Animal Biotechnology

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/labt20

Effects of pomegranate peel extract on ruminal and post-ruminal *in vitro* degradation of rumen inoculum of the dairy cow

Mohammad Javad Abarghuei, Yousef Rouzbehan, Abdelfattah Zeidan Mohamed Salem & Mohammad Javad Zamiri

To cite this article: Mohammad Javad Abarghuei, Yousef Rouzbehan, Abdelfattah Zeidan Mohamed Salem & Mohammad Javad Zamiri (2021) Effects of pomegranate peel extract on ruminal and post-ruminal *in vitro* degradation of rumen inoculum of the dairy cow, Animal Biotechnology, 32:3, 366-374, DOI: 10.1080/10495398.2020.1727492

To link to this article: https://doi.org/10.1080/10495398.2020.1727492

Published online: 14 Feb 2020.

Submit your article to this journal

Article views: 82

View related articles

View Crossmark data

Full Terms & Conditions of access and use can be found at
https://www.tandfonline.com/action/journalInformation?journalCode=labt20

ANIMAL BIOTECHNOLOGY
2021, VOL. 32, NO. 3, 366–374
https://doi.org/10.1080/10495398.2020.1727492

Taylor & Francis
Taylor & Francis Group

Check for updates

Effects of pomegranate peel extract on ruminal and post-ruminal *in vitro* degradation of rumen inoculum of the dairy cow

Mohammad Javad Abarghuei[a], Yousef Rouzbehan[a], Abdelfattah Zeidan Mohamed Salem[b], and Mohammad Javad Zamiri[c]

[a]Department of Animal Science, Faculty of Agriculture, Tarbiat Modares University, Tehran, Iran; [b]Facultad de Medicina Veterinaria Zootecnia, Universidad Autónoma del Estado de México, Toluca, México; [c]Department of Animal Science, College of Agriculture, Shiraz University, Shiraz, Iran

ABSTRACT

This experiment was carried out to study the effect of water extracted pomegranate peel extract (PE) on ruminal protein degradation and post-ruminal digestion in the dairy cow. PE was added at six levels of total phenolics (g/kg of the basal diet); 3.75 (PE1); 4.4 (PE2); 5.05 (PE3); 5.70 (PE4); and 6.35 (PE5). Rumen degradable crude protein (rdCP) decreased with PE addition ($L < 0.0001$), but total CP degradability (tdCP) was not affected. Compared to PE0, PE2, and PE3 diets showed higher ($L = 0.054$, $Q = 0.029$) digestibility of bypass CP (dBCP). Increasing levels of PE resulted in a decrease in proteolytic bacteria numbers ($p < 0.0001$). At PE4 and PE5 levels, total VFA and acetate concentrations linearly decreased compared to PE0. PE inclusion lowered the acetate:propionate ratio ($L = 0.0001$) and Ammonia-N production after 24 h ($L = 0.0008$) of incubation. The total number of protozoa, genera *Dasytricha* and *Isotricha*, and subfamilies *Entodiniinae*, *Diplodiniinae*, and *Ophrioscolecinae* decreased with increasing dietary PE concentration ($p < 0.0001$). The results suggest that all levels of PE addition reduce the protozoal population and Ammonia-N concentration. All PE levels slowed down protein degradation in the rumen but PE2 and PE3 showed the greatest effect.

KEYWORDS

Dairy cow; pomegranate-peel extract; protein; protozoa; rumen

Introduction

The cost of animal feeds, particularly protein supplements, has increased mainly due to changing climatic conditions and a shortage of water resources; yet these supplements may be metabolized less efficiently (i.e., losses of NH_3–N) in the rumen resulting in a reduction in animal performance.[1] Consequently, many studies have been carried out to improve the efficiency of nitrogen utilization, including the use of aqueous extracts of plant secondary metabolites (AE).[2] Plant secondary metabolites (PSM) in tree leaves such as *Salix babylonica* and *Leucaenaleucocephala*[3,4] were found to have a positive effect on ruminal fermentation parameters and to increase amino acid flow to the duodenum.[5] This led to greater muscle deposition and milk production.[6] PSM are a diverse group of molecules involved in the adaptation of plants to their environment but are not part of the primary biochemical pathways of cell growth and reproduction.[2] Other studies have shown that plant secondary metabolites decreased rumen proteolysis.[7] Research has shown that tannin extracts from *Cistus ladanifer* led to a reduction in effective rumen degradability of soybean protein and that linearly decreased with extract inclusion level.[7] In other research, grape pomace extract slowed soybean meal protein degradation in the rumen and the post-ruminal digestibility of this undegraded protein increased in proportion to the concentration of the extract.[8]

PSM extraction is generally carried out using organic solvents,[8] a relatively expensive procedure, but this work tests the effectiveness of a cheaper water extraction method (AE). Globally, the annual production of pomegranate peel (PP), a by-product of pomegranate juice extraction, is 15,000,000 t,[9] and it contains high levels of PSM such as polyphenols, tannins, saponins, and punicalaginan.[9] This study aims to determine the optimum levels of pomegranate peel

CONTACT Yousef Rouzbehan rozbeh_y@modares.ac.ir Department of Animal Science, Faculty of Agriculture, Tarbiat Modares University, P.O. Box14115-336, Tehran, Iran

© 2020 Taylor & Francis Group, LLC.

Table 1. The economics of water and organic solvent (OS) extraction of Total Phenolics (TP) from Pomegranate Peel (PP).

	Solvents Water	Ethanol + Methanol (50:50)(OS)
Total Extract obtained (mL)*	750 mL	500 mL
mg TP/mL extract	6.5 mg	10 mg
Cost ($)/total extract	0.1	20
Cost ($)/mg total phenolic	0.00002	0.004

*When mixing 1 kg of PP with either 1 L of water or 1 L OS, the total extract obtained was 750 mL and 500 mL respectively.

extract (PE) on the *in vitro* ruminal degradability and intestinal digestibility of a basal diet in the dairy cow.

Materials and methods

Pomegranate peel extract

The PP was sun-dried and then the AE was extracted using 1 g PP/mL of distilled water. This mixture was maintained at 40 °C for 72 h in a closed flask then the contents were strained and the extract kept at 4 °C until used. Preliminary work had been done to extract total phenolics (TP) using an ethanol/methanol mixture (50:50, v/v) (OS) by the same method to enable a cost comparison (Table 1).

Experimental diets

Six levels of PE were used in this study. A basal diet was formulated (Table 2) according to NRC[10] recommendations for dairy cows. 321 mg dry matter (DM) of diet was weighed into 120 mL serum bottles.[11] PE was added to the bottles at six levels of total phenolics (TP) (0, 0.65, 1.30, 1.95, 2.60, and 3.25 g TP/kg DM of basal diet). The basal diet (PE0) contains 3.1 g TP/kg DM, therefore, PE1 contains 3.75 g TP/kg DM with corresponding levels for PE2: 4.4 g, PE3: 5.05, PE4: 5.70, and PE5: 6.35. PE levels were selected for their secondary metabolite concentrations and impacts on protein degradability but kept within a range that would not negatively affect protein degradation and ruminal fermentation. Five sets of bottles were prepared for the determination of (i) ruminal *in vitro* DM and CP degradation, (ii) total *in vitro* DM and CP degradation, (iii) NH_3–N production and protozoa population after 12, 24, and 48 h of incubation, and VFA sampling and determination of proteolytic bacteria after 48 h of incubation (one set per incubation time).

In vitro gas production and related parameters

Nutrient degradation by ruminal microbes and by ruminal microbes plus HCl/pepsin were determined.[11]

Table 2. Ingredients and chemical composition of the basal diet[1].

Ingredients, g/kg DM	
Alfalfa hay	148.4
Corn silage	229.0
Wheat straw	17.7
Soybean meal, 44% CP	119.2
Canola meal	34.6
Cottonseed meal	41.4
Fish meal	9.9
Barley, rolled	121.8
Corn grain, ground, dry	111.1
Wheat grain, rolled	17.1
Wheat bran	68.1
Fat powder	18.3
Calcium carbonate	5.7
Sodium bicarbonate	15.0
Mineral and vitamin premix[2]	26.0
Salt	8.8
Magnesium oxide	8.8
Chemical composition, g/kg DM	
DM	955
OM	934
CP	160
EE	46
[3]NE_L, Mcal/kg DM	1.58
NDFom[a]	305
ADFom[b]	206
ADL[c]	57.60

[1]Basal diet Calculated from (NRC) National Research Council (2012); [2]Contained 196 g Ca, 96 g P, 71 g Na, 19 g Mg, 3 g Fe, 0.3 g Cu, 2 g Mn, 3 g Zn, 100 ppm Co, 100 ppm I, 0.1 ppm Se and 50×10^5 IU vitamin A, 10×10^5 IU vitamin D and 0.1 g vitamin E/kg; [3]NE_L calculated from (NRC) National Research Council (2012): NE_L (Mcal/kg) = 0.0245 × TDN (%) × 0.12; [a]NDFom: ash-free NDF; [b]ADFom: ash-free ADF; [c]ADL: lignin.

Extract effects were examined in three runs of *in vitro* gas production. The contents of one set of bottles were filtered after 48 h of incubation to measure rumen degradable dry matter and rumen degradable crude protein, and a second set was incubated for another 24 h in the presence of HCl/pepsin to measure the total degradability of dry matter and total degradability of crude protein.

Prior to their morning feed, rumen fluid was collected from three cannulated Holstein dairy cows fed three times daily on a TMR diet formulated according to NRC.[10] The cows' diet consisted of 80% forage to 20% concentrate with 12% CP/kg DM and 1.5 Mcal/kg DM of NEL (net energy of lactation). The rumen fluids were mixed and randomly assigned to each treatment group. The fluid was filtered through four layers of cheesecloth, transferred into a separator funnel, and gassed with CO_2. After standing for 15 min at 39 °C, the upper and lower parts of the fluid in the funnel were discarded. The residue was mixed with McDougall's buffer at a ratio 1:4 to obtain the inoculum. Fifty mL of the inoculum was added to the bottles with five bottles ($n = 5$) prepared per diet. After

gasifying with CO_2, the bottles were closed with rubber stoppers and incubated at 39 °C.

After 48 h, the incubation was stopped in one set of bottles by the addition of 1 mL of mercuric chloride solution (50 mg/mL). The second set of bottles had 6 mL of HCl solution (6.21 N) and 2 mL pepsin solution added (50 g/L; 2844-01, USA; 1:3000) and the incubation continued for 24 h, simulating post-ruminal digestion. After incubation, the contents of both sets of bottles were strained through N-free paper filters (no. 10300 012, S&S Whatman, Dassel, Germany). The residues were dried at 105 °C for 12 h, weighed and the nitrogen content measured using Kjeldahl analysis (method 984.13; AOAC 1990). For the first set of bottles, rdDM and rdCP were calculated by comparing DM and CP before and after incubation; the second set of bottles were similarly treated to calculate tdDM and tdCP. The difference between tdCP and rdCP was assumed to be equivalent to the degradable rumen escape CP (undegradable CP in the rumen but digested postruminally; pdBCP). From this value, the digestibility of bypass CP (dBCP) was calculated.

Total and individual VFA in samples were determined by gas chromatography. At the end of the incubation (48 h) 1 mL of the supernatant was collected in a microfuge tube containing 0.2 mL 20% orthophosphoric acid and 20 mM 2-ethyl butyric acid, as the internal standard, and then centrifuged at 15,000 *g* for 15 min at a temperature of 4 °C. The supernatant was collected and stored at −20 °C until analysis.[12] For VFA estimation, 1 ul supernatant was injected into a gas chromatograph (UNICAM 4600; SB Analytical, Cambridge, UK) equipped with a capillary column (Agilent J&W HP-FFAP, 10 m by 0.535 mm by 1.00 μm, 19,095 F-121; Agilent, Santa Clara, CA)[13]

To measure NH_3–N production during fermentation, sub-samples of 5 mL were taken after 12, 24, and 48 h of incubation from the respective sets of bottles and mixed with 1 mL of 0.2 *N* HCl for NH_3–N analysis using the phenol-hypochlorite method.[14] Sub-samples were frozen at −20 °C until analysis.

Total number and subfamily counts of protozoa were determined.[15] Two mL of rumen fluid was pipetted into a screw-capped test tube containing 5 mL of formalinized physiological saline (20 mL formaldehyde in 100 mL saline (0.85 g sodium chloride in 100 mL distilled water)). Total and differential counts of protozoa were made in 30 microscopic fields at a magnification of 200× in a Hemocytometer (Neubauer improved, Marienfeld, Germany).

A 5-mL sample from the content of each bottle was used for enumeration of proteolytic bacteria. Modified anaerobic techniques[16] were used in the preparation of the anaerobic culture media.

Analytical methods

Representative samples from basal diet and experimental diets were taken to carry out the following analysis: dry matter content was determined by oven drying at 105 °C for 16 h (AOAC, method 967.03).[17] Ash content was determined by incineration at 550 °C for 6 h, and the OM content was calculated as the difference between 100 and the percentage of ash (AOAC, method 942.05).[17] NDF was determined, with sodium sulfite in a neutral detergent solution (ND),[18] and ADFom was determined according to AOAC, method 973.18[17] and expressed exclusive of residual ash. The amount of ADL was determined by cellulose solubilization with sulfuric acid.[19] Nitrogen content was determined by the Kjeldahl method.[17]

Total phenolics (TP), Non-tannin phenolics (NTP) were measured using the Folin-Ciocalteau method.[20] Total tannins (TT) were calculated as the difference between TP and NTP. Tannic acid (Merck GmbH, Darmstadt, Germany) was used as the standard to express the amount of TP and TT. Condensed tannins (CT) were measured by the HCl-butanol method. Hydrolyzable tannins were analyzed using the Rhodanine assay.[20] Ten mL of the extract were prepared after TP separation and double the volume of n-butanol was added to fractionate saponins.[21] The GC-MS analysis of dihydromaltol and thymol isolated from PE was performed using a Perkin Elmer GC-MS (Model Perkin Elmer Clarus 500, USA) equipped with a VF-5 MS fused silica capillary column (30 m × 0.25 mm id., film thickness 0.25 μm). GC-MS spectroscopic detection, an electron ionization system with ionization energy of 70 eV was used. Pure helium gas (99.999%) was used as a carrier gas at a constant flow rate of ±1 mL/min. Mass transfer line and injector temperatures were set at 220 °C and 290 °C, respectively.

Statistical analysis

Incubation was done in three separate *in vitro* runs with five replicate test feed samples. Data on *in vitro* ruminal fermentation parameters of each of the three runs were averaged and used as the mean value for each individual sample within the diets. Data were subjected to one-way analysis of variance using the MIXED procedure of SAS version 9.0 (SAS Institute, Cary, NC, USA)[22] and the effects of the different

Table 3. Secondary metabolites levels (g/kg of DM) of the experimental diets ($n = 20$).

	Diets[1]					
Secondary metabolites	PE0	PE1	PE2	PE3	PE4	PE5
Total phenolics	3.10 ± 0.05	3.75 ± 0.06	4.40 ± 0.08	5.05 ± 0.03	5.70 ± 0.05	6.35 ± 0.09
Total tannins	0.80 ± 0.06	1.40 ± 0.09	2.00 ± 0.05	2.60 ± 0.08	3.20 ± 0.05	3.80 ± 0.07
Non-tannin phenolics	2.30 ± 0.05	2.35 ± 0.04	2.40 ± 0.08	2.45 ± 0.05	2.50 ± 0.07	2.55 ± 0.07
Condensed tannin, mg/kg	–	0.4 ± 0.02	0.8 ± 0.03	1.2 ± 0.03	1.6 ± 0.05	2.0 ± 0.04
Hydrolyzable tannin	–	0.045 ± 0.005	0.090 ± 0.004	0.135 ± 0.005	0.180 ± 0.002	0.225 ± 0.003
Saponins	11.00 ± 0.10	11.25 ± 0.100	11.50 ± 0.09	11.75 ± 0.10	12.00 ± 0.12	12.25 ± 0.12
Dihydromaltol, mg/kg	–	0.03 ± 0.002	0.06 ± 0.002	0.09 ± 0.003	0.12 ± 0.001	0.15 ± 0.003
Thymol, mg/kg	–	0.02 ± 0.001	0.04 ± 0.002	0.06 ± 0.001	0.08 ± 0.040	0.10 ± 0.030

[1]Diets: PE0, control, no additive = 3.1 g TP/kg DM of basal diet; PE1, control + 0.65 = 3.75 g TP/kg DM of basal diet; PE2, control + 1.30 = 4.4 g TP/kg DM of basal diet; PE3, control + 1.95 = 5.05 g TP/kg DM of basal diet; PE4, control + 2.60 = 5.70 g TP/kg DM of basal diet; PE5, control + 3.25 = 6.35 g TP/kg DM of basal diet.

Table 4. *In vitro* dry matter degradation (mg/g incubated DM) of the experimental diets incubated with pomegranate peel extract.

	Diets[1]							*p*-Value	
	PE0	PE1	PE2	PE3	PE4	PE5	SEM[2]	L[3]	Q[4]
rdDM[5]	681.17	681.39	691.34	686.28	560.68	550.37	34.253	0.039	0.048
tdDM[6]	716.91	716.45	714.63	712.32	700.28	691.42	6.450	0.079	0.337

[1]Diets: PE0, control, no additive = 3.1 g TP/kg DM of basal diet; PE1, control + 0.65 = 3.75 g TP/kg DM of basal diet; PE2, control + 1.30 = 4.4 g TP/kg DM of basal diet; PE3, control + 1.95 = 5.05 g TP/kg DM of basal diet; PE4, control + 2.60 = 5.70 g TP/kg DM of basal diet; PE5, control + 3.25 = 6.35 g TP/kg DM of basal diet.
[2]SEM: standard error of the mean; [3]L: linear; [4]Q: quadratic; [5]rdDM: Degradation of DM incubated in ruminal fluid.
[6]tdDM: Degradation of DM when incubated with ruminal fluid + HCl/pepsin.

levels of PE were partitioned into linear and quadratic components by orthogonal polynomials. Mean comparisons were performed using Duncan's test at $p < 0.05$.

$$Yij = \mu + Si + eij,$$

Where *Yij* is the general observation, *μij* the general mean, *Si* the ith effect of extracts on the observed parameters and *eij* the standard error term.

Results

Ingredients and chemical composition

Ingredients, chemical composition and net energy of lactation (NEL) in the basal diet, and secondary metabolites of the experimental diets are given in Tables 2 and 3, respectively.

Dry matter and crude protein degradability

The amount of rdDM did not differ between PE0, PE1, PE2, and PE3 diets but was lower in PE4 and PE5 compared to PE0 ($Q = 0.048$) (Table 4). The rdCP decreased linearly with increased addition of PE ($L < 0.0001$, $Q < 0.0001$), but tdDM and tdCP values were not affected by PE inclusion.

Addition of PE increased ($L = 0.001$, $Q = 0.002$) the proportion of ruminally un-degraded CP (rumen escape CP- Table 5). The amount of dBCP in PE2 and PE3 diets was higher compared to the PE0 diet ($L = 0.054$, $Q = 0.029$), but did not differ between PE1, PE4, and PE5 diets. The population of proteolytic bacteria decreased with increasing PE levels ($L < 0.0001$, $Q < 0.0001$).

The effect of pomegranate peel extract on volatile fatty acids and Ammonia-N production

The profile of VFA production is shown in Table 6. Total VFA and acetate concentrations did not differ between PE0, PE1, PE2, and PE3 diets but declined in PE4 and PE5 compared to PE0 ($L = 0.029$ and $L = 0.008$ respectively). Propionate, butyrate, isobutyrate and valerate concentrations were not influenced by the diets. The acetate to propionate ratio was lower ($L = 0.0001$) in PE3, PE4, and PE5 diets compared to the PE0 diet. The addition of PE decreased NH_3–N production ($L = 0.0038$ and $Q = 0.0113$) compared to the PE0 diet (Table 6).

Enumeration of rumen protozoa

Total numbers of protozoa ($L < 0.0001$, $Q < 0.0001$), genus *Isotricha* ($L < 0.0001$, $Q < 0.0001$), and *Dasytricha* ($L < 0.0001$, $Q < 0.0001$), subfamily of *Entodiniinae* ($L = 0.0003$, $Q = 0.0083$), *Diplodiniinae* ($L < 0.0001$, $Q < 0.0001$), and *Ophrioscolecinae* ($L < 0.0001$, $Q < 0.0001$) decreased with increasing levels of PE in the diet.

Table 5. *In vitro* crude protein degradation of the experimental diets incubated with pomegranate peel extract either in ruminal fluid or ruminal fluid followed by HCl/pepsin, and the proteolytic bacterial count (log_{10}/g digesta).

	Diets[1]							*p*-Value	
	PE0	PE1	PE2	PE3	PE4	PE5	SEM[2]	L[3]	Q[4]
rdCP[5]	132.77	119.23	120.80	119.40	119.10	119.60	0.171	<0.0001	<0.0001
tdCP[6]	136.81	133.07	135.42	133.89	132.91	133.47	1.142	0.077	0.704
pdBCP[7]	6.79	13.83	14.61	14.49	13.81	13.87	1.33	0.001	0.002
dBCP[8]	0.25	0.34	0.37	0.36	0.34	0.34	0.029	0.054	0.029
Proteolytic bacteria	7.42	6.42	6.40	6.38	6.28	6.26	0.014	<0.0001	<.0001

[1]Diets: PE0, control, no additive = 3.1 g TP/kg DM of basal diet; PE1, control + 0.65 = 3.75 g TP/kg DM of basal diet; PE2, control + 1.30 = 4.4 g TP/kg DM of basal diet; PE3, control + 1.95 = 5.05 g TP/kg DM of basal diet; PE4, control + 2.60 = 5.70 g TP/kg DM of basal diet; PE5, control + 3.25 = 6.35 g TP/kg DM of basal diet.
[2]SEM: standard error of the mean; [3]L: linear; [4]Q: quadratic; [5]rdCP: Degradation of CP incubated with ruminal fluid (mg/g incubated CP); [6]tdCP: Degradation of CP incubated with ruminal fluid + HCl/pepsin (mg/g incubated CP); [7]pdBCP: Protein un-degraded in ruminal fluid (bypass CP) but degraded with HCl/pepsin (mg/g incubated CP); [8]dBCP: Degradability of bypass CP in HCl/pepsin (mg/g rumen escape CP).

Table 6. Effect of pomegranate peel extract level on the total VFA (mmol), individual VFA (mol/100 mol) and NH_3–N production (mg/dL).

	Diets[1]							*p*-Value	
Item	PE0	PE1	PE2	PE3	PE4	PE5	SEM[2]	L[3]	Q[4]
Total VFA[5]	74.00	73.50	74.40	70.43	59.46	59.00	4.249	0.029	0.068
Individual VFA									
Acetate	46.58	47.18	45.45	42.72	34.52	31.69	3.122	0.008	0.104
Propionate	15.51	14.66	16.68	15.62	14.14	14.50	1.105	0.580	0.264
Butyrate	7.01	6.99	7.99	6.75	7.23	7.07	0.499	0.902	0.522
Isobutyrate	2.54	3.53	2.96	4.08	2.62	4.27	0.426	0.604	0.067
Isovalerate	0.84	0.88	1.02	1.00	0.71	1.17	0.090	0.649	0.032
Valerate	0.25	0.25	0.27	0.26	0.23	0.29	0.012	0.409	0.061
Acetate:Propionate	3.12	3.22	2.72	2.66	2.27	2.20	0.132	0.0001	0.301
NH_3–N[6]	77.66	52.00	50.52	49.49	51.58	47.39	5.676	0.0038	0.0113

[1]Diets: PE0, control, no additive = 3.1 g TP/kg DM of basal diet; PE1, control + 0.65 = 3.75 g TP/kg DM of basal diet; PE2, control + 1.30 = 4.4 g TP/kg DM of basal diet; PE3, control + 1.95 = 5.05 g TP/kg DM of basal diet; PE4, control + 2.60 = 5.70 g TP/kg DM of basal diet; PE5, control + 3.25 = 6.35 g TP/kg DM of basal diet.
[2]SEM: standard error of the mean; [3]L: linear; [4]Q: quadratic; [5]VFA: Volatile fatty acids; [6]NH_3–N: ammonia-N.

Discussion

Dry matter and crude protein degradability

Dietary supplementation with PE decreased rdDM in PE4 and PE5 compared to PE0. Similarly, it was reported that purified condensed tannins extracted from *Calliandra*, *Flemingia*, and *Leucaena* decreased rumen degradability of dry matter in soybean meal.[11] Other work indicated that the addition of chestnut (*Castanea spp.*) tannins to soybean meal decreased *in vitro* dry matter degradability.[23]

The tdDM was not influenced by the addition of PE, as in a previous study that found a reduction in DM digestibility of soybean meal at pH 7.0 with the addition of tannic acid whereas abomasal digestibility was not affected.[23] Other studies have shown a decrease in *in vitro* DM degradation by rumen bacteria plus pepsin with the addition of tannins to soybean meal,[24] suggesting that some tannins remain bound and other *in vivo* studies have shown that feeding tannins to sheep increased fecal nitrogen excretion.[25]

The effect of PE was greater for CP than DM, because of the strong hydrogen bond affinity of the phenolic groups for the carbonyl oxygen of the peptide group.[26] All PE levels decreased the extent of CP degradation. Other studies[11,27] have shown that quebracho-treated soybean meal decreased CP degradation but grape pomace tannins had no impact on ruminal CP degradability.[8] Total degradability of CP did not differ between diets, suggesting that tannins did not reduce proteolysis, degradation of peptides or deamination of amino acids.[8] PE-related effects were more noticeable in rumen fluid than under conditions simulating abomasal degradation due the stronger interaction between PE and proteins at a neutral pH, as the most stable tannin–protein complexes are formed between pH 4.0 and 7.0.[26] Inclusion of PE increased pdBCP due to its interaction with protein thus affecting ruminal protein digestibility. Studies have shown that 300, 600 and 900 mg condensed tannins per g soybean meal protein increased bypass CP.[11] Other work reported that the addition of PE in *in vitro* conditions decreased ammonia, large and small peptides and amino acid levels.[28] Inhibition of amino acid deamination is a significant effect as it increases dietary protein bypass improving nitrogen efficiency.[29]

Table 7. Effects of PE level on protozoal concentration (log_{10}/g digesta).

	Diets[1]							*p*-Value	
Protozoa	PE0	PE1	PE2	PE3	PE4	PE5	SEM[2]	L[3]	Q[4]
Total	5.84	5.40	5.18	5.02	5.03	5.18	0.058	<0.0001	<0.0001
Isotricha	3.52	0.19	0.00	0.00	0.00	0.00	0.318	<0.0001	<0.0001
Dasytricha	5.00	1.67	0.18	0.18	0.00	0.37	0.370	<0.0001	<0.0001
Entodiniinae	5.63	5.37	5.17	5.01	5.03	5.13	0.057	<0.0001	0.0083
Diplodiniinae	2.22	0.19	0.00	0.00	0.00	0.37	0.259	<0.0001	<0.0001
Ophrioscolecinae	3.33	0.00	0.00	0.00	0.00	0.00	0.340	<0.0001	<0.0001

[1]Diets: PE0, control, no additive = 3.1 g TP/kg DM of basal diet; PE1, control + 0.65 = 3.75 g TP/kg DM of basal diet; PE2, control + 1.30 = 4.4 g TP/kg DM of basal diet; PE3, control + 1.95 = 5.05 g TP/kg DM of basal diet; PE4, control + 2.60 = 5.70 g TP/kg DM of basal diet; PE5, control + 3.25 = 6.35 g TP/kg DM of basal diet; [2]SEM: standard error of the mean; [3]L: linear; [4]Q: quadratic.

Inclusion of PE increased dBCP compared to the control diet. Other research showed that increasing levels of tannin extract increased protein bypass degradability[8] whereas another study[11] reported that increasing dietary levels of condensed tannin could inhibit postruminal CP degradation partly explaining the increase in fecal nitrogen. Another study[27] found that soybean meal treated with annins 10–250 g/kg of quebracho tannins, reduced intestinal crude protein digestibility of un-degraded protein but only at the highest level. In the present study, intestinal crude protein digestibility of un-degraded protein decreased at the highest PE level which was lower than other studies.[27] The apparent differences in intestinal digestibility of protein treated with PE may be associated with differences in its chemical structure and how this affects biological activity.[11] Differences in tannins and their ability to bind with proteins suggests that the post-ruminal reversibility of the process could also vary[11] (i.e., variability in the tannin's ability to decrease the ruminal CP degradation leading to differences in bypass CP reaching the duodenum).

Proteolytic bacteria numbers decreased with the addition of PE probably due to direct inhibition of rumen microbial function caused by interaction with the bacterial cell wall, or indirectly by decreasing protein availability (i.e., reducing ammonia (Table 6)). Other researchers[30] reported that tanniniferous feed lowered proteolytic bacterial numbers. Other work reported that *Sesbania sesban* tannins[31] had no effect on proteolytic, peptidolytic or deaminative activity. Other studies indicate that the addition of concentrated pomegranate-residue extract increased total rumen bacteria population.[32]

The effect of pomegranate peel extract on volatile fatty acids and ammonia-N production

VFAs are the final products of rumen microbial fermentation, and the main supply of energy for the ruminant.[15] The PE3 level of 5.05 g TP/kg DM had no effect on total VFA and acetate concentrations suggesting that additives up to this level do not affect diet fermentability or energy availability, but higher levels decreased these parameters due to a decrease in DM degradation (Table 4). The major fermentation end products of protozoa are acetate and propionate[15] so a reduction in acetate concentration may be due to a decrease in protozoa numbers (Table 7). Studies using other plant metabolites (50 and 100 g/kg DM of quebracho tannin or tannic acid) had no effect on total VFA in *in vitro* fermentations.[33]

Results vary, the addition of *Leucaena leucocephala* and *Salix babylonica* extracts (0.6, 1.2, 1.8 mL extract/g DM) increased VFA levels[3] but Sainfoin hay extract (4.1 g catechin/kg DM substrate) and PE (2.8 g tannic acid/kg DM substrate) decreased total VFA production.[26]

PE inclusion linearly decreased the acetate to propionate ratio and other studies using *P. kurroa* aqueous extract[34] and purified hydrolyzable (Chestnut and Sumach) and condensed tannins (Mimosa and Quebracho)[35] also showed a lower ratio. In contrast, the ratio increased with the addition of 50 g gallic acid and 100 g tannic acid per kg DM,[33] but Sainfoin hay extract (4.1 g catechin/kg DM substrate) and PE (2.8 g tannic acid/kg DM substrate) had no effect.[28] These inconsistencies can be attributed to an adaptation of the bacterial population and experimental conditions of studies, including diet type, animal and plant species, dose levels and the chemical structure of extract and the rumen fluid pH values.[26]

The lower acetate to propionate ratio observed in this study was similar to that found with methane inhibitors.[29] Methane production was not directly measured but changes in fermentation end products suggest a reduction in methane production.

A reduction in NH_3–N concentration with increased levels of PE suggest an inhibitory effect on proteolytic activity (Table 5).[35] Additionally, decreased

ruminal NH_3–N concentrations are associated with the inhibition of protozoa,[36] probably as a consequence of reduced bacterial lysis.[37] *Entodinium* spp. protozoa are responsible for most ruminal bacterial breakdown[38] and in this study PE addition lowered *Entodiniu*m numbers (Table 7) leading to a decrease in NH_3–N concentration. NH_3–N concentration is also affected by ammonia binding to saponin-like compounds.[21] Other research showed that 15, 30 g TP/Kg DM of PE decreased NH_3–N concentration.[12]

Enumeration of rumen protozoa

Total protozoa numbers, genera *Dasytricha* and *Isotricha* and subfamilies *Entodiniinae, Diplodiniinae*, and *Ophrioscolecinae* decreased with increasing PE levels in the diet. The antiprotozoal effect of PE is probably due to the phenolic structure of active metabolites (i.e., tannins and saponins) that interrupt protozoal membranes and inactivate enzymes depriving protozoa of substrates and metal ions that are vital for cell metabolism.[39] Data on the effects of PE on protozoa populations are not consistent. *Castanea sativa* wood extract containing hydrolyzable tannins (0.5 and 2.5 g tannin/kg DM) in rumen simulation experiments had no effect on total protozoa populations, *Holotrichs* or *Entodiniomorphs*.[40] Another study[12] showed a decrease in total protozoa but in other research[41] total protozoa numbers increased with the addition of 200 mg/g of *Enterolobium cyclocarpum* (crude saponins, 19 mg/g) or 200 mg/g of *Pithecellobium saman* (crude saponins, 17 mg/g). *Sanguisorba officinalis* tannin extract (3 g tannin/kg DM substrate) increased *Entodiniomorph* sp. but did not affect *Holotricha*.[42] These inconsistencies could be explained by experimental differences such as *in vitro vs. in vivo*, level and bioactivity of tannins used and genetic variation in ruminal protozoa (i.e., ruminal protozoal species differ in their sensitivity to tannins).

Conclusion

All levels of PE in the diet decreased ruminal degradation of protein without detrimentally affecting its intestinal digestion. Post-ruminal digestibility of ruminally un-degraded protein increased with the addition of PE with PE2 and PE3 levels conferring the best results. This study showed that water extraction of PE is as effective as the more expensive solvent extraction (Table 7). However, *in vivo* studies are needed to confirm the influence of PE on the efficiency of nitrogen utilization in ruminant animals.

Acknowledgments

We acknowledge the Iran National Science Foundation for the kind financial support for carrying out the trial, and gratefully acknowledge Mr. Gary Easton for his assistance in the English editing of the manuscript.

Disclosure statement

The authors wish to confirm that there are no known conflicts of interest associated with this publication and there has been no financial support for this work that could have influenced its outcome.

ORCID

Yousef Rouzbehan http://orcid.org/0000-0001-6818-4465
Abdelfattah Zeidan Mohamed Salem http://orcid.org/0000-0001-7418-4170

References

1. Dschaak CM, Williams CM, Holt MS, Eun JS, Young AJ, Min BR. Effects of supplementing condensed tannin extract on intake, digestion, ruminal fermentation, and milk production of lactating dairy cows. *J Dairy Sci*. 2011;943:2508–2519.
2. Durmic Z, Blache D. Bioactive plants and plant products: effects on animal function, health and welfare. *Anim Feed Sci Technol*. 2012;176(1–4):150–162.
3. Jiménez-Peralta FS, Salem AZM, Mejia-Hernández P, et al. Influence of individual and mixed extracts of two tree species on *in vitro* gas production kinetics of a high concentrate diet fed to growing lambs. *Livest Sci*. 2011;136(2–3):192–200.
4. Salem AZM, Olivares M, Lopez S, et al. Effect of natural extracts of *Salix babylonica* and *Leucaena leucocephala* on nutrient digestibility and growth performance of lambs. *Anim Feed Sci Technol*. 2011;170(1–2):27–34.
5. Mueller-Harvey I. Review, unraveling the conundrum of tannins in animal nutrition and health. *J Sci Food Agric*. 2006;86(13):2010–2037.
6. Vasta V, Nudda A, Cannas A, Priolo A. Alternative feed resources and their effects on the quality of meat and milk from small ruminants. *Anim Feed Sci Technol*. 2008;147(1–3):223–246.
7. Dentinho MTP, Moreira OC, Pereira MS, Bessa RJ. The use of a tannin crude extract from *Cistus ladanifer* L. to protect soya-bean meal protein from degradation in the rumen. *ANM*. 2007;1(05):645–650.
8. Alipour D, Rouzbehan Y. Effects of several levels of extracted tannin from grape pomace on intestinal digestibility of soybean meal. *Lives Sci*. 2010;128(1–3):87–91.
9. Rajabi M, Rouzbehan Y, Rezaei J. A strategy to improve nitrogen utilization, reduce environmental impact, and increase performance and antioxidant capacity of fattening lambs using pomegranate peel extract. *J Anim Sci*. 2017;95(1):499–510.
10. NRC. *Nutrient Requirements of Dairy Cattle*. 7th revised ed. Washington, DC: National Academy Press; 2001.

11. Cortés JE, Moreno B, Pabón ML, et al. Effects of purified condensed tannins extracted from *Calliandra*, *Flemingia* and *Leucaena* on ruminal and postruminal degradation of soybean meal as estimated *in vitro*. *Anim Feed Sci Technol*. 2009;151(3–4):194–204.
12. Abarghuei MJ, Rouzbehan Y, Salem A. The influence of pomegranate-peel extracts on *in vitro* gas production kinetics of rumen inoculum of sheep. *Turk J Vet Anim Sci*. 2014;38:212–219.
13. Cottyn BG, Boucque CV. Rapid method for the gas-chromatographic in rumen fluid. *J Agric Food Chem*. 1968;16(1):105–107.
14. Broderick GA, Kang JH. Automated simultaneous determination of ammonia and total amino acids in ruminal fluid and *in vitro* media. *J Dairy Sci*. 1980; 63(1):64–75.
15. Dehority BA. *Rumen Microbiology*. Nottingham, UK: Nottingham University Press; 2003.
16. Bryant MP. Commentary on the Hungate technique for culture of anaerobic bacteria. *Am J Clin Nutr*. 1972;25(12):1324–1328.
17. AOAC. *Association of Official Analytical Chemists. Official Methods of Analysis*. Vol. 1, 185th ed. Arlington, VA: AOAC; 1990.
18. Van Soest PJ, Robertson JB, Lewis BA. Methods for dietary fiber, neutral detergent fiber, and nonstarch poly-saccharides in relation to animal nutrition. *J Dairy Sci*. 1991;74(10):3583–3597.
19. Robertson JB, Van Soest PJ. The detergent system of analysis. In: James WPT, Theander O, eds. *The Analysis of Dietary Fibre in Food*. Vol. 158. New York, NY; Basel, Switzerland: Marcel Dekker; 1981: 123, Chapter 9.
20. Makkar HPS. Quantification of Tannins in Tree Foliage. A Laboratory Manual for the FAO/IAEA Co-ordinated Research Project on Use of Nuclear and Related techniques to Develop Simple Tannin Assays for Predicting and Improving the safety and Efficiency of Feeding Ruminants on Tanniniferous Tree Foliage. Joint FAO/IAEA, FAO/IAEA of Nuclear Techniques in Food and Agriculture. Animal Production and Health Sub-programme, FAO/IAEA Working Document. IAEA, Vienna, Austria; 2000.
21. Makkar HPS, Sen S, Blummel M, Becker K. Effects of fractions containing saponins from *Yucca schidigera*, *Quillajasaponaria* and *Acacia auriculoformis* on rumen fermentation. *J Agric Food Chem*. 1998;46(10): 4324–4328.
22. SAS. *User's Guide: Statistics*. Version 9 ed. Cary, NC: SAS Institute; 2002.
23. Driedger AR, Hatfield EE. Influence of tannins on the nutritional value of soybean meal for ruminants. *J Anim Sci*. 1972;34(3):465–468.
24. González S, Pabón ML, Carulla J. Effects of tannins on *in vitro* ammonia release and dry matter degradation of soybean meal. *Arch Latinoamericanos de Pro Anim*. 2002;10:97–101.
25. Barry TN, Manley TR, Duncan SJ. The role of condensed tannins in the nutrition value of *Lotus pedunculatus* for sheep. 4. Sites of carbohydrate and protein digestion as influenced by dietary reactive tannin concentration. *Br J Nutr*. 1986;55(1):123–137.
26. McSweeney CS, Palmer B, McNeill DM, Krause DO. Microbial interactions with tannins: nutritional consequences for ruminants. *Anim Feed Sci Technol*. 2001; 91(1–2):83–93.
27. Frutos P, Hervas G, Giraldez FJ, Fernandez M, Mantecon AR. Digestive utilization of quebracho-treated soya bean meal in sheep. *J Agric Sci*. 2000; 134(1):101–108.
28. Refat B, Anele U, He ZX, Bassiony SM ,Abdel-Rahman GA ,Yang WZ. Effect of sainfoin hay and pomegranate peel extracts on *in vitro* fermentation and protein degradation using the RUSITEC technique. *Can J Anim Sci*. 2015;95(3):417–423.
29. Van Nevel CJ, Demeyer DI. Manipulation of rumen fermentation. In: Hobson PN, ed. *The Rumen Microbial Ecosystem*. New York, NY: Elsevier Science Publishing; 1988:387–443.
30. Abarghuei MJ, Rouzbehan Y, Alipour D. Effect of oak (*Quercus libani* Oliv.) leaf tannin on ruminal fermentation of sheep. *J Agr Sci Technol*. 2011;13:021–1032.
31. Newbold CJ, El-Hassan SM, Wang J, Ortega ME, Wallace RJ. Influence of foliage from African multipurpose trees on activity of rumen protozoa and bacteria. *Br J Nutr*. 1997;78(2):237–249.
32. Jami E, Shabtay A, Nikbachat M, Yosef E, Miron J, Mizrahi I. Effects of adding a concentrated pomegranate-residue extract to the ration of lactating cows on *in vivo* digestibility and profile of rumen bacterial population. *J Dairy Sci*. 2012;95(10):5996–6005.
33. Getachew G, Pittroff W, Putnama DH, Dandekar A, Goyal S, Depeters EJ. The influence of addition of gallic acid, tannic acid, or quebracho tannins to *Alfalfa hay* on *in vitro* rumen fermentation and microbial protein synthesis. *Anim Feed Sci Technol*. 2008;140(3–4):444–461.
34. Alexander G, Singh B, Sahoo A, Bhat TK. *In vitro* screening of plant extracts to enhance the efficiency of utilization of energy and nitrogen in ruminant diets. *Anim Feed Sci Technol*. 2008;145(1–4):229–244.
35. Jayanegara A, Goel G, Makkar HPS, Becker K. Divergence between purified hydrolysable and condensed tannin effects on methane emission, rumen fermentation and microbial population in vitro. *Anim Feed Sci Technol*. 2015;209:60–68.
36. Williams AG, Coleman GS. *The Rumen Protozoa*. New York, NY: Springer-Verlag New York Inc; 1991.
37. Hristov AN, McAllister TA, Van Herk FH, Cheng KJ, Newbold CJ, Cheeke PR. Effect of *Yucca schidigera* on ruminal fermentation and nutrient digestion in heifers. *J Anim Sci*. 1999;77(9):2554–2563.
38. Belanche A, De La Fuente G, Moorby JM, Newbold CJ. Bacterial protein degradation by different rumen protozoal groups. *J Anim Sci*. 2012;90(12):4495–4504.
39. Calsamiglia S, Busquet M, Cardozo PW, Castillejos L ,Ferret A. Invited review: essential oils as modifiers of rumen microbial fermentation. *J Dairy Sci*. 2007;90(6): 2580–2595.

40. Sliwinski BJ, Soliva CR, Machmüller A, Kreuzer M. Efficacy of plant extracts rich in secondary constituents to modify rumen fermentation. *Anim Feed Sci Technol*. 2002;101:101–114.
41. Hess HD, Kreuzer M, Diaz TE, et al. Saponin rich tropical fruits affect fermentation and methanogenesis in faunated and defaunated rumen fluid. *Anim Feed Sci Technol*. 2003;109(1–4):79–94.
42. Cieslak A, Zmora P, Matkowski A, et al. Tannins from *Sanguisorba officinalis* affect in vitro rumen methane production and fermentation. *J Anim Plant Sci*. 2016;26:54–62.