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Ruminal fermentation, microbial protein synthesis and nitrogen balance in sheep fed pistachio by-product silage

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Abstract This study was conducted to evaluate the effect of feeding several levels of pistachio by-product silage (PBS) on feed intake, nitrogen metabolism, protozoa population and purine derivative excretion in sheep. The isoenergetic and isonitrogenous diets were: 1) control (without PBS), 2) 7% PBS, 3) 14% PBS, and 4) 21% PBS. Eight rams (40±2.5 kg live weight) were used in a replicated Latin square design with four diets and four periods of 35 days each. Feeding 14% of PBS enhanced significantly (P<0.05) dry matter intake, nitrogen intake and nitrogen retention in sheep. Purine derivative excretion and microbial protein synthesis increased (P<0.05) when diets contained PBS. Ruminal pH increased and Holotrich protozoa population decreased linearly with increasing levels of PBS in the diets (P<0.05). By increasing PBS in the experimental diets, total protozoa population and *Entodinium* sp. changed quadratically (P<0.05). The results showed that PBS up to 14% can be used as a dietary ingredient for sheep, especially when conventional feeds are in short supply.

Keywords: purine derivatives, ruminal pH, ammonia N, ruminal protozoa

Introduction

The high cost of animal feed in semi-arid countries, is a major problem that limits livestock production, including the sheep which are raised predominantly under extensive systems. Therefore, using agricultural by-products as alternative feed is a logical and suitable strategy in low input systems. The use of agricultural by-products in animal feed will reduce the feed costs and environmental pollution (Vasta et al., 2008). However, due to the effects of many factors like environment conditions, processing methods and local technologies their nutritional value fluctuates considerably (Ajila et al., 2012). Some by-products contain antinutritional factors including the tannins, which interfere with the utilization of dietary nutrients. Tannins are water-soluble polyphenolic polymers of high molecular weights with many phenolic hydroxyl groups that are capable of forming complexes, especially with proteins and then with carbohydrates, metal ions and amino acids (Makkar, 2003).

Even, low dietary concentrations of condensd tannins were shown to be antinutritional in diets, increasing the flow of non-ammonia nitrogen and essential amino acids from the rumen to small intestine (McNabb et al., 1993).

Positive effects of tannins on protein performance, practically are important, because these effects limit proteolysis and (or) the ruminal deamination of amino acids (Schofild et al., 2001). In Iran, more than 551 thousand tons of pistachio are produced annually (Food and Agricultural Organization, 2018), and a large volume of by-products are produced as a result of pistachio dehulling. Pistachio by-products contain the upper shell, branches, leaves, wooden hull and nuts, and contain (DM basis) 13.5% crude protein, 5.38% ether extract, 9.24% ash, 31.8% NDF and 4.5% (Bohluli et al., 2010). The high moisture contents in the by-product can create several problems, including rapid spoilage when it is exposed to the air; thus becoming an environmental pollutant (Gholizadeh, 2010). Therefore, the high levels of tannins and moisture limit the use of pistachio by-products in animal feeding. Shakeri et al. (2014), feeding pistachio by-product silage (at 6 and 12% levels in the diet on DM basis) to calves, found no effects on digestibility and ruminal fermentation parameters. Feeding growing lambs on pistachio epicarp silage up to 25% of total DM intake, affected wool characteristics (Salehi et al., 2012). The growing lambs fed pistachio hulls silage with waste dates (in a ratio of 5:1) recorded increased final body weight, weight gain and weights of warm and cold carcass (Soltaninejad et al., 2016). The aim of this study was to determine the effects of feeding pistachio by-product silage (PBS) on dry matter intake, nitrogen metabolism and ruminal fermentation in sheep.

Materials and methods

Pistachio by-products (include upper shells, leaves, branches, wooden shells and a small amount of nuts) were ensiled in a trench silo by trampling and sealed by polyethylene sheets and a layer of mud. The silage was prepared without any additive and opened after 60 days. The silage was removed as needed daily and covered again.

Animals and diets

The *in vivo* trial was conducted using 8 rams (2-year-old; and mean live weight of 40±2.5 kg). The animals were allocated to four treatments in a replicated Latin square design with four 35-day periods. There were 30 days for adaptation to diets and 5 days for collecting samples in each period. They were housed in metabolic cages allowing separate collection of feces and urine. The animals were fed TMR diets *ad libitum* twice a day (08:00 and 17:00 h) and the amounts of feed offered per animal were collected and adjusted according to feed refusals daily. Fresh and clean water was available at all times. The animals were maintained according to the guidelines set by the Iranian Council on Animal Care (1995). The rams were shorn and treated externally with albendazole for parasites and vaccinated for enterotoxaemia.

Four isoenergetic (as metabolizable energy) and isonitrogenous diets (40% forage and 60% concentrates), were formulated to meet the sheep requirements (NRC, 2007); including, the control diet (without PBS), and diets containing 7, 14, and 21% PBS, respectively (Table 1). Samples of diets were ground (1-mm screen) and analyzed for DM, CP, EE, ash (AOAC, 2005), and NDF and ADF (Van Soest, 1991).

Sample collection and measurements

Ruminal fluid was sampled by stomach tube from each sheep at 0, 3 and 6 h after feeding on the last day of each period. The pH of ruminal fluid was measured with a portable pH meter (AZ, Model 8601) immediately after collection. A sub-sample (10 mL) of ruminal fluid was mixed with 0.1 mL 50% sulfuric acid and kept frozen at -20 ◦C. The samples were thawed at 4 ◦C, centrifuged at 12,000 rpm for 10 min, and analyzed for ammonia N concentration, using phenol-hypochlorite reaction (Broderick and Kang, 1980)

Ciliated protozoa were enumerated, in duplicate, using an improved bright-line Neubauer hemocytometer (0.1mm depth, Hausser Scientific, Horshman, PA, USA) in samples of the ruminal fluid which had been preserved with methylgreen-formalin-saline (Ogimoto and Imai, 1981). Protozoal counts of different genera were recorded and grouped as *Entodinium* sp., Holotrichs (*Isotricha* and *Dasytricha* sp.) and cellulolytic protozoa (*Polyplastron*, *Diplodinium* and *Enoploplastron* sp.).

Purine derivatives (uric acid, urea nitrogen and creatinine) were measured in the urine samples collected during the last 5 days of each period. A bucket containing 100 mL of 10% (vol/vol) sulfuric acid, to keep the final pH below 3, was placed under the cage for urine collection. Urine samples collected daily for 5 d were weighed, and a sample of 20 mL was stored at -20 °C for later analysis. The amount of purines (mmol/day) absorbed by the animal were estimated using the model described by Chen and Gomez (1992). The supply of microbial N was then calculated based on microbial purine digestibility of 0.83 and purine nitrogen to total microbial N ratio of 0.116 to 1.00 (Chen and Gomez, 1992):

Microbial nitrogen (g/day) = (microbial purine absorbed ×70) / (0.116×0.83×1000)

Statistical analysis

Data on feed intake, rumen fermentation parameters and microbial protein synthesis were subjected to analysis of variance using the MIXED procedure (SAS, 2005), and according to the following models:

$$Y_{ijk} = \mu + T_i + P_j + C_k + Z_m + ZT_{mi} + e_{ijk}$$

$$Y_{ijk} = \mu + T_i + P_j + C_k + e_{ijk}$$

in which, Y_{ijk} is the dependent variable, μ the overall mean, T_i the fixed effect of treatment i, P_j the random effect of period j, Ck the random effect of animal, Z_m the effect of time, ZT_{mi} the interaction effect of $Z_m \times T_i$ and e_{ijk} is the random error. All mean separations were performed using the LSD procedure. Polynomial contrasts were used to determine the linear and quadratic components of the dietary treatment response.

Table 1. Ingredients and nutrient composition of the experimental diets (DM basis)

Ingredients	Percent pistachio by-product silage (PBS) in diet			
	0	7	14	21
Alfalfa hay, chopped	25	22	17	12
Wheat straw, chopped	15	11	9	7
Pistachio by-product silage (PBS)	0	7	14	21
Barley grain, ground	29.5	28.5	26.5	26
Corn grain, ground	15	15	15	15
Soybean meal	4.5	3.5	3.5	3
Wheat bran	9	11	13	14
Vitamin A, D and E Premix[a]	0.6	0.6	0.6	0.6
Trace-mineralized salt[b]	0.6	0.6	0.6	0.6
Sodium bicarbonate	0.4	0.4	0.4	0.4
Limestone	0.4	0.4	0.4	0.4
Dietary chemical composition				
Metabolizable energy (Mcal/kg DM)	2.51	2.54	2.55	2.55
Crude protein (g/kg DM)	12.21	12.25	12.34	12.25
Dry matter (g/kg DM)	892.2	783.2	631	620
Organic matter (g/kg DM)	926.8	926.4	925.35	924.9
Ether extract (g/kg DM)	26.1	33.1	40.0	46.5
NDF[c] (g/kg DM)	332.6	320.09	315.1	307.0
ADF[d] (g/kg DM)	240.8	223.0	208.1	192.4
Phenolic compounds (g/kg DM)	0	1.28	2.54	3.84

[a]Contains 5,000,000 IU of Vitamin A; 5,000,000 IU of Vitamin D and 500,000 IU of Vitamin E per kg.
[b]Composition: 75.15% NACL, 3.046% Mn, 1.025% cu-sulphate, 0.253% Zn-sulphate, 0.015% EDDI-80 and 0.011% Na-selenide.
[c]Neutral detergent fiber.
[d]Acid detergent fiber.

Results

Dry matter intake, digestibility and nitrogen balance

Pistachio by-product silage (PBS) at 7 and 14% levels in the diet significantly (P<0.05) increased the dry matter intake (DMI) and nitrogen intake (Table 2). Nitrogen intake changed quadratically (P<0.05), being highest at 14 %, intermediate at 7 %, and lowest at 21 % PBS) and control diet. Excretion of N in feces did not differ among diets, but urinary N excretion and then nitrogen retention was higher and lower in the sheep fed the control diet (P<0.05) in comparison with other groups, respectively. Dry matter and protein digestibility was not influenced by dietary PBS.

Ruminal pH and ammonia N concentration

Sheep fed 21% PBS recorded higher ruminal pH (P<0.05) in zero time (before feeding) than animal fed other experimental diets. Also, the lowest ruminal pH was found in the sheep fed on the control diet (Figure 1). With increasing levels of PBS in the diets, concentration of ruminal ammonia N decreased significantly (Figure 2, P<0.05). The sheep feeding the control diet recorded greater ammonia N after feeding than animals fed PBS-containing diets.

Table 2. Dry matter intake, nitrogen intake, excretion and retention in sheep fed diets containing pistachio by-product silage (PBS)

Parameters	PBS level in diet DM (%)				SEM	Contrast	
	0	7	14	21		Linear	Quadratic
Dry matter intake (kg/day)	1.23[b]	1.36[a]	1.37[a]	1.24[b]	0.03	0.81	0.01
Dry matter digestibility (%)	63.11	65.90	67.85	68.41	5.83	0.20	0.70
Protein digestibility (%)	61.47	64.99	64.84	64.23	4.07	0.66	0.62
N intake (g/day)	24.56[c]	27.26[ab]	28.07[a]	25.06[bc]	0.75	0.82	0.008
N excretion (g/day) in:							
Urine	4.16[a]	4.25[a]	3.17[ab]	2.66[b]	0.47	0.05	0.69
Feces	11.53	11.59	11.87	11.07	1.07	0.86	0.70
N retention (g/day)	8.82[b]	11.40[ab]	13.01[a]	11.32[ab]	1.21	0.14	0.12

[a,b] Within rows, means with common superscript (s) are not different (P>0.05).
SEM: standard error of the mean.

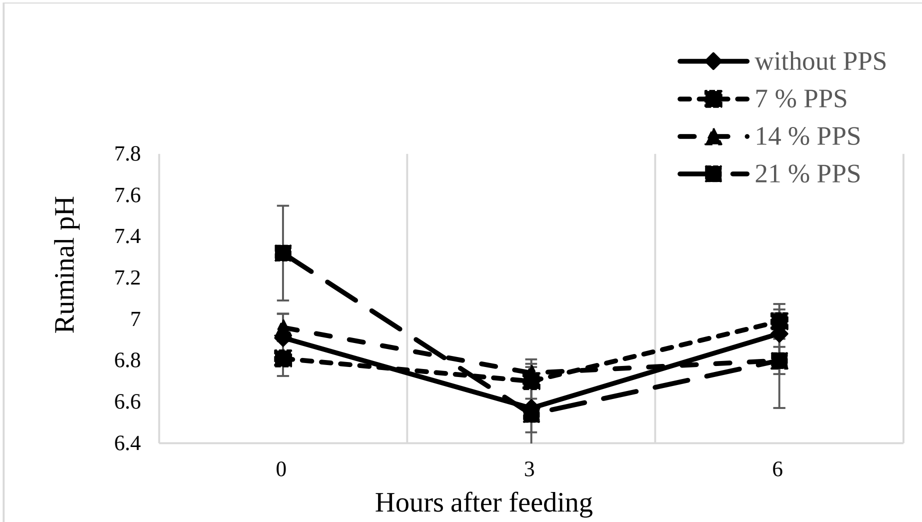

Figure 1. Ruminal pH in sheep feeding pistachio by-product silage (PBS) relative to feeding time

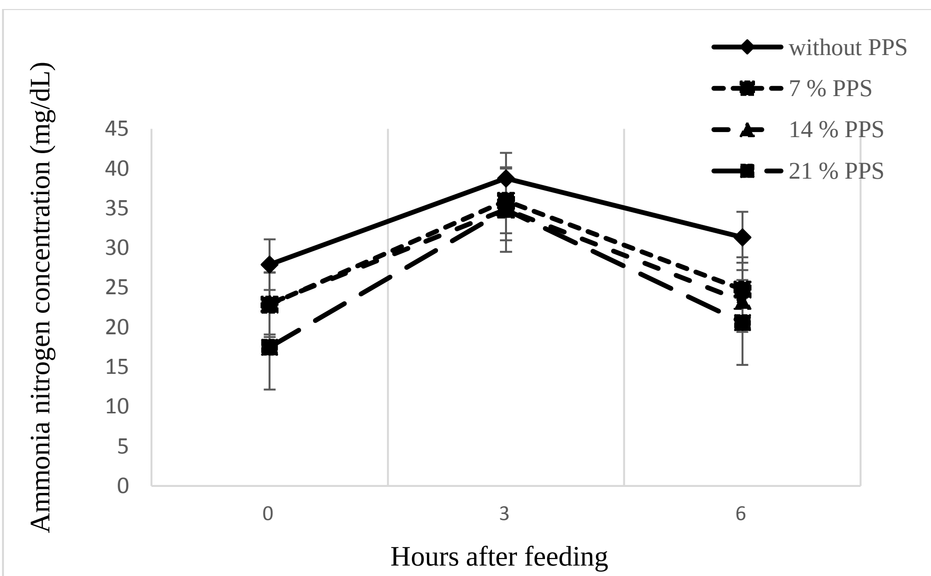

Figure 2. Ammonia nitrogen concentration (mg/dL) in sheep feeding pistachio by-product silage (PBS) relative to feeding time

Protozoal population

The population of *Holotrichs* in the ruminal fluid decreased linearly (P<0.05), being highest for the control and 7% PBS, intermediate for 14% PBS, and lowest for 21% PBS diets. (Table 3). The number of total protozoa and *Entodinium* species changed quadratically (P<0.05), being higher in sheep fed 14% PBS than in other animals. Total number of *cellulolytica* was not affected by the diets.

Table 3. Protozoal population in sheep ruminal fluid (×10^5 cells/mL) on diets containing pistachio by-product silage (PBS)

	PBS level in diet DM (%)				SEM	Contrast	
	0	7	14	21		Linear	Quadratic
Entodinium	10.52[b]	11.41[b]	14.99[a]	11.01[b]	1.12	0.90	0.02
Holotricha	0.52[a]	0.50[a]	0.38[ab]	0.28[b]	0.07	0.01	0.54
Cellulolytica	0.36	0.28	0.29	0.35	0.06	0.76	0.20
Total protozoa	11.40[b]	11.99[b]	15.66[a]	11.64[b]	1.20	0.28	0.02

[a,b] Within rows, means with common superscript (s) are not different (P>0.05).
SEM: standard error of the mean.

Purine derivative excretion and microbial protein synthesis

Urinary allantoin excretion tended to change quadratically (P=0.06), with no difference between PBS-containing diets and the control one. Allantoin excretion was higher in 14% PBS diet vs. 21% PBS diet, but there was no difference between 7% and 21% PBS diets (Table 4). As a result, microbial protein synthesis tended to increase (P<0.05) in these animals.

Table 4. Urinary excretion of purine derivatives in sheep fed diets containing pistachio by-product silage (PBS)

	PBS level in diet DM (%)				SEM	Contrast	
	0	7	14	21		Linear	Quadratic
Allantoin (mmol/day)	8.8[ab]	9.05[ab]	12.35[a]	6.93[b]	1.64	0.69	0.06
Uric acid (mmol/day)	0.68	0.50	0.65	0.43	0.08	0.43	0.01
Creatinine (mmol/day)	3.2	3.19	5.10	4.3	0.93	0.12	0.98
Purine (mmol/day)	10.50[ab]	10.77[ab]	14.48[a]	8.40[b]	1.39	0.69	0.06
Microbial nitrogen (g/day)	8.89[ab]	9.02[ab]	12.5[a]	7.03[b]	1.6	0.72	0.06
Microbial protein (g/day)	55.56[ab]	56.40[ab]	78.15[a]	43.98[b]	10.24	0.72	0.06

[a,b] Within rows, means with common superscript (s) are not different (P>0.05).
SEM: standard error of the mean.

Discussion

Dry matter intake and nitrogen balance

The DMI changed quadratically. Sheep fed 7 and 14% of PBS consumed more DMI than those fed 21% PBS and the control diet. With increasing PBS, the NDF and ADF contents in the diets decreased. Mertens (2009) suggested that the NDF concentration was negatively correlated with DMI. The decrease in DMI in the sheep feeding on the 21% PBS diet was probably due to higher tannin content in this diet (Silanikove et al., 2001; Shakeri, 2016). Total concentration of tannins and phenolic compounds in the diet containing 21% PBS was 2% and 3% (DM basis) higher than in diets containing 7 and 14% PBS, respectively. Kumar and Singh (1984) showed that the minimum amount of tannin in the ruminants feed that can negatively affect the free consumption of dry matter was 2% in DM. Dry matter intake in lambs was reduced at 30% sun-dried pistachio by-product in the diet (Shakeri et al., 2016), whereas Mahdavi et al. (2010) and Norouzian and Ghiasi (2012) observed no changes in the feed intake by feeding pistachio by-product male lambs. Lambs fed 21% and 14% pistachio by-product silage and date waste, consumed more DM than control diet (Soltaninejad et al., 2017). Adding PBS to the sheep diets had not significant effect on dry matter and crude protein digestibility. Similarly, inclusion of up to 25% dried pistachio hull in the dairy cow diet had not significant effect on apparent nutrient digestibility (Bohluli et al., 2010).

The increase in N intake and retention rate, and the decrease in urinary N excretion in the sheep feeding on PBS-containing diets may be related to the presence of tannin in PBS (Al-Dobaib, 2009). In general, decreased urinary nitrogen excretion compared to fecal excretion improves the nitrogen utilization efficiency. Thus, tannins in PBS may be generating undegradable rumen escape protein. Increased in nitrogen intake and retention as a result of adding pistachio by-product to sheep diet is consistent with reports in sheep (Ghasemi et al., 2012), lambs (Rajaei Sharifabadi and Naserian, 2014) and dairy goats (Mokhtarpour et al., 2017).

Ruminal pH and nitrogen

Ruminal fluid pH increased in the sheep feeding on PBS. Ensiling of pistachio by-product, decreased soluble carbohydrate (Bagheripour et al., 2012); therefore, the increase in ruminal pH in sheep fed on 21% PBS diet may be associated with decreasing soluble carbohydrate in this diet compared with other diets. Beside, highly condensed tannins could change the ruminal fermentation pattern (Ghasemi et al., 2012). Previous studies reported different responses in ruminal pH to the feeding of pistachio by-product. Contrary to our results, Rezaeenia et al. (2012) reported no effects on ruminal pH by feeding pistachio by-product silage in Holstein dairy cows. However, ruminal pH increased in Baluchi male lambs fed 40% pistachio hulls (Ghasemi et al., 2012).

Inclusion of 21% of PBS in the diet had significant effect on ruminal concentration of ammonia N. The sheep feeding the control diet recorded greater ammonia N after feeding. Tannins reduced ruminal degradation of protein (Min et al., 2003) and thus decreased ammonia N concentration in the ruminal fluid. Other studies reported a decrease in ruminal ammonia concentration when pistachio by-product was fed to sheep (Ghaffari et al., 2014) and dairy cows (Bohluli et al., 2010). Generally, inclusion of pistachio by-products in the diet of ruminants decreased ammonia emission without negatively impacting on protein digestibility (Alkhtib et al., 2017).

Protozoal population

Ensiling of pistachio by-product in previous studies, decreased water soluble carbohydrates (Bagheripour et al., 2012). *Holotricha* consumes non starch carbohydrates and soluble sugars, therefore, a decrease in the *Holotricha* population with addition of PBS to experimental diets was expected. While *Endodinium* species consume both structural and non-structural carbohydrates (Williams and Coleman, 1988). On the other hand, tannins and phenolic structures disrupt protozoa membrane and in high dosage, inactivate protozoal enzymes. Among protozoa population, *Holotrich* species are more sensitive to tannins than *Endodinium* (Makkar, 2003; Patra and Saxena, 2009).

The high *entodiniomorphs* number in sheep fed the 14% PBS diet was related to the increase in DMI. There are studies reporting no change in rumen protozoal population when pistachio by-products were included in the diet (Rahimi et al., 2012; Mirheidari et al., 2019). Plant tannins do not have a consistent effect on protozoa, which may depend on the other dietary components and forage to concentrate ratio (Yanez-Ruiz and Belanche, 2020). It was reported that moderate levels of tannins in dietary concentrate could increase the ruminal protozoal population (Belanche et al., 2011).

Purine derivative excretion and microbial protein synthesis

The results of this experiment showed that 14% PBS in the diet increased the supply of microbial protein to sheep. Uric acid and creatinine concentrations were high numerically in sheep fed 14% PBS, too. The increase in allantoin concentration and microbial protein synthesis might be due to the higher dry matter intake in sheep fed diet containing 14% PBS. It has been reported that low condensed tannins cause higher ratio of available nutrients to be allocated to microbial protein synthesis and less to volatile fatty acid production (Makkar et al., 1997). Provision of high levels of pistachio by-product, in lieu of lucerne hay, decreased purine derivatives excretion and microbial nitrogen supply in sheep (Ghasemi et al., 2012). Shakeri et al. (2012) indicated that 12 and 18% of pistachio by-product silage in Holstein fattening bulls decreased the microbial protein synthesis because of the lack of synchronization in carbohydrate and protein availability in the rumen.

Conclusions

Inclusion of pistachio by-product silage, up to 14% of dry matter, in the diet of Kermani sheep increased microbial protein synthesis and N retention without negatively affecting the fermentation parameters. Therefore, whenever conventional feedstuffs are in limited supply, part of the dietary forage could be replaced with pistachio by-product.

Disclosure statement

The authors declare that there is no conflict of interest regarding the publication of this article.

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