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ORIGINAL ARTICLE

Effects of pistachio by-products in replacement of alfalfa hay on populations of rumen bacteria involved in biohydrogenation and fermentative parameters in the rumen of sheep

M. H. Ghaffari[1], A.-M. Tahmasbi[1], M. Khorvash[2], A.-A. Naserian[1], A. H. Ghaffari[1] and H. Valizadeh[1]

1 Department of Animal Science, Faculty of Agriculture, Ferdowsi University of Mashhad, Mashhad, Iran, and
2 Department of Animal Sciences, Faculty of Agriculture, Isfahan University of Technology, Isfahan, Iran

Summary

The objective of this study was to investigate the effect of sundried pistachio by-products (PBP) as a replacement of alfalfa hay (AH) on blood metabolites, rumen fermentation and populations of rumen bacteria involved in biohydrogenation (BH) in Baluchi sheep. Four adult male Baluchi sheep (41 ± 1.3 kg, BW) fitted with ruminal cannulae were randomly assigned to four experimental diets in a 4 × 4 Latin square design. The dietary treatments were as follows: (i) control, (ii) 12% PBP (0.33 of AH in basal diet replaced by PBP), (iii) 24% PBP (0.66 of AH in basal diet replaced by PBP) and (iv) 36% PBP (all of AH in basal diet replaced by PBP). The basal diet was 360 g/kg dry matter (DM) alfalfa hay, 160 g/kg DM wheat straw and 480 g/kg DM concentrate. The trial consisted of four periods, each composed of 16 days adaptation and 4 days data collection including measurement of blood metabolites, rumen fermentation and population of bacteria. No differences were observed in rumen pH among the treatments, while rumen ammonia-N concentrations were decreased ($p< 0.05$) with increasing PBP by up to 36% DM of the diets. Using of 36% PBP in the diet reduced ($p < 0.05$) total volatile fatty acids (VFA) concentrations and the molar proportion of acetate, while the concentration of propionate, butyrate and acetate to propionate ratio were similar to all other treatments. The concentration of blood urea nitrogen (BUN) decreased ($p < 0.01$) with increasing PBP by up to 36% DM in the diets of sheep. However, other blood metabolites were not affected by the experimental diets. It was concluded that PBP in replacement of AH had no effects on the relative abundance of *Butyrivibrio fibrisolvens* and *Butyrivibrio proteoclasticus* in relation to the control diet.

Keywords pistachio by-products, biohydrogenation, rumen bacteria, real-time PCR, sheep

Correspondence Dr. Morteza Hosseini Ghaffari, Department of Animal Sciences, Ferdowsi University of Mashhad, PO Box 91775-1163, Mashhad, Iran. Tel: +98 935 781 4911; Fax: +98 511 879 6845; E-mail morteza.h.g@gmail.com

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Introduction

The use of locally available feed resources has great potential for improving animal production in developing countries (Argüelloa, 2011). Iran is considered to be the largest producer of pistachio worldwide with an annual production of approximately 500 000 tonnes of fresh pistachio by-products (PBP) (Shakeri et al., 2012). Pistachio by-products consist of 64.5% soft external hull, 25% twigs, 10% leaves and 0.5% kernel and bony shells (Vahmani et al., 2006; Bagheripour et al., 2008). While PBP is high in crude protein (158.2 g/kg) and ether extract (EE) (69.5 g/kg) and hence a valuable feed for ruminants (Behgar et al., 2009), its nutritive value is subject to variation due to de-hulling process, pistachio cultivar and growing conditions (Bagheripour et al., 2008). Pistachio by-products (sun-dried or as silage) have recently come to be used in diets for dairy cows (15% DM, PBP) (Mokhtarpour et al., 2012), growing calves (18% DM, PBP) (Shakeri et al., 2012) and sheep (30% DM, PBP) (Norouzian et al., 2011). Phenolic compounds and tannins have been defined as secondary metabolites of PBP (Bohluli et al., 2009; Ghasemi et al., 2012b). The total phenolic compounds and tannins of sun-dried PBP have been reported to be 7.6–15.6% DM and 3.4–10.2% DM respectively (Shakeri and Fazaeli, 2007; Bagheripour et al., 2008; Bohluli et al., 2009). Previous studies have reported that the last step of the rumen biohydrogenation (BH) process was inhibited by tannin supplementation (Khiaosa-Ard et al., 2009; Vasta et al., 2009a,b). This selective

activity of tannins to rumen bacteria could be beneficial nutritionally by altering the rumen BH process and hence enhancing conjugated linoleic acid (CLA) content of ruminant-derived products. Despite many efforts to show the effects of plants containing tannins on ruminal BH and fermentation characteristics (Khiaosa-Ard et al., 2009; Vasta et al., 2009a; Toral et al., 2011), authors could not find any information on possible effects of PBP (containing phenolic compounds and tannins) on rumen bacteria involved in BH. The objective of the present investigation is to determine the effect of PBP on ruminal fermentation characteristics and blood metabolites. As its second objective, the study aims to determine whether dietary PBP affects the rumen bacteria responsible for the BH of fatty acids.

Materials and methods

Animals, experimental diets and management

The experiment was conducted at the Research Farm of the Faculty of Agriculture, Ferdowsi University of Mashhad (Iran), in 2012. The experimental protocols were reviewed and approved by the Animal Care Committee of Ferdowsi University of Mashhad, Iran. Four adult male Baluchi sheep (41 ± 1.3 kg, body weight) fitted with ruminal cannulae (2.5 cm i.d.) were assigned at random to four experimental diets in a 4 × 4 Latin square design. The dietary treatments were as follows: (i) control, (ii) 12% PBP (0.33 of AH in basal diet replaced by PBP), (iii) 24% PBP (0.66 of AH in basal diet replaced by PBP) and (iv) 36% PBP (all of AH in basal diet replaced by PBP). The basal diet was 360 g/kg DM alfalfa hay, 160 g/kg DM wheat straw and 480 g/kg DM concentrate. The DM intake for each sheep was 1190 g per day on average. The diets were formulated for maintenance requirements according to AFRC (1993). The trial consisted of four periods, each composed of 16 days adaptation and 4 days data collection including measurement of blood metabolites, rumen fermentation and population of bacteria. All diets were supplied as a total mixed ration (TMR) twice daily in equal portions at 08:00 and 20:00 h. Clean water was freely available adlibitum at all times. The sunflower oils were stored at 4 °C and mixed evenly with the concentrate each morning. Sun-dried PBP which contained soft external hull, twigs, leaves and bony shells were collected from Bardaskan Town (Babakhan, Khorasan-e-Razavi Province, Iran). The ingredients and chemical composition of the diets and sundried PBP are presented in Tables 1 and 2 respectively.

Table 1 Ingredients and chemical composition of experimental diets (DM basis)

	Treatments			
	0PBP	12PBP	24PBP	36PBP
Diet ingredients (% of DM)				
Alfalfa hay (AH)	36	24	12	0
Pistachio by-product (PBP)	0	12	24	36
Wheat straw	16	16	16	16
Barley	20	20	20	20
Canola meal	5	6	7.5	9
Wheat bran	19	18	16.5	15
Sunflower oil	3	3	3	3
Common salt	0.2	0.2	0.2	0.2
Vitamin/mineral premix*	0.6	0.6	0.6	0.6
Limestone	0.2	0.2	0.2	0.2
Chemical composition				
ME (MJ/kg)	10.89	10.93	10.97	10.97
Dry matter (%)	96.5	97.1	97.2	97.4
Crude protein (%)	13	12.8	12.7	12.6
Acid detergent fibre (%)	25.3	24.5	23.8	23
Neutral detergent fibre (%)	41.9	40.5	39.3	38.1
Total phenols (%)	0.23	1.34	2.64	3.52
Total tannins (%)	0.19	0.95	1.65	2.46

ME, metabolizable energy.

*Each kg of the vitamin–mineral premix contained (DM basis): vitamin A (50 000 IU), vitamin D3 (10 000 IU), vitamin E (0.1 g), calcium (196 g), phosphorus (96 g), sodium (71 g), magnesium (19 g), iron (3 g), copper (0.3 g), manganese (2 g), zinc (3 g), cobalt (0.1 g), iodine (0.1 g), selenium (0.001 g).

Table 2 Chemical composition of pistachio by-products

Item	Pistachio by-products (g/kg DM)
DM	910.0
Ash	127.0
Ether Extract	64.5
CP	114.2
ADF	205.8
NDF	332.6
NFC	361.7
Total phenolic	99.5
Simple phenolic	32.7
Total tannin	66.8

Measurements and sampling procedures

Rumen and blood sampling

On day 18 of each period, rumen fluid was collected by suction through the rumen cannula before morning feeding (0.0 h) to 8 h after feeding at 30-min intervals for determination of rumen pH. Ruminal pH was measured with a portable pH meter (Metrohm 744, Herisau, Switzerland). On day 19 of each period,

the rumen fluid samples were collected, strained through four layers of cheesecloth and prepared for subsequent ammonia-N and volatile fatty acids (VFA) analyses. The rumen fluid samples were taken at 0, 1, 2, 3, 4, 6 and 8 h after feeding for determination of ammonia-N concentrations and VFAs at 4 h after morning feeding. For stabilizing, 10 ml of rumen fluid was acidified with 10 ml of 0.2 N HCl for ammonia-N determination and each ml of rumen fluid was acidified with 20 ml of 50% H_2SO_4 and frozen at −18 °C until VFA analyses by gas chromatography. On day 20 of each period, blood samples from all the sheep were obtained from the jugular vein 0, 2 and 4 h after the morning feeding (10 ml into sterile tubes containing EDTA solution). The samples were immediately placed on ice for processing in the laboratory. Blood samples were centrifuged (3000 g for 15 min at 5 °C), and plasma was stored at −20 °C for later analysis.

DNA extraction

On day 17 of each period, the rumen digesta were collected before the morning feeding and frozen (−20 °C) until further analysis. Stored rumen samples were freeze-dried, and 80 mg sample was crushed with a mortar and pestle. Recently, it has been reported that freeze-drying improves DNA extraction yield and quality from gut contents (Ruiz and Rubio, 2009). After disruption, freeze-dried samples were transferred to 1.5 ml tubes containing three 0.5-mm-diameter glass beads and 200 ml tissue lysis buffer. Samples were vortexed twice for 2 min and incubated on ice between shakings. This work allowed disruption of bacterial cell wall and detached bacteria from feed particles. Tubes were centrifuged at 2000 g for 5 min at 4 °C for the sedimentation of feed particles. The supernatants (200 μl) were transferred to fresh 1.5 ml microtubes. DNA extraction was performed using a commercial DNA isolation kit, AccuPrep Genomic DNA Extraction Kit (Bioneer, Daejeon, South Korea) according to the manufacturer's protocol. The concentration of DNA was measured by Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). DNA concentrations ranged between 30 and 50 ng/μl. All extracted DNA samples were stored at −20 °C until subsequent analysis. DNA samples were used as templates for real-time PCR assay.

Primers and real-time PCR

The relative abundance of *B. fibrisolvens* and *B. proteoclasticus* in digesta samples from sheep was measured by real-time PCR and the SYBR Green PCR Master Mix Kit (SYBR Green I qPCR Master Mix, Syntol, Moscow, Russia) according to Valizadeh et al. (2010). Species-specific primers used to amplify partial 16S rDNA regions used in this study are described in Table 3. The reaction was in a final volume of 25 μl, containing the following: 11.4 μl of SYBR Green PCR Master Mix Kit, 0.6 μl of primer mixture containing 10 pmol of each primer, 1 μl of template DNA and 12 μl of deionized water. SYBR Green I qPCR Master Mix contained KCl, Tris-HCL (pH 8.8), 6.25 mM $MgCl_2$, dNTP, Taq DNA polymerase, Tween and SYBR Green I. The DNA samples were not adjusted for differences in DNA concentrations, but all relative comparisons were made on basis of a constant volume of DNA-extract to achieve optimal relative expression results in real-time PCR. A no-template (sterile distilled water) negative control tube was loaded on each plate run to check for contamination and primer dimer formation and normalizing for background fluorescence. Real-time PCR was performed on an ABI 7300 Sequence Detection System (Applied Biosystems, Foster City, CA, USA), under the following conditions: initial denaturation at 95 °C for 10 min was followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 59 °C for 30 s, extension at 72 °C for 30 s. Amplicon specificity was performed via dissociation curve analysis of PCR end products by increasing the temperature at a rate of 1 °C every 30 s from 65 to 95 °C. The threshold cycle (i.e. the amplification cycle in which product formation exceeds background fluorescence) of each sample was determined during the exponential phase of amplification. Each real-time PCR reaction was performed in duplicate. The relative abundances of *B.*

Table 3 Polymerase chain reaction primers used for amplifying target bacteria in the rumen contents of sheep

Target species	Primer Sequence 5′ to 3′	Product size (bp)	References
Total bacteria	F: GTGSTGCAYGGYTGTCGTCA R: ACGTCRTCCMCACCTTCCTC	146	Maeda et al. (2003)
Butyrivibrio fibrisolvens	F: ACCGCATAAGCGCACGGA R: CGGGTCCATCTTGTACCGATAAAT	65	Paillard et al. (2007b)
Butyrivibrio proteoclasticus	F: TCCGGTGGTATGAGATGGGC R: GTCGCTGCATCAGAGTTTCCT	185	Paillard et al. (2007b)

fibrisolvens and *B. proteoclasticus* were determined using total bacteria as reference using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). All samples have been measured in a single run. The fold change in specific bacteria species DNA in sheep fed the experimental diets compared with the control diet was calculated by normalizing specific bacteria species DNA to total bacterial DNA in the experimental groups and relating that ratio to that of the control sheep. Change in certain bacteria species are reported as fold change in genomic DNA per 1 μl of extracted DNA compared with control. The relative abundances of *B. fibrisolvens* and *B. proteoclasticus* were determined using total bacterial amplification as the reference gene (Ghasemi et al., 2012a).

Chemical analysis

Samples of feed were analysed for dry matter (DM), organic matter (OM), ether extract (EE) and nitrogen by standard procedures (AOAC, 2000). Neutral detergent fibre (NDF) and acid detergent fibre (ADF) were determined according to Van Soest et al. (1991). The total phenolics content was determined according to the Folin-Ciocalteu assay, and total tannins were determined by methods previously described (Makkar, 2000). Tannic acid (Merck GmbH, Darmstadt, Germany) was used as the standard to express the amount of total phenols and total tannins. Rumen ammonia concentrations were determined using the distillation method (Kjeltec Auto 1030 Analyzer, Tecator, Hoganas, Sweden). Rumen samples were analysed for VFAs by gas chromatography (0.25 × 0.32, 0.3 mm i.d. fused silica capillary, model no. CP-9002 Vulcanusweg 259 a.m., Chrompack, Delft, the Netherlands) as described by Bal et al. (2000). In blood samples, the concentration of cholesterol, blood urea nitrogen (BUN), total protein, albumin and glucose was determined by an automated biochemical analyzer (Biotecnica, Targa 3000, Rome, Italy) using commercial kits [Pars Azmoon Company, Catalogue Numbers: glucose (1-500-017), albumin (1-500-001), total protein (1-500-028), BUN (1-400-029), cholesterol (1-500-010), triglyceride (1-500-032), Tehran, Iran] according to the manufacturer's instructions.

Statistical analysis

The data were analysed as a 4 × 4 Latin square design using the PROC MIXED procedure of SAS (SAS, 2003). The statistical model included sheep as random effect, and period and treatment (diet) as fixed effects. The data were analysed according to the following model:

$$Y_{ijk} = \mu + P_i + T_j + C_k + e_{ijk}$$

where Y_{ijk} is the dependent variable, μ is overall mean, P_i is fixed effect of period (I = 1, 2, 3 and 4), T_j is fixed effect of treatment (j = 1, 2, 3 and 4), C_k is the random effect of sheep, and e_{ijk} is the residual error. For the statistical analysis of ruminal fluid characteristics (pH, VFAs, ammonia-N) and bacteria populations, sampling time and sampling time × treatment were added to the model and analysed using repeated measures. Subsequent comparison of means was performed according to Tukey–Kramer. Effects of dietary treatments are declared significant at $p < 0.05$, and trends are declared at $p < 0.10$. Results are reported as least squares means.

Results

Ruminal fermentation parameters

The mean of ruminal fermentation parameters is presented in Table 4. The different treatments had no effects on ruminal pH. Ruminal pH over the 8 h after feeding is shown in Fig. 1. For all treatments, rumen pH decreased after the morning feeding and reached its minimum values approximately 1.5–2.5 h after the morning feeding and then increased (Fig. 1). Rumen ammonia-N concentrations decreased ($p < 0.05$) with increasing PBP by up to 36% DM in the diets of sheep (Table 4). Lower concentration of ammonia-N in rumen ($p < 0.05$) was observed with PBP diets than with AH, but no significant differences were detected in the concentration of rumen ammonia-N among the

Table 4 Ruminal fermentation characteristics in Baluchi male sheep (n = 4) fed different levels of pistachio by-products (PBP)

	Treatments					
Item	0PBP	12PBP	24PBP	36PBP	SEM	p-value
Rumen pH	6.31	6.33	6.26	6.38	0.06	0.52
Rumen NH_3-N (mg/dl)	19.41^{a}	17.46^{ab}	16.31^{b}	15.18^{b}	0.78	0.02
Total VFA (mmol/l)	103.30^{a}	104.63^{a}	103.01^{ab}	100.33^{b}	0.87	0.04
Individual VFA (mmol/100 mol)						
Acetate	70.93^{a}	69.71^{a}	69.44^{a}	66.99^{b}	0.70	0.02
Propionate	21.14	23.79	22.35	22.25	0.91	0.30
Butyrate	11.24	11.13	11.23	11.11	0.29	0.98
C2:C3*	3.36	2.96	3.11	3.02	0.14	0.24

0PBP, 0% PBP, 36% AH; 12PBP, 12% PBP, 24% AH; 24PBP, 24% PBP 12% AH; 36PBP, 36% PBP, 0% AH.

Means within a row with different superscripts letters are significantly different ($p < 0.05$).

*Acetate to propionate ratio.

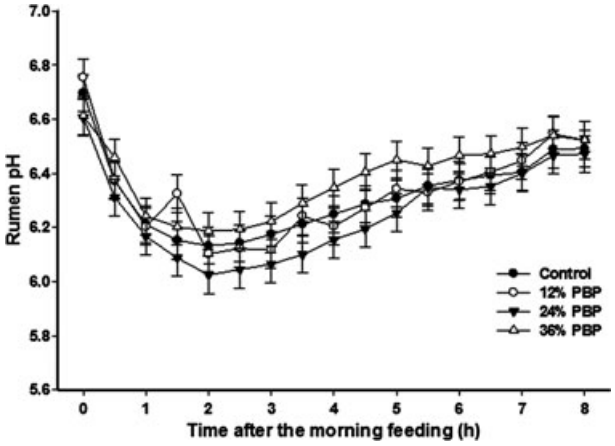

Fig. 1 Ruminal pH of Baluchi male sheep fed different levels of PBP (Treatment effect: not significant; Time effect: $p < 0.01$; Treatment × Time effect: not significant). Bars indicate standard error.

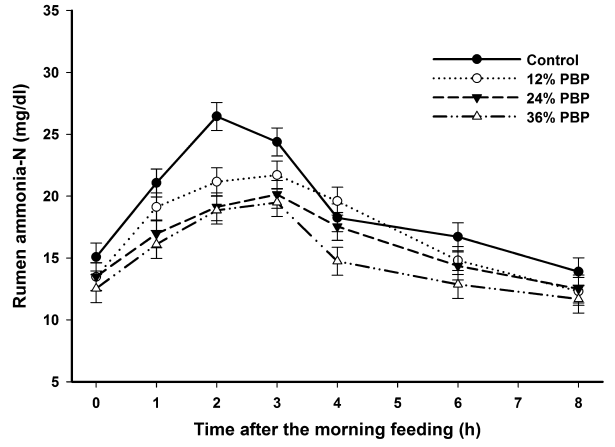

Fig. 2 Ruminal ammonia-N concentrations of Baluchi male sheep fed different levels of PBP (Treatment effect: $p < 0.05$; Time effect: $p < 0.01$; Treatment ×Time effect: $p = 0.05$). Bars indicate standard error.

PBP treatments (Table 4). Moreover, fluctuations in ruminal ammonia-N concentrations after the morning feeding followed a similar pattern for all treatments (Fig. 2). Use of 36% PBP in the diet reduced total VFA concentrations and molar proportion of acetate ($p < 0.05$), while the concentration of propionate, butyrate and acetate to propionate ratio was similar to all other treatments (Table 4).

Population of rumen bacteria involved in biohydrogenation

Figure 3 shows the dissociation curves of specific primers in amplification of two rumen bacteria involved in BH, namely *B. fibrisolvens* and *B. proteoclasticus*. The single peak of these curves confirms the specificity of primers in quantification of the bacteria species reported by Paillard et al. (2007b). Results of the relative abundance of *B. fibrisolvens* and *B. proteoclasticus* in rumen are presented in Table 5. The use of PBP in the diets had no effects on the relative abundance of *B. fibrisolvens* and *B. proteoclasticus* compared with the control diet.

Blood metabolites

The mean of blood metabolites is presented in Table 6. There were no significant differences among the treatments for glucose, cholesterol, albumin and total protein concentrations in the blood of sheep. The results showed that different levels of PBP had effect ($p < 0.01$) on BUN (Table 6). Blood urea nitrogen concentrations decreased ($p < 0.01$) with increasing the level of PBP in the diets.

Discussion

Ruminal fermentation parameters

Research shows that tannins can modify microbial populations, consequently altering variables like nutrient digestibility, rumen ammonia-N concentrations, VFA profiles, with potential effects on animal metabolism (Jones et al., 1994; Al-Dobaib, 2009; Krueger et al., 2010). Consistent with previous findings, using PBP in the diets had no effect on ruminal pH (Bohluli et al., 2009; Gholizadeh et al., 2010; Rezaeenia et al., 2012). This is while Ghasemi et al. (2012b) reported that ruminal pH increased from 6.03 to 6.33 ($p < 0.01$) in Baluchi lambs as a result of including PBP by up to 50% DM of their diets. It is generally agreed that tannins reduce the rate of protein degradation in the rumen (Min et al., 2005; Ghasemi et al., 2012b). In the current study, reduction in ruminal ammonia-N concentrations with the use of PBP in the diet of sheep could probably resulted from a greater concentration of tannins bound to proteins and decreased proteolysis of feed protein (Min et al., 2003; Frutos et al., 2004). The results in our study are consistent with those of Ghasemi et al. (2012a) who obtained lower ruminal ammonia-N concentrations in sheep fed 40% PBP than those fed the control diet. Inclusion of quebracho tannins (20–30 g/kg DM) decreased the rate of degradation and effective degradability of protein of lucerne hay in sheep (Al-Dobaib, 2009). However, the lowest ruminal ammonia-N concentrations measured in the current study were still above the minimum concentrations required (≥5 mg/dl) for rumen microbial growth (Satter and Roffler, 1975). Previous studies suggest that feeding PBP at low (≤6%) or medium (≤15%) levels had no adverse

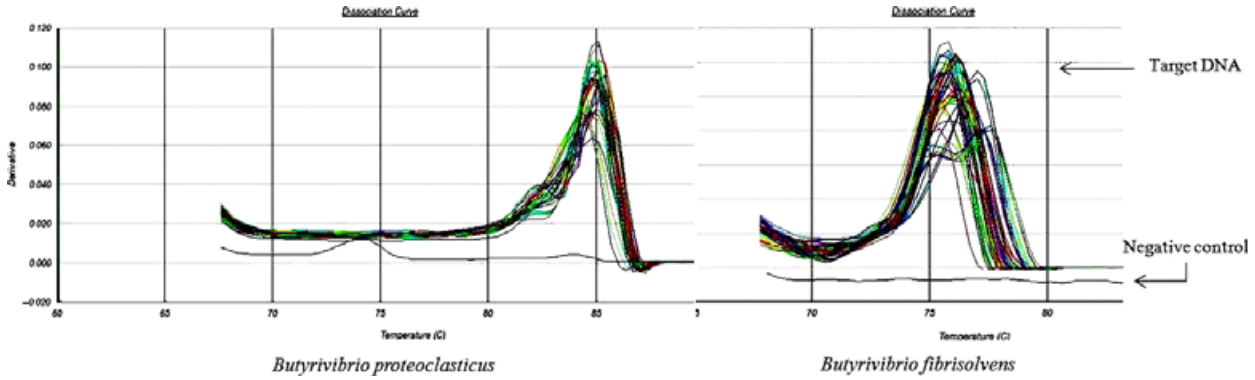

Fig. 3 Dissociation curves showing specificity of real-time PCR in quantifying biohydrogenating bacteria in the rumen of sheep.

Table 5 Effect of PBP on populations of rumen bacteria* involved in biohydrogenation in Baluchi male sheep (n = 4).

	Treatments					
Bacteria	0PBP	12PBP	24PBP	36PBP	SEM	p-value
Butyrivibrio fibrisolvens	1.000	1.764	0.955	0.909	0.318	0.591
Butyrivibrio proteoclasticus	1.000	0.962	1.026	1.020	0.306	0.999

0PBP, 0% PBP, 36% AH; 12PBP, 12% PBP, 24% AH; 24PBP, 24% PBP 12% AH; 36PBP, 36% PBP, 0% AH.
*Fold change compared to control.

Table 6 Blood metabolites in Baluchi male sheep (n = 4) fed different levels of pistachio by-products (PBP)

	Treatments					
Item	0PBP	12PBP	24PBP	36PBP	SEM	p-value
Glucose (mg/dl)	59.33	61.02	58.00	55.83	1.72	0.28
Cholesterol (mg/dl)	78.23	85.61	78.76	86.35	3.85	0.34
Albumin (g/l)	32.00	32.29	30.75	33.61	1.83	0.72
Total Protein (g/l)	68.67	68.82	68.42	69.19	2.99	0.99
BUN (mg/dl)	19.13^{a}	18.17^{b}	16.24^{c}	16.04^{c}	0.33	0.001

0PBP, 0% PBP, 36% AH; 12PBP, 12% PBP, 24% AH; 24PBP, 24% PBP 12% AH; 36PBP, 36% PBP, 0% AH.
Means within a row with different superscripts letters are significantly different ($p < 0.05$).

effects on rumen fermentation, digestion or performance (Ghasemi et al., 2012b; Mokhtarpour et al., 2012; Shakeri et al., 2012). However, higher PBP levels may adversely affect voluntary feed intake and growth performance (Shakeri et al., 2004). Ghasemi et al. (2012b) reported that the concentration of total and individual VFA in the rumen of sheep fed PBP by up to 50% DM was lower ($p < 0.05$) than those fed AH. Beauchemin et al. (2007) also reported that the concentration of total VFA and acetate tended to decrease ($p < 0.08$) with increasing quebracho tannins by up to 2% DM in the diets of dairy cows. The effects of PBP on rumen bacteria have also been investigated by Ghasemi et al. (2012a) who found that using 40% pistachio hulls (30.7 g/kg DM, tannins) in the diets of sheep decreased ($p < 0.05$) the rumen population of total and *cellulolytic* bacteria. In the current study, depression in VFA concentrations with using PBP in the diet might be related to the lower rumen microbial fermentation in presence of tannins.

Population of rumen bacteria involved in biohydrogenation

Many ruminal bacteria species of the genera *Butyrivibrio, Fusocillus, Eubacterium, Micrococcus, Megasphaera, Ruminococcus and Clostridium* are known to participate in ruminal BH of fatty acids (Harfoot and Hazlewood, 1997; Maia et al., 2007). Kemp (1984) has classified bacteria involved in the different steps of the BH pathway into two groups, A and B. *Butyrivibrio fibrisolvens* is the most active species among the group A and plays a major role in hydrogenating unsaturated fatty acids to vaccenic acid (VA), while few species of bacteria such as *B. proteoclasticus* (group B) hydrogenate the last step of the BH process (Maia et al., 2007; Paillard et al., 2007a,b). Recent studies have shown that dietary tannins (4% DM, tannins) decrease the last step of the rumen BH process due to their ability to inhibit the activity of some ruminal bacteria (Vasta et al., 2009a, 2010). The results obtained in the current study on the population of rumen bacteria involved in BH are in contrast to the findings of Vasta et al. (2010) who reported that use of quebracho tannin (9.6% of DM intake) in the diets of sheep increased the relative abundance of *B. fibrisolvens* (8.76 vs. 4.22% of total

bacteria), while the abundance of *B. proteoclasticus* (2.77 vs. 3.99% of total bacteria) decreased. However, the total number of bacteria was not affected in their study. Durmic et al. (2008) reported that *Acacia mearnsii* tannins extract had a selective inhibitory effect towards *B. proteoclasticus* without affecting *B. fibrisolvens* in an *in vitro* study. Results from the study of Kronberg et al. (2007) indicated that the addition of quebracho condensed tannin (200 g/kg of flaxseed) reduced BH of LNA in flaxseed (13 vs. 43%) in the *in vitro* batch culture. In another study, addition of condensed tannins (600 μg CT/ml) from sainfoin leaves (*O. viciifolia*) inhibited the growth and protease activity of *B. fibrisolvens* (Jones et al., 1994). Effect of tannin supplementation on rumen microbial population is variable and mostly depends on the type of tannins (hydrolysable, condensed, protein-bound or fibre-bound), their origin and supplementation levels (Patra and Saxena, 2011). Wallace et al. (2006) reported that *B. proteoclasticus* hydrogenate the last step of the rumen BH only during the growing phase, and therefore, according to Kim et al. (2008), the activity of *B. proteoclasticus* [and therefore stearic acid (SA) production] may not be proportional to concentrations of 16S rRNA gene copies. In the present study, using PBP in the diets had no effects on the abundance of rumen bacteria involved in BH, which might be related to the low tannin doses and tannin source. However, more research is needed to investigate on the role of other bacteria involved in rumen BH.

Blood metabolites

The findings of the current study are consistent with those of Ghasemi et al. (2012b) who reported that use of PBP by up to 50% DM in the diets of sheep had no effects on their blood metabolites. Similarly, Rezaeenia et al. (2012) reported that use of 15% PBP silage (5.5% DM, tannins) in the diet of early lactation dairy cows had no effects on blood glucose and cholesterol. Condensed tannins have been reported as it caused a decrease in lipid digestibility to be responsible for the inhibition of the enzymatic activity of the lipase (Chung et al., 1998). Tannins have also been reported to exert other physiological effects, such as a reduction in blood cholesterol levels (Chung et al., 1998). Gholizadeh et al. (2010) also observed no changes in the concentration of blood metabolites (i.e. cholesterol, glucose, triglyceride and BUN) of dairy cows when using 10% PBP in the diets. Moreover, we found that use of PBP in the diet of sheep decreased ($p < 0.01$) BUN concentrations without affecting other blood metabolites. Consistent with our findings, Shakeri et al. (2012) reported that use of PBP silage in the diet decreased ($p < 0.01$) the concentration of BUN in growing male calves. The decrease in the concentration of BUN in the present study might be related to lower ruminal degradation of protein in the presence of tannins (McNabb et al., 1996; Min et al., 2005; Ghasemi et al., 2012b).

Conclusion

The present study revealed that use of PBP in the diet of sheep decreased BUN concentrations without affecting other blood metabolites. It was also found that use of PBP, especially at higher inclusion rates, decreased the concentration of total VFA, acetate and ammonia-N in the rumen. In addition, use of PBP in the diets did not affect the relative abundance of rumen bacteria involved in BH. Further research is needed using tannin-binding agents (e.g., PEG) and extracted tannins from PBP to define the effects of tannins on the growth and activity of rumen microbes.

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