1t	0.049	0.061	0.008	0.233
15:0	0.493	0.479	0.093	0.822
15:0iso	0.220	0.237	0.032	0.497
15:0anteiso	0.461	0.505	0.063	0.535
16:0	7.208	10.208	0.609	0.028
16:1c7	0.065	0.123	0.070	0.224
16:1c9	0.049	0.065	0.015	0.132
16:0iso	0.309	0.357	0.068	0.495
17:0	0.208	0.214	0.037	0.820
17:0iso	0.239	0.252	0.046	0.674
17:0anteiso	0.504	0.503	0.117	0.996
18:0	7.892	14.209	1.720	0.005
18:1t5	0.030	0.034	0.009	0.605
18:1t6	0.018	0.064	0.024	0.014
18:1t9	0.019	0.042	0.014	0.012
18:1t10	0.016	0.072	0.034	0.036
18:1t11	1.336	1.815	0.132	0.030
18:1c6	0.127	0.316	0.076	0.002
18:1c9	1.855	2.603	0.288	0.050
18:1c11	0.159	0.231	0.019	0.028
18:1c12	0.072	0.135	0.021	0.001
18:1c13	0.006	0.007	0.002	0.559
18:1c14	0.017	0.054	0.017	0.005
18:2t8c13	0.012	0.007	0.020	0.112
18:2t9c12	0.011	0.011	0.009	1.000
18:2t9c13	0.094	0.085	0.020	0.490
18:2t10c12	0.022	0.017	0.009	0.502
18:2c9t11	0.095	0.076	0.029	0.110
18:2c9c12	1.698	2.215	0.285	0.136
18:3c9c12c15	0.346	0.358	0.005	0.593
20:0	0.192	0.254	0.030	0.085
20:1t11	0.044	0.056	0.016	0.206
20:1c11	0.007	0.007	0.050	0.826
20:2n-6	0.002	0.001	0.040	0.905
20:4n-6	0.056	0.036	0.017	0.001
21:0	0.025	0.022	0.008	0.519
22:0	0.129	0.155	0.016	0.103
22:5n-6	0.005	0.010	0.013	0.557
22:6n-3	0.021	0.018	0.013	0.750
23:0	0.104	0.105	0.027	0.950
24:0	0.132	0.157	0.019	0.098
$\sum$SFA[3]	16.032	25.449	1.170	0.006
$\sum$MUFA[4]	4.042	5.873	0.428	0.008
$\sum$PUFA[5]	2.614	3.110	0.369	0.202
$\sum$OBCFA[6]	2.678	2.792	0.446	0.746
$\sum$PUFAn-6	1.916	2.385	0.303	0.166
$\sum$PUFAn-3	0.348	0.358	0.057	0.639

[1] Dietary treatment consisted of hay *ad libitum* + 800 g/head/day of a control concentrate (CTRL) or a concentrate containing 11.7% cocoa bean shell (CBS).
[2] Probability of significant effect ($P \leq 0.05$).
[3] Saturated Fatty Acids.
[4] Monounsaturated Fatty Acids.
[5] Polyunsaturated Fatty Acids.
[6] Odd and Branched Chain Fatty Acids.

4.5% of the total DM intake. That percentage of inclusion was chosen in order to maximize the dietary administration of CBS and to respect the limit imposed by the current European regulation on the presence of theobromine in animal feeds.

The biohydrogenation of dietary PUFA represents one of the most investigated issues of ruminant metabolism due to its implications on the quality of ruminant products. It is known that the chemical composition, the physical status of the diet as well as the presence of bioactive compounds may greatly affect both the extent and the pathways of PUFA biohydrogenation, which in turn

Table 4
Effect of the inclusion of cocoa bean shell in the diet of lactating ewes on the fatty acid profile of milk (g/100 g of total fatty acids).

| Items | Dietary treatment[1] (D) | | | P-value[2] | | |
	CTRL	CBS	SEM	D	Time (T)	D × T
4:0	1.88	1.98	0.084	0.016	0.194	0.363
6:0	2.05	2.05	0.099	0.491	0.085	0.910
8:0	2.36	2.31	0.170	0.109	0.023	0.806
10:0	7.87	7.39	0.582	0.001	0.002	0.336
12:0	4.63	4.12	0.391	<0.001	0.001	0.184
12:1	0.19	0.16	0.025	0.001	0.007	0.123
14:0	11.7	10.9	0.450	0.001	0.016	0.484
14:1c9	0.22	0.17	0.019	<0.001	<0.001	0.989
15:0	1.02	1.09	0.09	0.921	0.089	0.955
15:0iso	0.28	0.28	0.025	0.968	0.048	0.139
15:0anteiso	0.50	0.51	0.041	0.904	0.700	0.998
16:0	27.3	26.6	0.344	0.002	0.315	0.005
16:1c9	1.07	0.91	0.072	<0.001	0.067	0.932
17:0	0.50	0.54	0.031	0.009	0.854	0.864
17:0iso	0.44	0.40	0.039	<0.001	0.007	0.746
17:0anteiso	0.54	0.49	0.054	<0.001	0.143	0.766
18:0	7.51	9.05	0.611	<0.001	0.011	0. 860
18:1t6	0.11	0.13	0.013	0.190	0.009	0.817
18:1t9	0.23	0.25	0.227	0.751	0.062	0.265
18:1t10	0.37	0.33	0.052	0.005	0.043	0.375
18:1t11	0.83	0.86	0.840	0.614	0.053	0.346
18:1c6	0.42	0.40	0.026	0.008	0.008	0.860
18:1c9	18.5	19.6	1.36	<0.001	0.017	0.361
18:1c11	0.77	0.76	0.745	0.947	0.001	0.483
18:1c12	0.30	0.27	0.021	<0.001	0.000	0.050
18:2c9t11	0.43	0.40	0.419	0.150	0.517	0.981
18:2c9c12	3.30	2.96	0.325	0.069	<0.001	0.084
18:3n-6	0.06	0.04	0.009	0.003	0.123	0.242
18:3c9c12c15	0.51	0.50	0.041	0.247	0.000	0.271
20:0	0.34	0.38	0.028	0.004	0.001	0.453
20:1c11	0.07	0.05	0.010	<0.001	0.004	0.077
20:2n-6	0.02	0.02	0.008	0.698	0.132	0.772
20:3n-6	0.02	0.02	0.008	0.113	0.111	0.449
20:4n-6	0.22	0.18	0.013	<0.001	0.003	0.216
20:5n-3	0.03	0.02	0.010	0.010	0.703	0.699
22:0	0.19	0.20	0.026	0.134	0.006	0.838
22:2n-6	0.06	0.04	0.011	0.068	0.000	0.327
22:6n-3	0.03	0.03	0.009	0.413	0.309	0.032
24:0	0.06	0.08	0.014	0.013	0.019	0.452
∑SFA[3]	65.9	65.2	0.926	0.086	0.030	0.478
∑MUFA[4]	22.8	24.0	0.724	<0.001	0.008	0.417
∑PUFA[5]	4.79	4.34	0.185	<0.001	0.000	0.068
∑OBCFA[6]	4.08	4.87	0.196	0.033	0.757	0.753
∑PUFAn-6	3.68	3.30	0.015	<0.001	0.000	0.073
∑PUFAn-3	0.68	0.64	0.049	0.615	0.000	0.194
PUFAn6/n3	5.73	5.19	0.345	0.001	0.001	0.70

[1] Dietary treatment consisted of hay *ad libitum* + 800 g/head/day of a control concentrate (CTRL) or a concentrate containing 11.7% cocoa bean shell (CBS).
[2] Probability of significant effect ($P \leq 0.05$).
[3] Saturated Fatty Acids.
[4] Monounsaturated Fatty Acids.
[5] Polyunsaturated Fatty Acids.
[6] Odd and Branched Chain Fatty Acids.

Table 5
Effect of the inclusion of cocoa bean shell in the diet of lactating ewes on the cheese fatty acid profile (g/100 g of total fatty acids).

| Item | Dietary treatment[1] (D) | | | P-value[2] | | |
	CTRL	CBS	SEM	D	Time (T)	D × T
4:0	1.79	1.96	0.031	0.002	0.103	0.737
6:0	2.13	1.93	0.070	0.169	0.439	0.312
8:0	2.32	2.15	0.029	<0.001	0.244	0.691
10:0	8.08	7.22	0.121	<0.001	0.412	0.637
12:0	4.84	4.08	0.097	<0.001	0.105	0.640
12:1	0.19	0.16	0.004	<0.001	0.113	0.287
14:0	11.66	10.88	0.098	<0.001	0.226	0.661
14:1c9	0.21	0.18	0.042	<0.001	0.077	0.429
15:0	1.09	1.13	0.008	0.039	0.436	0.031
15:0iso	0.30	0.30	0.005	0.473	0.002	0.809
15:0anteiso	0.52	0.52	0.004	0.991	0.001	0.878
16:0	26.76	27.43	0.127	<0.001	0.001	0.926
16:1c9	1.00	0.91	0.135	<0.001	0.043	0.209
17:0	0.57	0.61	0.019	0.255	0.559	0.104
17:0iso	0.48	0.43	0.012	0.021	0.135	0.512
17:0anteiso	0.71	0.66	0.125	0.027	0.148	0.877
18:0	7.85	9.60	0.222	<0.001	0.181	0.283
18:1t6	0.11	0.12	0.005	0.430	0.635	0.609
18:1t9	0.22	0.24	0.007	0.234	0.575	0.807
18:1t10	0.34	0.29	0.019	0.256	0.334	0.633
18:1t11	0.79	0.84	0.016	0.125	0.974	0.595
18:1c6	0.41	0.42	0.008	0.921	0.377	0.153
18:1c9	17.61	19.01	0.198	<0.001	0.411	0.784
18:1c11	0.86	0.81	0.016	0.045	0.143	0.033
18:1c12	0.34	0.28	0.018	<0.001	0.176	0.078
18:2c9t11	0.39	0.40	0.008	0.493	0.928	0.402
18:2c9c12	3.31	2.80	0.064	<0.001	0.427	0.215
18:3c6c9c12	0.04	0.07	0.004	0.002	0.230	0.644
18:3c9c12c15	0.48	0.50	0.007	0.010	0.143	0.015
20:0	0.35	0.41	0.010	<0.001	0.555	0.643
20:1c11	0.07	0.06	0.004	0.323	0.182	0.897
20:2n-6	0.03	0.01	0.01	0.002	0.466	0.591
20:3n-6	0.02	0.02	0.007	0.250	0.148	0.526
20:4n-6	0.01	0.01	0.002	0.466	0.591	0.844
20:5n-3	0.11	0.10	0.004	0.217	0.036	0.844
22:0	0.18	0.22	0.008	0.011	0.439	0.233
22:2n-6	0.05	0.04	0.004	0.925	0.114	0.217
22:6n-3	0.02	0.02	0.003	0.350	0.230	0.632
24:0	0.06	0.08	0.004	0.001	0.042	0.783
∑SFA[3]	66.02	65.98	0.120	0.844	0.326	0.440
∑MUFA[4]	21.87	23.12	0.190	<0.001	0.400	0.777
∑PUFA[5]	4.75	4.10	0.082	<0.001	0.848	0.262
∑OBCFA[6]	3.80	3.79	0.030	0.746	0.105	0.020
∑PUFA n-6	3.65	3.07	0.073	<0.001	0.936	0.496
∑PUFAn-3	0.65	0.61	0.009	0.001	0.530	0.053
PUFAn6/n3	5.58	5.08	0.094	0.007	0.806	0.345
AI[7]	3.00	2.80	0.031	0.001	0.528	0.977
TI[8]	3.20	3.01	0.014	<0.001	0.084	0.865

[1] Dietary treatment consisted of hay *ad libitum* + 800 g/head/day of a control concentrate (CTRL) or a concentrate containing 11.7% cocoa bean shell (CBS).
[2] Probability of significant effect ($P \leq 0.05$).
[3] Saturated Fatty Acids.
[4] Monounsaturated Fatty Acids.
[5] Polyunsaturated Fatty Acids.
[6] Odd and Branched Chain Fatty Acids.
[7] Atherogenic index calculated as $(12:0 + 4 * 14:0 + 16:0)/(MUFA + PUFAn\text{-}6 + PUFAn\text{-}3)$.
[8] Thrombogenic index $(14:0 + 16:0 + 18:0)/[(0.5 * 18:1) + (0.5 * other MUFA) + (0.5*PUFA\text{-}6) + (3*PUFAn\text{-}3) + (PUFAn\text{-}3/PUFAn\text{-}6)]$.

may affect animal performance and fatty acid composition (Vasta and Luciano, 2011). Considering that CBS is a good source of polyphenols, especially tannins, an impairment of rumen BH could have been expected. However, our data on the fatty acids of rumen content indicate a lacking inhibitory effect of the tannins on the biohydrogenation (**BH**). BH is operated by the rumen microflora as a detoxification mechanism to escape the bacteriostatic effects of PUFA (Maia et al., 2010). Specifically, dietary PUFAs (mainly 18:2c9c12 and 18:3c9c12c15) are progressively converted into 18:0 with the production of several intermediates products (Buccioni et al., 2012). Although the exact mechanism of action is still controversial, tannins may affect the microbial metabolism through a direct inhibitory effect or by inducing a shift in the rumen microflora composition, which in turn may result in an impairment of BH (Costa et al., 2017, Frutos et al., 2020). The inhibition of the first step of BH protects PUFA from the saturation, resulting in an increase of PUFA both in the rumen content and in the ruminant products. Conversely, the impairment of the following steps may produce an accumulation of BH intermediate fatty acids, such as 18:1t11 and 18:2c9t1. In this study, the fatty acid composition of the rumen content seems to reflect that of

the ingested diet, probably suggesting that the inhibitory effect of the tannins on the BH did not occur or occurred minimally. Indeed, the quantity of the total PUFA and the principal substrates of the BH (i.e., 18:2c9c12 and 18:3c9c12c15) were comparable between the rumen content of the two groups, mirroring the quantity of 18:2c9c12 and 18:3c9c12c15 ingested by the two groups of ewes. Additionally, the hypothesis of a lacking or weak effect of tannins seems also supported by the findings on other fatty acids involved in the BH. In particular, the greater quantity of 18:0 and 18:1 isomers in the CBS rumen content seems to be related with the greater intake of 18:0 and 18:1c9 rather than with an effect of tannins on BH. Indeed, the ingestion of 18:0 was 3-fold greater in the CBS than CTRL group (1.51 v. 0.49 g/day). Regarding 18:1 isomers in the rumen, Van de Vossenberg and Joblin (2003) observed that cis-18:1 isomers can be converted to 18:0 through the synthesis of trans isomers. In our experimental conditions, the CBS ewes ingested 150% more 18:1c9 than the CTRL ewes (4.29 vs 2.85 g/day), which may justify both the greater presence of 18:1c9 and the higher presence of total and trans-18:1 isomers in the CBS rumen content. Our results are consistent with a previous study where dairy ewes received a high amount of dietary 18:1c9 (Campione et al., 2020). Though our data seem to indicate a negligible effect of tannins on BH, it could be stressed that the greater quantity of dietary fibre and lignin in the CBS diet could have partially masked the effect of tannins on this process. In particular, the greater quantity of lignin ingested by the CBS ewes could have increased the retention time of feed in the rumen of CBS ewes, increasing the time available for the microorganisms to perform their metabolism on unsaturated dietary fatty acids.

The milk fatty acid profile partially confirmed the differences observed in the rumen content between CTRL and CBS groups. Consistent with the rumen content, the sum of MUFA was greater in the CBS milk, mainly due to the contribution of 18:1c9. Moreover, 18:0 was greater in the milk of the same group. Preformed 18:0 and 18:1c9 fed with the diet or originating from rumen microbial metabolism greatly contribute to their presence in ruminant milk (Frutos et al., 2020). In addition, 18:1c9 can be synthetized from 18:0 by the action of delta-9 desaturase in the mammary gland (Mosley et al., 2002). In our trial, CBS group ingested a greater quantity of 18:0 and 18:1c9, which may explain the greater percentage of both these fatty acids observed in the CBS milk. In contrast with rumen content, the sum of SFA was found at comparable percentage in the milk of the two groups. However, among individual SFA, the proportion of 10:0–16:0 was greater in the CTRL milk. The reduction of short and medium chain fatty acids is a common result when long chain fatty acids are fed to ruminants as they negatively affect the de novo fatty acid synthesis in the mammary gland (Shingfield et al., 2003). The presence of OBCFA in milk should reflect that of the rumen content as the most part of milk OBCFA originate from rumen microflora (Vlaeminck et al., 2006). However, we have found that the proportion of total OBCFA was greater in the milk of CTRL ewes despite the concentration of these fatty acids in the rumen did not differ between groups. Nevertheless, it should be noticed that when the fatty acid composition of rumen content was expressed as g/100 g of total fatty acids, OBCFAs were greater in the rumen of CTRL group (data not shown). Vlaeminck et al. (2006) reported that amylolitic bacteria are richer in 15:0 and 15:0anteiso than fibrolytic bacteria. These fatty acids were found at comparable level in the milk of the two groups, which is consistent with the high consumption of hay by both the groups. Conversely, 17:0iso and 17:0anteiso were greater in the milk of CTRL group, while 17:0 was greater in the CBS milk. Therefore, further study is needed to assess if dietary CBS may have had an effect on the composition of rumen microflora. Regarding PUFA, several feeding strategies have been proposed with the aim of enhancing their concentration in ruminant products, due

to their potential in preventing chronic diseases or reduce inflammatory status in humans (Marventano et al., 2015). In our experiment, dietary CBS did not affect the milk PUFAn-3 and lowered milk total PUFA as compared with CTRL. However, it should be underlined that this result was principally due the greater percentage of PUFAn-6 found in the CTRL milk. PUFAn-6, such as arachidonic acid and its precursor 18:2c9c12, may be potentially harmful for human health as they may exert pro-inflammatory activity. For this reason, the PUFAn-6-to-n-3 ratio of the ingested diet should not be greater than four (Husted & Bouzinova, 2016). The milk from both the groups moderately exceeded the recommended value, but CBS showed a lower PUFAn-6-to-n-3 ratio in comparison with CTRL milk. Almost the entire sheep milk produced worldwide is not consumed as such, but it enters in the human diet as transformed products (i.e. yoghurt, cheese, etc.). Therefore, the relevance of the chemical and nutritive characteristics is greater if referred to these products. In the present study, the collected milk was used for cheese production. Both the CBS and CTRL cheese contained a high quantity of fat (more than 30%), which is not recommended for a balanced human diet. Moreover, CBS cheese contained 3% more fat than CTRL cheese. However, the macronutrients provided by a single food should not be considered alone but in the context of the total diet and, in the case of fat, its quality also evaluated. Usually, the fatty acid composition of cheese is similar to that of the milk used for the cheese production. Therefore, it is not surprising that the fatty acid composition of cheese largely reflects that of the milk. However, differing from milk, the sum of PUFAn-3 was greater in the CTRL as compared to CBS cheese. Nevertheless, the increased total PUFAn-3 was not enough to counterbalance the greater percentage of PUFAn-6 in CTRL cheese, thus resulting in greater PUFAn-6-to-n-3 ratio than CBS. Moreover, CTRL cheese showed greater atherogenic and thrombogenic indexes than CBS. Therefore, the worst nutritional properties of CBS cheese due to the greater quantity of fat were partially mitigated by improved healthy indexes.

As regards the effect of dietary CBS on animal performance and major components of milk, consistent with Carta et al. (2020), we did not observe an effect of dietary CBS on DM intake, milk yield and fat and protein percentage. In contrast with the same authors, we observed that urea concentration was lower in the CBS milk. The rumen protein metabolism largely affects the milk and protein yield. Licitra et al. (1996) reported that the C fraction of the dietary protein is supposed to be insoluble and unavailable in the rumen, thus it would not contribute to provide available amino acids at small intestine level. Similar to analogous by-products from the agro-industry, such as the hazelnut skin, the crude protein of CBS used in our study was rich in C fraction, which is related also to the presence of lignin. Though the experimental feeds given to the animals were formulated to provide both the groups with an isoproteic diet, the CBS concentrate showed a double concentration of C protein fraction in comparison with the CTRL concentrate, which may have determined a different ratio between available protein and readily available energy in CBS diet. The ammonia concentration in the rumen usually increases when a diet characterized by a lower energy-to-protein ratio is administered (Miller et al., 2001). In this study we did not measure the rumen ammonia content. However, the concentration of urea in milk can give information consistent with the rumen protein metabolism. In particular, it has been observed that in presence of a lower energy-to-protein ratio, a greater quantity of ammonia produced by the rumen bacteria during the degradation of dietary protein is excreted in the form of urea with milk, urine and faeces, instead of being incorporated into microbial protein (Miller et al., 2001). Consistently, we found a lower urea content in the milk from CBS ewes. A role of the greater quantity of phenols and tannins contained in the CBS concentrate in reducing milk urea could have been supposed as moderate doses of dietary

tannins may improve rumen protein metabolism. At rumen pH, tannins create stable complex with the protein, preventing its conversion to ammonia and resulting in a greater duodenal flux of protein nitrogen (Orlandi et al., 2015). However, the lower casein index observed, even if not significant (P = 0.055), in the CBS milk seems to suggest that the worse quality of protein fed to the CBS group played a major role in determining the rumen protein metabolism. Finally, considering cheese, the numerical difference observed in the chemical composition of milk could have been hampered by the concentration of milk components during cheese making and ageing, thus contributing to explain the greater percentage of protein found in CTRL group.

Conclusions

Our results indicate that, within the limit imposed by the current regulation, cocoa bean shell could be included in the diet of lactating ewes without detrimental effects on milk yield and protein and the fat percentage. Interestingly, the milk obtained from the ewes fed with cocoa bean shell diet showed a lower urea concentration, which could be the result of a different efficiency in nitrogen utilization at rumen level. Results from this experiment support the idea that biohydrogenation pathways of dietary PUFA were weakly impaired by the dietary treatment and protective effects of tannins of cocoa bean shell on dietary PUFA occurred minimally. Nevertheless, the lower proportion of odd and branched chain fatty acids observed in the rumen content of CBS group could be related with changes in the metabolism or in the rumen microbial population, which deserves further investigations. Dietary CBS negatively affected the nutritive value of cheese by reducing protein and increasing fat percentage, but improved the health indexes of fat. Considering the role of fat in determining the flavour of cheese, it would be of interest to evaluate the acceptability by the consumers of the cheese produced with the milk obtained from ewes fed CBS.

Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.animal.2021.100243.

Ethics approval

This study was not regulated by the Directive 2010/63/EU on the protection of animals used for scientific purposes because no practice causing lasting pain, suffering, distress or harm was used. Nevertheless, the animals were handled only by skilled personnel and in accordance with the Italian guidelines for the protection of experimental animals.

Data and model availability statement

None of the data were deposited in an official repository.

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Declaration of interest

The authors declare no conflict of interest.

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