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Abstract. Carob (*Ceratonia siliqua*) is commonly found in the Mediterranean region and may be used as an alternative feed resource in livestock production. However, carob contains plant secondary compounds, such as polyphenols, which limit its use due to potential toxicity problems. This study aimed to investigate whether the substitution of barley by carob pulp at a relatively high level of up to 35% causes production-level reduction and has detrimental effects on animal welfare. Lamb performance parameters such as feed intake, liveweight and carcass weight were recorded and feeding behaviour was monitored. Blood metabolites and protein profiles were determined to detect signs of metabolic distress. The inclusion of carob pulp resulted in similar level of performance by animals in all the experimental diets. However, the feeding pattern was different with feed intake being significantly lower during the first 90 min post feed supply in the carob-fed lambs compared with the Control animals, although total daily intake was similar. In terms of welfare indicators, the inclusion of carob pulp in the lambs' diet reduced blood cholesterol while increasing both non-esterified fatty acid and urea levels. These indicate that the animals were probably under some form of metabolic stress but not at a level to cause concerns, as confirmed by the similar serum protein profile especially in terms of albumin to globulins ratio.

Additional keywords: metabolic welfare, polyphenols, serum metabolites, serum protein profile.
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Introduction

Carob (*Ceratonia siliqua*) is a leguminosae plant representing an important component of the Mediterranean vegetation (Battle and Tous 1997). The carob pod is exploited by the food industry: the carob gum, a common food thickener and stabiliser, is extracted from the seeds; the pulp, the carob pod by-product, is traditionally used as food and feed (www.feedipedia.org, verified 24 November 2014). Carob pulp presents a high sugar content [~44% of the dry matter (DM); Calixto and Cañellas 1982; Marakis *et al*. 1997], a low protein content (3–5% DM; Avallone *et al.* 1997; Marakis *et al*. 1997) and a variable amount of tannins (3–20% DM; Avallone *et al.* 1997; Priolo *et al*. 2000; Silanikove *et al*. 2006). Carob pulp and pods can be potentially used as a source of energy for growing lambs and kids but the low protein content and the presence of tannins could limit their extensive use (Guessous *et al.* 1989; Karabulut *et al.* 2006; Silanikove *et al.* 2006). Obeidat *et al*. (2012) reported that the inclusion of carob pods up to 25% in lamb diets did not affect nutrient intake and digestibility in Awassi lambs whereas Priolo *et al*. (2000) showed that lambs given a diet containing 56% of carob pulp exhibited lower performance with the halving of the feed efficiency index compared with lambs fed with a conventional diet based on cereals. Since the 1900s several tools for animal welfare measurement have been developed and discussed (Broom 1991). Metabolic distress is a situation arising from metabolic imbalances in the blood, which can lead to discomfort, impaired feed intake and eventually sickness, and is one of the means for measuring wellbeing in animals (Broom 1991; Ohl and Van der Staay 2012). Blood metabolite profile can assist in appreciation of the animals' welfare status, in particular in relation to their nutritional and health status. For example, it is well known that high blood level of non-esterified fatty acids (NEFA) is a consequence of body fat reserve mobilisation (Hatfield *et al.* 1999) and can be observed in starving animals, although other factors may also lead to a rise in blood NEFA. Also the blood protein

profile, which relates to the proportions of the various fractions of albumin and globulins, provides farmers with a welfare indicator for their animals. Alteration of serum protein profile may occur in the case of liver disorders, acute inflammation and other physiological disorders. Apaydin and Dede (2010) for instance observed this phenomenon in sheep affected by a protozoon disease. Protein profile may also vary with age (Piccione *et al.* 2014) and nutrition; however, the changes due to nutrition are often subtle and difficult to detect and interpret (Eckersall 1997).

Most studies dealing with the use of non-conventional feeds focus on animal performance or product quality (Devendra 1988; Ben Salem *et al.* 2004; Vasta *et al.* 2008) but very few papers study their impact and implications on animal welfare. The feeding of non-conventional feeds rich in plant secondary compounds, such as carob, may cause malnutrition leading to increased vulnerability by animals to disease condition (Mahgoub *et al.* 2008). Tannin consumption can affect animal welfare as does many other plant secondary compounds (Durmic and Blache 2012). Tannins include a wide variety of chemicals, some of which may reduce feed intake (Waghorn 2008), cause astringency (Mueller-Harvey 2006) and metabolic discomfort, indicated for instance by high blood urea concentration in sheep urine (Mahgoub *et al.* 2008). However, some tannins could lead to reduction of gastro-intestinal worms thus improving animal performance and welfare (Waghorn 2008).

The aim of this study was to assess the feasibility of partially substituting cereals by carob pulp in concentrate-based diets for growing lambs and to assess whether there are any metabolic disorders arising from intake of these diets. Additionally, the effect of carob-rich diets on the feed ingestion behaviour was also investigated.

Materials and methods

Animal management

Twenty-six Comisana male lambs of 90 days of age (10 days) were selected. The animals were born on the same farm; during the pre-experimental stage, lambs had free access to their mother's milk (until 60 days) and to a ration composed of faba bean, wheat, barley and lucerne hay. At the age of 60 days all the animals were dewormed by injection of a broad spectrum antiparasitic drug of the avermectin family.

The animals were divided into three homogeneous groups, according to their weight [20.3 kg 4.4 kg (s.e.)], and randomly assigned to three experimental diets. The Control group (eight animals) was fed a total mixed diet consisting of barley, lucerne hay, wheat bran and soya bean meal that were coarsely ground. Two groups received a mixed diet with the same ingredients as in the Control group but with the addition of different proportions of carob pulp (24% and 35%, on an as-fed basis, respectively for Ca24 and Ca35 groups; nine animals in each group). The diets were formulated in order to supply an equivalent crude protein (CP) allowance. The ingredients and chemical composition of the diets are shown in Table 1.

The animals were placed in individual boxes (1.2 m · 1.8 m) equipped with a feeding trough and a plastic bucket for drinking water. An adaptation period of 10 days was observed during which the pre-experimental diet was gradually replaced by the experimental one. The diets (Control, Ca24 and Ca35) were given on an *ad-libitum* basis from 9 a.m. to 6 p.m. throughout the56daysoftheexperimentalfeedingtrial.Thedailyintakewas measured by weighing the refused feed at the end of the day.

The pattern of the individual feed intake was carried out on Days 20, 33, 47 and 53. On these days, the amount of feed left in

Table 1. Ingredients and chemical composition of the diets (Control, Ca24 and Ca35 groups)

Item	Diet		
	Control	Ca24	Ca35
Ingredients (% as fed)			
Barley	60	33	23
Carob pulp	0	24	35
Dehydrated lucerne	20	20	17
Soya bean meal	9	13	16
Wheat bran	11	10	9
Chemical composition			
Dry matter, g/100 g fresh weight	88.9	88.2	87.8
Crude protein[A]	18.0	19.6	19.2
Neutral detergent fibre[A]	34.6	34.4	34.6
Acid detergent fibre[A]	13.7	18.0	22.7
Hemicellulose[A]	20.9	15.6	11.9
Cellulose[A]	5.4	7.9	11.3
Acid detergent lignin[A]	8.3	10.9	11.4
Ether extract[A]	2.0	3.3	2.2
Total phenols[B]	8.6	14.2	16.7
Metabolisable energy (MJ/kg)[C]	10.49	10.41	10.48

[A]Expressed as g/100 g of dry matter.
[B]Expressed as mg of tannic acid equivalents/g of dry matter. [C]Expressed as fresh weight basis.

the feeder was weighed at 1030 hours, 1200 hours, 1500 hours and 1800 hours. On the basis of the total individual dry matter intake (DMI) of the day, the proportions in the four periods of the day (Period 1 = 0900–1030 hours; Period 2 = 1030–1200 hours;Period3=1200–1500hours;Period4=1500–1800hours) were calculated and expressed as % of the total DMI.

Theanimalswereweighedregularlyonaweeklybasiswithan electronic weighing scale before feeding and slaughtered after 56 days of experimental feeding trial by captive bolt followed by exsanguination. The lambs were slaughtered in a public slaughterhouse 100 km away from the farm and 24-h lairage time was observed.

The trial was conducted at an experimental farm of the University of Catania (Italy). The experimental protocol used was approved by the University of Catania in which the animals were handled by specialised personnel following the European Union Guidelines (2010/63/EU Directive).

Feed and blood sampling

Fresh feed samples were collected four times during the trial (on Days 9, 30, 44 and 51, respectively), and stored at 30C; analyses were done on a pooled sample for each diet.

Individual blood samples (10 mL) were collected, in the morning before feed allocation, from the external jugular vein usingVacutainertubes(TerumoCorporation,Tokyo,Japan)with no additive. Trained professionals were assigned to carry out this operation to minimise stress. The blood samples were collected at Day –10 (i.e. before the commencement of the adaptation period) and Day 55 of the trial. The blood samples were allowed to clot at room temperature (20C) and centrifuged at 2081*g* for 15 min at 4C to separate the serum. The serum samples were neither lipemic nor haemolysed and were dispensed into 2-mL capped centrifuge tubes and stored at 80C before analyses, performed within 2 months. At the time of analysis, serum samples were thawed at 20C for 30 min before assessing protein concentrations.

Laboratory analyses Feed analyses

Feeds were analysed for DM, whereas the AOAC (1995) methods were used for the analyses of CP (method 984.13) and crude fat (CF; method 935.38) extracted with petroleum ether. Neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid detergent lignin (ADL) were determined according to Van Soest *et al*. (1991), with sodium sulfite, without a amylase and expressed with residual ash method. Diet metabolisable energy (ME) was estimated by means of a commercial software (ASSIST.T Alimentazione, version 1.3.1, developed by CRPA spa, Italy, www.CRPA.it, verified 24 November 2014).

For the analysis of total phenols in the feed, samples were first treated as described by Makkar *et al*. (1993) with minor modifications. Briefly, 200 mg of finely ground feeds was extracted with 5 mL of diethyl ether containing 1% acetic acid to remove pigments and the supernatant was discarded. For extraction of total phenolic compounds, 10 mL of 70% (v/v) acetone was added and samples were subjected to ultrasonic treatment for 30 min in a cold water bath. Samples were then extracted for 2 h using a rotating device and then centrifuged at 2500*g* for 10 min at 4C. The supernatant was collected for subsequent analyses. The above extraction procedure was repeated and the supernatant collected. The residue from the acetone extraction was subjected to a further extraction using a modification of the method described by Silanikove *et al*.
(2006). Briefly, 9 mL of citrate-phosphate buffer containing 0.5 mg/mL of urea (pH 4.7) was added to the residue and samples were incubated at 90C for 2 h. A clear supernatant was obtained by centrifugation at 2500*g* for 20 min at 20C.

In all the above extracts, total phenols were determined using the Folin–Ciocalteau reagent. The concentration of total phenols in feeds was calculated as the sum of the concentration measured in each extract. The assays were calibrated using standard solutions of tannic acid and results were expressed as mg of TA equivalents/g of feed (on a DM basis).

Blood analyses

Two types of analysis were done on the blood serum, namely haematochemical and protein profile. The haematochemical profile included total protein, cholesterol, triglycerides, total bilirubin, iron, NEFA and urea; the protein profile determined albumin and globulins (a1, a2, b and g globulins).

Total protein was measured by biuret method using an automated analyser (Knoelab20, Dasit, Helsinki, Finland). The protein standard was albumin (0.5 g/mL; Dasit, Milano, Italy). Electrophoresis was performed using a semi-automated AGE system (Helena Laboratories, Helena Biosciences, Gateshead, Tyne and Wear, UK) according to the manufacturer's procedure. For each serum sample 10 mL were applied to numbered sample wells containing agarose gel G previously prepared. Each gel could accommodate up to 24 samples. Films were electrophoresed for 28 min at 450 V. After electrophoresis, films were simultaneously fixed using an automated system (SAS2, Helena Biosciences), stained in blue stain acid solution (Coomassie Blue Brillant R250, Helena Biosciences) for 10 min, and then dried at 37C. After distaining in acetic acid and drying completely for 15 min films were scanned on a densitometer (EZ-Scan, Helena Biosciences). Using the computer software Phoresis (Helena Biosciences), electrophoretic curves plus related quantitative specific protein concentrations foreach sample were displayed. All samples were analysed by the same individual and relative protein concentrations within each fraction were determined as the optical absorbance percentage, and absolute concentrations (g/100 mL) were calculated using the total serum protein concentration. The concentration of protein fractions were finally expressed as g/L.

Triglycerides and total cholesterol were assessed by means of a spectrophotometer (SEAC, Florence, Italy). Triglycerides and total cholesterol were determined after enzymatic hydrolysis by means of an enzymatic colourimetric test. Briefly, triglycerides were determined after enzymatic hydrolysis with lipoprotein lipase. The indicator was a coloured phenazone formed from hydrogen peroxide, 4-aminoantipyrine, and 4-chlorophenol under the catalytic influence of peroxidase. Total cholesterol was determined after enzymatic hydrolysis and oxidation. Hydrogen peroxide produced formed a red dyestuff by reacting with 4-aminoantipyrine in the

presence of phenol and peroxidase. The colour intensity is directly proportional to the concentration of cholesterol. NEFA were measured enzymatically with a commercially available kit (Randox Laboratories, Crumlin, UK). Samples exhibited parallel displacement to the standard curve; the intra-assay coefficient of variation was less than 8%. Urea, total bilirubin and iron were determined with the use of commercial kits (Centronic GmbH, Wartenburg, Germany) and finally measured using the UV Spectrophotometer (SEAC, Slim).

Statistical analyses

ANOVA was used to determine the effect of dietary treatment on performance indicators (feed intake, liveweight gain, feed efficiency and carcass yield) and serum haematochemical and protein composition. Regarding intake and feed efficiency data, the individual average value for the whole experimental period hasbeenincludedinthedatabaseforANOVAanalysis.Datawere analysed as a completely randomised design, with a model that included the diet as fixed effect. When the ANOVA was significant ($P < 0.05$), means were separated by pairwise comparison by means of the Tukey's method. Individual data of the feed intake pattern (expressed as % of the total DMI during the day) were analysed by including the fixed effects of animal (nested within the diet), diet (Control, Ca24 and Ca35), experimental day (20, 33, 47 and 53) and period of the day (Interval 1 = 0900–1030 hours; Interval 2 = 1030–1200 hours; Interval3=1200–1500hours;Interval4=1500–1800hours)and their interactions. The effect of the experimental day was not significant; therefore, factors in the model were reduced to only animal (nested within the diet), diet, period of the day and their interaction. Means were separated by Tukey's test pairwise comparison ($P < 0.05$).

Results

Dietary composition and lamb performance

The experimental diets were similar in terms of CP and NDF contents but differed in the proportion of the fibre fractions (Table 1). Total phenols content, as expected, was higher in the carob diets as compared with the Control due to the incorporation of carob pulp.

DMI was not significantly affected by the dietary treatment (Table 2). CP and NDF ingestion were not significantly affected by the experimental treatment. However, a different nutrient intake has been obtained for fibre fractions: the Ca35 group showed significantly ($P < 0.05$) lower hemicellulose intake compared with Control and Ca24 groups. Lambs in the Control group showed the lowest level of daily cellulose and ADL intake (Table 2). Total phenols consumption was also affected by the experimental diet, as expected, showing the lowest values in Control lambs compared with Ca24 and Ca35 ($P < 0.05$).

Regarding animals' performance, none of the parameters measured during the *in vivo* phase and at slaughtering were affected by the experimental diet (Table 2). This implies that no detrimental effect on animal performance indicators has been obtained even at the highest value of carob pulp inclusion.

Pattern of feed intake

The distribution of the DMI throughout the day is shown in Fig. 1. This parameter was significantly affected by the period of the day ($P < 0.0005$) and by the diet · period interaction ($P < 0.0005$), while the effect of the diet was not significant ($P > 0.05$).

On average (data not shown), in the three experimental groups, lambs showed the highest percentage (35.6%) of the total daily DMI in the Interval 1, i.e. in the first 90 min after feed supply, and the lowest values (10.7%) in the second interval of observations(1030–1200hours).Inthethirdandfourthintervals, a significant increase in the proportion of the daily DMI was observed (respectively 22.0% and 31.8% for Intervals 3 and 4). All the average values of the four considered intervals were significantly different ($P < 0.05$).

Figure 1 shows a different DMI pattern in the Control lambs compared with those fed the carob-containing diets. Indeed, the proportion of the ingestion in the first 90 min following feeding was significantly ($P < 0.05$) lower in both Ca24 and Ca35 groups compared with the Control group.

In the other periods of the day the percentage of the total daily DMI was less affected by the experimental diet. Indeed, a different percentage of ingestion was observed exclusively in the interval 1200–1500 hours: Ca35 lambs ingested more DM as compared with Control lambs (24.3% vs 18.2%; $P < 0.05$).

In the last period of feed availability (1500–1800 hours), the Controlgroup showedasignificantlylower($P<0.05$)proportion of DMI compared with that observed in the first period of the day. On the contrary, in both Ca24 and Ca35 groups the percentage of DMI reached the same level as in the first period ($P > 0.05$). Overall, in our experimental conditions, the high levels of inclusion of carob in the diets offered to both Ca24 and Ca35 groups did not affect total DMI (Table 2) but affected feed ingestion behaviour (i.e. the distribution of the total DMI

Table 2. Performance and nutrient intake of lambs fed on Control diet or two carob pulp-based diets (Ca24 and Ca35) a, b, Within a row, means with different letters differ ($P < 0.05$)

	Control	Carob 24 (Ca24)	Carob 35 (Ca35)	s.e.m.	*P*-value
		Performance			
No. of lambs	8	9	9		
Bodyweight Day 0 (kg)	19.3	18.7	18.6	0.73	0.923
Bodyweight Day 57 (kg)	29.6	30.0	28.3	1.08	0.797

Average daily gain (Day 0 to Day 56) (g/day)	181	198	170	8.8	0.418
Dry matter intake (g/day)	749	843	809	31	0.474
Feed efficiency (kg weight gain/ kg dry matter intake)	0.24	0.23	0.21	6.86	0.126
Carcass yield (%)	43.8	42.3	43.8	0.32	0.111
Carcass weight (kg)	13.0	12.7	12.4	0.50	0.901
	Nutrient intake (g/day)				
Crude protein	135.1	165.4	155.6	6.2	0.133
Neutral detergent fibre	259.4	290.0	279.8	10.5	0.511
Hemicellulose	156.7b	131.7b	96.1a	6.7	0.0005
Cellulose	40.6a	66.3b	91.7c	4.9	0.0005
Acid detergent lignin	62.2a	92.1b	92.0b	4.2	0.001
Ether extracts	18.4a	22.0a	31.8b	1.3	0.0005
Total phenols (mg/day)[A]	6458a	11967b	13479b	731	0.0005

throughout the day), by lowering the rate of DM consumption in the period immediately following feeding.

Blood analyses

The values observed for the blood serum metabolites in all the experimental groups before the feeding trial (Day –10) and at the end of the trial (Day 55) are shown in Table 3. The consumption of the three experimental diets did not affect

[A] Expressed as tannic acid equivalents.

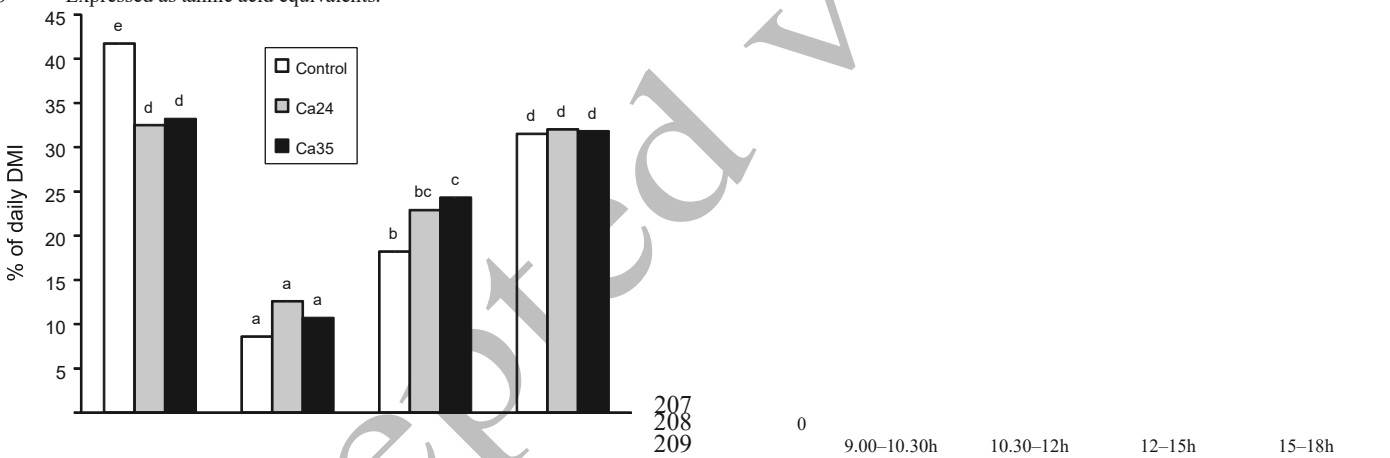

Fig. 1. The pattern of dry matter intake (DMI) throughout the day in terms of percent of the total daily intake. a, b, Means with unlike letters differ ($P < 0.05$).

serum total protein and bilirubin but there was tendency in serum iron levels ($P = 0.091$), with the Control group showing the highest value. However, urea ($P < 0.001$), cholesterol ($P < 0.0005$), triglycerides ($P < 0.05$) and NEFA ($P = 0.0005$) were all significantly affected by the diets. Urea blood level was significantly higher ($P < 0.05$) in both carob groups compared with the Control, whereas the opposite pattern was observed for cholesterol, which showed significantly lower ($P < 0.05$) values in Ca24- and Ca35-fed animals in comparison with those supplied with the Control diet. Triglycerides values were also affectedbythehigherlevelofcarobinclusioninthediet,showing Ca35 lambs significantly lower value ($P < 0.05$) compared with Control-fed animals, whereas in Ca24 lambs triglycerides were not different compared with both Control and Ca35 groups. NEFA blood content reached the highest values in Ca35 lambs, which showed significantly ($P < 0.05$) higher value compared with Control and Ca24 lambs.

Blood serum protein fractions are depicted in Table 4. Apart from the blood b and g globulins, there was an effect of the diet on all the other parameters, though of variable amplitude. There was a marked effect of the diet on a1 and a2 globulins: a1 globulins were significantly lower in the Ca24 lambs, compared with Ca35 and Control ($P < 0.05$) although a2 globulins significantly ($P < 0.05$) differed between Ca24 and Control lambs. There was also a tendency ($P < 0.1$) in the ratio of albumin to globulins (A/G) proteins to rise with the inclusion of carob in the diets compared with the animals in the Control group. All these observations tend to indicate some effect of carob pulp ingestion in modifying the serum protein profile.

Discussion

Intake and performance

Carob pulp usually contains high level of total phenols with someofthesebeingrepresentedbytannins(Avallone*etal.*1997). Such high level of tannins in the feeds could potentially reduce the nutritive value of feed, especially as tannins bind with the proteins making them unavailable to the animal (Kumar and Singh 1984; Provenza 1995), and negatively affect intake due to the astringency associated with tannins (Bate-Smith 1973).

Table 3. Blood metabolic indicators before (Day –10) and at the end (Day 55) of the feeding experimental trial a, b, Within a row, means with unlike letters differ ($P < 0.05$)

	Control	Carob 24 (Ca24)	Carob 35 (Ca35)	s.e.m.	*P*-value
Total protein at Day –10 (g/100 mL)	5.89	5.93	6.14	0.09	0.520
Total protein at Day 55 (g/100 mL)	6.61	6.77	6.64	0.08	0.749
Urea at Day –10 (mmol/L)	6.65b	6.09ab	5.14a	0.26	0.043
Urea at Day 55[A] (mmol/L)	7.20a	9.40b	10.30b	0.37	0.001
Cholesterol at Day –10 (mg/100 mL)	44.25	56.56	67.60	4.87	0.156
Cholesterol at Day 55 (mg/100 mL)	46.13b	30.78a	27.56a	2.01	0.0005
Triglycerides at Day –10 (mg/100 mL)	17.63	23.22	25.44	1.41	0.066
Triglycerides at Day 55 (mg/100 mL)	24.88b	20.78ab	18.56a	1.00	0.030
Non-esterified fatty acids at Day –10 (mmol/L)	0.11	0.14	0.12	0.005	0.164
Non-esterified fatty acids at Day 55 (mmol/L)	0.18a	0.19a	0.46b	0.036	0.0005
Total bilirubin at Day –10 (mM/L)	0.28	0.31	0.32	0.03	0.876
Total bilirubin at Day 55 (mM/L)	0.33	0.37	0.47	0.04	0.333
Iron at Day –10 (mg/100 mL)	146.5	171.2	178.8	6.52	0.114
Iron at Day 55 (mg/100 mL)	238.6	181.0	199.2	10.9	0.091

[A] Statistical analysis includes the value at Day –10 as covariate; adjusted means are shown.

Table 4. Blood protein profile before (Day –10) and at the end (Day 55) of the feeding experimental trial a, b, Within a row, means with unlike letters differ ($P < 0.05$)

	Control (C)	Carob 24 (Ca24)	Carob 35 (Ca35)	s.e.m.	*P*-value
Albumin at Day –10 (g/100 mL)	3.36	3.04	3.16	0.07	0.159
Albumin at Day 55 (g/100 mL)	2.79a	3.30b	3.14ab	0.08	0.026
a1 globulins at Day –10 (g/100 mL)	0.24	0.32	0.33	0.18	0.069
a1 globulins at Day 55 (g/100 mL)	0.23ab	0.16a	0.30b	0.02	0.001
a2 globulins at Day –10 (g/100 mL)	0.75	0.78	0.84	0.02	0.266
a2 globulins at Day 55 (g/100 mL)	0.93b	0.81a	0.85ab	0.02	0.048
b globulins at Day –10 (g/100 mL)	0.40	0.43	0.45	0.02	0.680
b globulins at Day 55 (g/100 mL)	0.50	0.44	0.39	0.02	0.142
g globulins at Day –10 (g/100 mL)	1.13	1.36	1.36	0.07	0.377
g globulins at Day 55 (g/100 mL)	2.16	2.06	1.97	0.07	0.617
Ratio albumin/globulin, Day –10	1.38	1.07	1.16	0.07	0.156
Ratio albumin/globulin, Day 55	0.75	0.97	0.91	0.04	0.060

Surprisingly, in our experimental conditions, a positive correlation has been observed between DMI and total phenols intake (R^2 = 0.53; P = 0.0005) and there was no effect of the diet on the various *in vivo* parameters (Table 2), despite the ~1.8–2.0 times higher ingestion of total phenolic compounds in carob groups. It is probable that the level of proteins and energy in the diets were more than sufficient such that the interaction of nutrients-toxins (tannins) was not detrimental in agreement with Freeland and Janzen (1974) and Priolo *et al*. (2000). Even if the diet effect was not significant, it is worthy to note that the carob groups showed a higher daily DM consumption and a lower feed efficiency than the Control group but with very similar carcass weights. This is in line with the findings of Priolo *et al*. (1998) who observed that the inclusion of 20% of carob increased feed intake, did not affect animals' growth and worsened feed efficiency. Similarly, Guessous *et al*. (1989) observed that at the rate of 200 g/kg of inclusion, carob pulp-fed lambs reached a gain comparable to the Control diet. The ability of animals eating carob to maintain similar performance to those not eating carob is an indication of the ability of the animal to cope with anti-nutritional factors in the diet, such as tannins. In this study, it has been observed that even at the level of 35%, carob pulp allowed to maintain a level of growth and carcass weight and yield comparable to animals fed on a conventional diet probably due to the high sugar content in carob, which offsets the detrimental effect of tannins by allowing a sufficient amount of energy.

The effect of carob secondary compounds on lamb feeding behaviour has been studied by measuring the proportion of the diet consumed during the day and by limiting the access to feed during the night, when small ruminants usually show a negligible eating activity (Avondo *et al*. 2013). Similar setup has been implemented by Villalba *et al*. (2006) in lambs fed diets containingtannins.Asaconsequenceofthis,assoonasthefeedis given in the morning, the animals are stimulated to ingest. Under these conditions, the effect of carob pulp secondary compounds on feeding behaviour is expected to be highlighted. Indeed, in the present study, the inclusion of carob in the diet appeared to affect the feeding behaviour of the animal. A clear pattern seems to be defined with a lower rate of ingestion immediately after feed administration being evident when carob is included in the diet. Thus, the animals in the Control group ate more than 40% of the whole daily intake in the first 90 min of feeding compared with only 33% in the carob groups. A similar behaviour has been observed in heifers supplemented with tannins extracted from the quebracho tree (Landau *et al.* 2000). It could be inferred that, athighlevelofinclusion,theastringencyincarobpulpovercomes the palatability due to its sweetness, making the feed less appealing and inducing animals to differently modulate the daily feed consumption compared with the Control group. This is further confirmed by the recovery in intake observed in our study that occurred in the later monitoring periods of the day, in agreement with Provenza *et al*. (1992). It is also interesting to note that throughout the study, the overall pattern of feeding was unchanged as the date factor was not significantly different (P > 0.05) indicating that the animals were comfortable with that pattern. This finding is quite useful as it is generally regarded that to avoid reducing DMI it is advisable to follow a type of pattern with supply of tannins at specific times or in a particular sequence with other non-tanniferous feeds, mainly depending on the type of feeds used in the diets (Mote *et al.* 2007). In this study, carob-containing diets were supplied steadily and in a mixed diet, in order to avoid feed selection, resulting in no reduction in final intake. This is a valuable observation as it indicates that farmers, when using carob pulp at relatively high level, need not take demanding steps, like alternating two types of feeds, to maintain similar animal performance but should allow the animal sufficient time to consume the ration.

Blood metabolites

The metabolic response of the animals was a major concern in this study. Blood cholesterol level decreases with carob ingestion, in agreement with Silanikove *et al*. (2006) who found low levels of blood cholesterol in kids fed carobcontaining diets. To our knowledge, it has been observed for the first time here in sheep. This effect could be related to the presence of tannins in carob, as the hypocholesterolemic effect of condensed tannins has been documented in humans (Chung *et al.* 1998; Bele *et al.* 2010). Few studies reported that in case of infections, blood cholesterol level goes down with amplitude depending on factors like breeds and physiological state (Wellde *et al.* 1989; Adamu *et al.* 2008). Urea concentration in plasma may increase due to overfeeding, resulting in higher level of protein intake and subsequent reflection in the level of urea excretion, or to underfeeding, when animal mobilises the body reserves and tends to recycle the nitrogen andminimiseitsexcretioninurine(Nozière*etal*.2000).Caldeira *et al*. (2007) reported that animals with extreme body condition score have higher blood urea level compared with animals with an intermediate score. In this trial, blood urea increased when animals were fed the carob diets, which is contrary to the observations reported by Priolo *et al*. (2000) and Fernández *et al*. (2012) but complies with those reported bySilanikove*etal*.(2006)andWhitney*etal*.(2014).Thisissueis quite complex. Indeed, a possible relationship between carob tannins ingestion and blood urea level could be accounted for by the tannins effect in ruminal nitrogen metabolism. However, when two carob diets were supplied to kids, one with polyethylene glycol, which inhibits the effects of tannins in the rumen, and the other without, blood urea level in kids remains higher in both cases, when compared with kids fed the Control diet; unfortunately this issue was not elaborated further (Silanikove *et al.* 2006). In the absence of other parameters (e.g. faecal nitrogen, urinary nitrogen and hepatic function indicators), which are not available in this study, we can only hypothesise that carob could have altered the site of nitrogen metabolism, as suggested by Whitney *et al*. (2014) who studied the effect of redberry juniper, as a source of secondary compounds, in lamb diets.

In this study, the animals fed 35% level of carob had a significantly higher level of blood NEFA compared with the other groups. High levels of NEFA generally indicate a

mobilisation of body fat reserves to meet metabolic needs. Ospina *et al*. (2010) and Huzzey *et al*. (2012) showed that animals under metabolic stress and having disease problems have elevated levels of blood NEFA. Lapierre *et al*. (2000) also found that higher blood NEFA corresponded to lower feed intake. NEFA may also increase in cases of physiological stress like inflammation (Bell 1995; Waldron *et al.* 2003; Pethick *et al.* 2005). Interestingly NEFA did not increase in animals fed the Ca24 diet, which may indicate that dietary carob

pulp does not affect this metabolic parameter when it is included in the diet at levels up to 24%. In conclusion, in this study carob ingestion impaired both protein and energy metabolic parameters with a greater effect at the highest level of inclusion.

Protein electrophoresis

Serum protein electrophoresis is a tool used in the diagnosis of serum protein disorders (O'Connell *et al.* 2005). Plasma protein levels in terms of albumin and globulins (a1, a2 b and g) reflect, with a good fidelity, changes associated with the body response to injury; a1 and a2 globulins are considered as moderate indicators of health status in sheep (Cray *et al.* 2009). Albumin is one of the proteins in the plasma known as acute phase proteins (APP), whose levels change whenever the animal is subjected to external or internal challenges like trauma, inflammation and stress (Murata *et al.* 2004). Depending on whether the level increases or decreases, the various APP are termed as 'positive' or 'negative. In ruminants, APP are used as biomarkers of disease conditions (Ceciliani *et al.* 2012). For example, albumin is considered as a negative APP as it decreases in cases of health disorders (Ceciliani *et al.* 2012); thereby the application of the measurement of APP is a tool to indicate herd health (Cray *et al.* 2009). In this study it was found that the diet had some effects on the protein fractions with carob pulp-fed animals having higher albumin levels ($P = 0.026$) and a tendency for higher A/G ($P = 0.06$). Albumin level is a good indicator of nutritional status (Hoaglund *et al.* 1992; Hoffman *et al.* 2001). High A/G ratio may indicate low protein utilisation efficiency and liver dysfunction; conversely, low A/G ratio is due to some sort of mild infection (Farver 1997; Ndlovu *et al.* 2009) which, in the case of the present study, was not diagnosed. El-Sherif and Assad (2001) reported that ewes in lactation have higher A/G ratio than dry ones due to higher nutritional stress. Our results may indicate that the animals eating carob pulp had some nutritional stress compared with the Control ones; however, it may be inferred that they were able to cope with it as the performances were not affected by the diet.

Conclusions

This study showed that the inclusion of locally available carob pulp was pertinent in substituting barley in the diet of fattening lambs without causing metabolic distress. The measured indicators, some blood metabolites and serum protein profile, showed that carob inclusion did not heavily affect physiological welfare of the animals. This indicates that the animals were able to cope with a diet including up to 35% of carob pulp without hindrance to intake and performance. The animals adopted a different feeding behaviour, modulating the rate of ingestion throughout the day. The specific experimental conditions, supplying of mixed diets in order to avoid feed selection, could represent a feeding strategy that farmers could implement when dealing with feeds containing high level of carob pulp.

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