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The influence of pomegranate-peel extracts on in vitro gas production kinetics of rumen inoculum of sheep

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Abstract: Different levels of pomegranate peel extracted by either water (PEH) or solvent mixture (PEM) were evaluated for their effect on in vitro gas production and ruminal fermentation patterns. Two levels of PEH or PEM (15 and 30 mg total phenolics (TP)/g DM of basal diet) were compared to a control (0) level. Ruminal fermentation parameters such as 24-h partitioning factor (PF_{24}), gas yield (GY_{24}), in vitro organic matter disappearance (IVOMD), metabolizable energy (ME), and microbial protein (MP) were calculated. The addition of PEH or PEM had no effect ($P > 0.05$) on asymptotic gas production, apparent degraded substrate (ADS), IVOMD, ME, PF_{24}, GY_{24}, MP, or total volatile fatty acids (VFAs). However, fermentation rate ($P = 0.032$) and the proportion of butyrate ($P < 0.0001$) decreased. The addition of either PEH or PEM with substrate resulted in lower acetate concentration ($P = 0.002$) and acetate to propionate ratio ($P = 0.007$) in comparison to the control. Inclusion of PEH or PEM led to a lower ammonia (NH_3-N) concentration ($P = 0.0002$) and protozoa counts in comparison to the control. In conclusion, the addition of PEH or PEM positively modified some rumen parameters such as acetate to propionate ratio, NH_3-N concentration, and protozoa population.

Key words: Sheep, pomegranate peel extract, gas production, ruminal parameters

1. Introduction

Many chemical feed additives such as antibiotics, ionophores, methane inhibitors, and defaunating agents have been used in ruminant feeding to improve rumen fermentation with the aim of improving the efficiency of milk and meat production in ruminants (1). However, the inclusion of some of these additives in animal rations has been limited due to the occurrence of multidrug resistant bacteria, which may be a risk to human health. Since 2006, the use of antibiotics as feed additives in animal feeds has been banned in the EU because of the possibility of residues in milk and meat (1). Increasing interest in organic farming and the contribution of ammonia and methane released by ruminants to climate change have compelled ruminant nutritionists and microbiologists to explore natural alternatives to these chemical feed additives for ecofriendly animal production. A group of natural products (plant secondary metabolites (PSMs)) such as saponins or phenols in the ruminant diet have shown some promise as a nutritional strategy in recent years (1–3). These bioactive compounds with rumen modifying capability may be of interest in ruminant production (4). Recently, numerous studies have attempted to exploit these PSMs to improve the efficiency of nitrogen and energy in the rumen (1). However, the usefulness of plant extracts having high content of saponins, flavonoids, and tannins varies depending upon the source, type, and level of PSMs.

Pomegranate peel (*Punica granatum*) is a by-product of the pomegranate juice industry and contains substantial amounts of polyphenols such as saponins, ellagic tannins, ellagic acid, and gallic acid (5). It is commonplace for PSMs to be extracted from pomegranate peel using organic solvents. However, this method is relatively expensive, which is encouraging the use of water extraction (6). Therefore, there is a need to investigate the effectiveness of a promising cheap technique at farmer level using water as the solvent. Hence, this study was carried out to assess the impact of 2 doses of pomegranate-peel extract (15 and 30 mg TP/g DM of basal diet), extracted either by water or a solvent mixture, on in vitro gas production and ruminal fermentation of sheep as ruminal inoculum donor.

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2. Materials and methods

2.1. Preparation of extract

Pomegranate peel was obtained from 2 main factories in Saveh city (Iran), which used similar pomegranate varieties and processing methods. The collected peels were sun-dried and water extraction was carried out using 1 g of dried peel/mL of distilled water, whereas solvent extraction used 1 g of dried peel/10 mL of solvent mixture. The solvent mixture consisted of methanol, ethanol, and distilled water in the ratio of 3:3:4.

2.2. Analytical methods and secondary metabolites analysis of pomegranate peel extract

Dry matter content was determined by oven drying at 60 °C for 48 h (7). Ash content was determined by incineration at 550 °C overnight, and the OM content was calculated as the difference between 100 and the percentage of ash (7). The ash-free neutral detergent fiber (NDFom) was determined, with sodium sulfite in ND, and ash-free acid detergent fiber (ADFom) was determined according to the literature (7) and expressed exclusive of residual ash. Lignin was determined by solubilization of cellulose with sulfuric acid. Nitrogen was determined by the Kjeldahl method (7).

Ten milliliters of each pomegranate extract was fractionated by funnel separation with a double volume of ethyl acetate to determine total phenolics (TPs) by drying and quantifying the TP layer in the funnel (8). After TP separation, a double volume of n-butanol was added to fractionate the saponins (8). The remaining solution was considered to be the aqueous fraction (AF) that has the other secondary compounds such lectins, polypeptides, and starch (9).

2.3. In vitro fermentation

Three adult rumen cannulated sheep were used as donors for rumen fluid. The animals were fed alfalfa hay, barley grain, soybean meal, pomegranate peel, and mineral/vitamin premix twice daily, at 0800 and 1700, with free access to a mineral block and water. To examine the effect of each extract (PEH or PEM) on fermentation parameters, 2 doses, either 15 or 30 mg TP/g DM of basal diet (Table 1), were used. The effects of extracts were examined in 3 runs of in vitro gas production. In vitro gas production kinetic was determined as described by Menke and Steingass (10). Rumen fluid of each sheep was collected 1 h before morning feeding, strained through 4 layers of cheesecloth, and kept under flushing CO_2 using a magnetic stirrer fitted on a hot plate at 39 °C. Approximately 500 mg of a standardized feedstuff, alfalfa hay (638 g/kg), barley grain (183.9 g/kg), soybean meal (139.1 g/kg), and mineral/vitamin premix (39 g/kg) ground to pass a 1-mm sieve, was accurately weighed in into 100 mL-glass gas syringes (11). There were 4 syringes prepared per treatment. Syringes were pre-warmed (39 °C) for 1 h before addition of 40 mL of rumen buffer mixture into each syringe, and incubated in a water bath maintained at 39 ± 0.1 °C as described by Menke and Steingass (10). Volume of gas produced was recorded at incubation times of 3, 6, 8, 12, 16, 24, 48, 72, 96, and 120 h.

After 24 h of incubation, for the second set of syringes the volume of gas production (GP_{24}) was recorded and the contents of syringes were transferred to centrifuge tubes and centrifuged at 20,000 × *g* for 20 min at 4 °C. Samples of supernatants (5 mL) were immediately preserved with 1 mL of HCl 0.2 N and stored at –20 °C prior to analysis of ammonia (12). For analysis of VFAs, 2 mL of supernatants was preserved, at –20 °C, with 0.5 mL of an acid solution containing 20% orthophosphoric acid and 20 mM 2-ethyl-butyric acid. Total VFAs were measured by gas liquid chromatography using ethyl-butyric acid as the internal

Table 1. Secondary compound levels of pomegranate-peel extract, ingredients, and chemical composition of diet.

Secondary compounds	PEH	PEM
	mg TP/g DM	
Total phenolics (TP)	170.5	200
Saponins	57.5	70
Aqueous fraction[a]	369.6	400
Diet (substrate) (g/kg DM)		
Alfalfa hay	638	
Soya bean meal	139.1	
Barley grain	183.9	
Mineral/vitamin premix[b]	39	
Chemical composition	PEH	PEM
Dry matter	902.7	902.7
Organic matter	926.3	926.3
Crude protein	190.9	190.9
ME	9.6	9.6
Ether extract	22.4	22.4
Neutral detergent fiber (om)	330.3	330.3
Acid detergent fiber (om)	240.5	240.5
Acid detergent lignin	54.0	54.0

ME is estimated (MJ/kg DM) (NRC, 2001). [b]Mineral/vitamin premix: 39 g/kg minerals and vitamins premix, which contained (per kg) 185 g Ca, 104 g Mg, 2.25 g Co, 44.0 g Mn, 36.4 g Zn, 1.3 g I, 10,000,000 iu retinol, 2,000,000 iu vit. D3, and 40,000 iu β-tocopherol); PEH: pomegranate-peel extract by water. PEM: pomegranate-peel extract by solvent mixture.

standard. Fermentation residues were oven-dried at 60 °C for 48 h to estimate potential DM disappearance. Loss in weight after drying was the measure of degradable DM.

2.4. Calculations

For a more precise estimation of gas production throughout the duration of in vitro fermentation, a nonlinear equation was used to analyze the kinetic data using SAS software (13).

$A = b \times (1 - e^{-\mu (t - L)})$,

where A is the volume of gas production at time t, b is the asymptotic gas production (mL/g DM), μ is the rate of gas production (/h), and L is the discrete lag time prior to gas production.

The DM degradability at 24 h of incubation (apparent degraded substrate, ADS; mg/g DM) was calculated as the difference between DM content of substrate before incubation and its undegradable DM after incubation (11).

ADS = DM content of substrate before incubation – undegradable DM after incubation

Metabolizable energy (ME, MJ/kg DM) and in vitro organic matter disappearance (IVOMD) were estimated according to Menke et al. (14) as:

OMD (g/kg OM) = 148.8 + 8.89 GAS + 4.5 CP + 0. 651 XA and

ME (MJ kg/DM) = 2.20 + 0.136 GAS + 0.057 CP,

where OMD is OM disappearance, ME is metabolizable energy, CP is crude protein in g/100 g DM, XA is ash in g/100 DM, and GAS is the net gas production (mL) for 200 mg of sample. Gas yields (GY_{24}) were calculated as the volume of gas produced after 24 h (mm gas/g DM) of incubation divided by the amount of ADS (g) as:

Gas yields (GY_{24}) = mL gas/g DM/g ADS

GP: net gas production at 24 h (mL/200 mg DM)

Microbial protein production (MP) was calculated (11) as:

MP (mg/g DM) = mg ADS − (mL gas × 2.2 mg/mL),

where the 2.2 mg/mL is a stoichiometric factor that expresses milligrams of C, H, and O required for the volatile fatty acids' gas associated with production of 1 mL of gas (15). The ratio of organic matter truly degraded (mg) to produced gas (mL) after 24 h of incubation was used to estimate the partitioning factor (11).

2.5. Protozoa population

Total numbers and generic composition of ciliate protozoa were determined according to the procedures described by Dehority (16). From each run, a sub-sample for protozoal counts was taken with 2 mL of syringe content pipetted into a screw-capped test tube containing 5 mL of formalinized physiological saline (containing 20 mL of formaldehyde in 100 mL of distilled water). Thereafter, 2 drops of brilliant green dye (2 g of brilliant green and 2 mL of glacial acetic acid diluted to 100 mL with distilled water) were added to the test tube; the contents were mixed thoroughly and allowed to stand overnight at room temperature. Total and differential counts of protozoa were made in 30 microscopic fields at a magnification of 20× in a hemocytometer (Neubauer-improved, Marienfeld, Germany).

2.6. Statistical analyses

Incubation was done in 3 separate in vitro runs with 4 replicate test feed samples. Data on in vitro ruminal fermentation parameters of each of the 3 runs within sample replicates were averaged and used as the mean value of each individual sample within diets (4 samples). Data of in vitro ruminal gas production, fermentation parameters, and protozoa were analyzed as a randomized complete design using the "GLM" option of SAS (17) to determine differences due to PEH or PEM extract and levels.

$Yij = \boldsymbol{\mu} + S_i + e_{ij}$,

where Yij is the general observation, $\boldsymbol{\mu}_{ij}$ the general mean, S_i the ith effect of extracts on the observed parameters, and e_{ij} the standard error term. Means were tested using Duncan's test at $P < 0.05$.

3. Results

Pomegranate peel secondary metabolites' (i.e. total phenolics, saponins, and aqueous fraction) concentrations are presented in Table 2. Pomegranate peel extract had no effect on gas production (Figure). Extracts had no effect on asymptotic gas production (Table 3), but fermentation rate was decreased ($P = 0.032$) with increasing dose of extract. The value of ADS (mg/g DM) was lower in PEH30 and PEM30 compared to the control ($P = 0.010$). The addition of PEH or PEM had no effect on IVOMD (g/kg), ME (MJ/kg DM), PF_{24} (mg ADS/mL gas), GY_{24} (mL gas/g ADS), or MP (mg/g DM). No differences in total VFAs were noted (Table 4). The addition of either PEH or PEM with substrate resulted in lower acetate ($P = 0.002$), butyrate concentration ($P < 0.0001$), acetate to propionate ratio ($P = 0.007$), and NH_3-N concentration ($P = 0.0002$) in

Table 2. Secondary metabolites content of diets.

	PEH			PEM		
	0	15	30	0	15	30
Total phenolics	-	15	30	-	15	30
Saponins	-	5	10	-	5.2	10.4
Aqueous fraction[a]	-	32.5	65	-	30	60

[a]Aqueous fraction (lectins, polypeptides, starch; (9). PEH: pomegranate-peel extract by water, PEM: pomegranate-peel extract by solvent mixture.

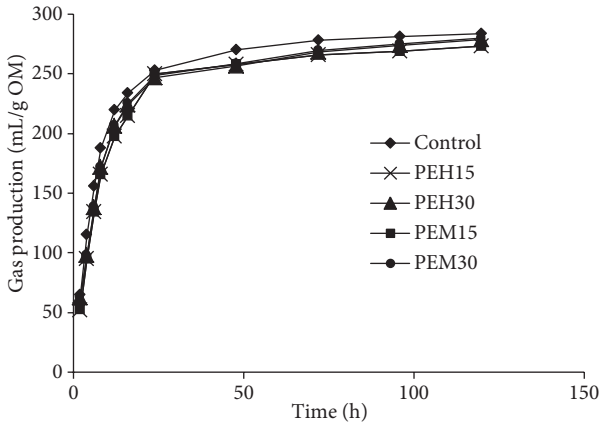

Figure. Cumulative gas production profiles (mL gas/g DM) from in vitro fermentation of the diet at different doses of pomegranate-peel extract. PEH: pomegranate-peel extract by water, PEM: pomegranate-peel extract by solvent mixture.

comparison to the control. The amount of total protozoa and subfamily *Entodiniinae* by using either PEH or PEM declined at 3, 12, and 24 h of incubation (Table 5). When either of the extracts was added, the populations of *Dasytricha* spp., *Isotricha* spp., and subfamilies *Diplodiniinae* and *Ophrioscolecinae* had completely disappeared at 24 h of incubation.

4. Discussion

There is limited information in the literature on the use of pomegranate extract extracted by water on ruminal fermentation parameters. Using either PEH or PEM had no effect on *b,* but μ decreased. Similar findings were obtained when tannic and gallic acids reduced the rate of fermentation in vitro (18). When either PEH or PEM are included at either 15 or 30 level, the decrease in μ value and no effect on *b* might suggest that rumen microbes are either capable of degrading tannins in extract or are able to tolerate the effects of tannins (18).

In this research, the ADS was lower in PEH30 and PEM30 compared to the control, but the IVOMD, ME, PF_{24}, GY_{24}, and MP were not influenced by either PEH or PEM supplement. In contrast, other research (19) noted that addition of different extracts of *Leucaena leucocephala* and *Salix babylonica* extracts, (0.6, 1.2, 1.8 mL extract/g DM) containing a low TP concentration (<5% of DM) and saponin increased gas volume GP_{24}, TDS, and MP versus the control. Another study (20) indicated that using extracts of *Moringa oleifera* aqueous methanol and *Picrorhiza kurroa* aqueous decreased apparent DM degradability and GP_{24} but had no effect on MP. It is likely that the variation between extract effects could be due to the chemical nature of their active compounds, their activities, and their concentration (2).

As VFAs are the end products of rumen microbial fermentation and represent the main supply of metabolizable energy for the ruminant (21), a reduction in their production would be nutritionally unfavorable for the animal. However, total VFA concentration was not affected compared with the control, which suggests that these additives did not modify diet fermentability and energy availability. In agreement with the present study, it has been reported that addition of tannic acid to alfalfa hay had no influence on total VFAs (18). In contrast, in another study (20) it was reported that total VFAs were increased by addition of *Plumbag zeylanica* and *Terminalia bellerica* aqueous extracts. The increase in propionate concentration with the addition of either PEH15 or PEM30 could be

Table 3. In vitro rumen fermentation of diets at different doses of pomegranate-peel extract.

	b	μ	GP_{24}	ADS	IVOMD	ME	PF_{24}	GY_{24}	MP
Control	273.6	0.136^{a}	253	802.6^{a}	67.2	9.4	2.7	157.6	55.4
PEH15	264.4	0.122^{b}	250	795.0^{ab}	66.7	9.3	2.6	157.2	56.0
PEH30	265.4	0.119^{b}	248.4	775.5^{bc}	66.4	9.2	2.6	158.0	48.6
PEM15	265.5	0.115^{b}	249.6	781.6^{ab}	66.6	9.3	2.6	157.5	49.7
PEM30	267.1	0.122^{b}	246.6	755.5^{c}	66.1	9.2	2.5	159.7	40.5
SEM	3.189	0.004	1.63	7.504	0.579	0.088	0.036	1.841	4.066
P value	0.317	0.032	0.726	0.010	0.726	0.727	0.232	0.184	0.121

b: asymptotic gas production (mL); μ: fermentation rate (/h); $GP_{24:}$ Gas production at 24 h (mL); ADS: Apparent degraded substrate (mg/g DM); IVOMD: in vitro organic matter disappearance (g/kg); ME: metabolizable energy (MJ/kg DM); PF_{24}: partitioning factor at 24 h of incubation (mg ADS/mL gas); GY_{24}: gas yield at 24 h (mL gas/g ADS); MP: microbial protein production (mg/g DM); PEH: pomegranate-peel extract by water, PEM: pomegranate-peel extract by solvent mixture.

Table 4. Effect pomegranate-peel extract on total and individual VFA production (mmol/g OMD) and NH_3-N (mg/100 mL rumen fluid) concentration and at 24 h.

Parameters	Control	PEH15	PEH30	PEM15	PEM30	SEM	P value
Total VFA	10.95	11.09	10.56	10.77	10.50	0.149	0.079
Individual VFA							
Acetate	5.04^{a}	4.88^{ab}	4.60^{cd}	4.78^{bc}	4.50^{d}	0.070	0.002
Propionate	2.28^{c}	2.73^{a}	2.52^{abc}	2.60^{ab}	2.40^{bc}	0.085	0.032
Butyrate	2.09^{a}	2.08^{a}	1.90^{c}	1.98^{b}	1.79^{d}	0.022	<0.0001
Isovalerate	0.98^{a}	0.99^{a}	1.01^{a}	0.88^{b}	0.90^{b}	0.011	<0.0001
Valerate	0.55^{a}	0.42^{b}	0.51^{a}	0.38^{b}	0.51^{a}	0.022	0.001
Acetate/Propionate	2.22^{a}	1.79^{b}	1.83^{b}	1.83^{b}	1.90^{b}	0.067	0.007
NH_3-N	43.54^{a}	36.90^{b}	26.80^{c}	35.90^{b}	25.89^{c}	1.752	0.0002

PE: pomegranate-peel extract; VFA: volatile fatty acid; NH_3-N: ammonia concentration at 24 h. PEH: pomegranate-peel extract by water, PEM: pomegranate-peel extract by solvent mixture.

due to the inhibitory effect of phenolic compounds and saponins on protozoa, which is in agreement with previous studies (22,23). A reduced protozoa number is sometimes associated with increasing propionate (24). In the present trial, butyrate was lower with the extract compared to the control. Lower protozoal counts (Table 5) are expected to be associated with a reduced net butyrate proportion (25). Other authors have observed similar decreases in the butyrate proportion using 50 and 100 g/kg tannic acid and quebracho tannin (18). In contrast, other researchers have detected increases in the butyrate proportion by *Zingiber officinale* aqueous and *Moringa oleifera* aqueous methanol extracts (2 mg/mL) (20). Acetate to propionate ratio was decreased by addition of either PEH or PEM compared to the control. This is consistent with a study (20) that showed that using *P. kurroa* aqueous extract increased propionate production and decreased acetate to propionate ratio. In contrast to our result, in another study (18) it was reported that acetate to propionate ratio was increased by addition of 50 and 100 g of gallic acid and tannic acid per kg DM compared to the control. Other researchers (20) stated that using *Plumbago zeylanica* aqueous and *Zingiber officinale* aqueous extracts (2 mg/mL) had no influence on acetate to propionate ratio. Differences in studies may be due to the fact that these effects may vary with diet, chemical composition, and the dose used (22).

In the current study, a reduction in NH_3-N concentration with increased level of either PEH or PEM suggests an inhibitory effect on proteolytic activity in the rumen (26). Moreover, reduced ammonia concentrations in the rumen are typical when protozoa are inhibited (Table 5), presumably as a result of a reduction in bacterial lysis (27). Another possible explanation for the reduction in NH_3-N concentration when either PEH or PEM was added may have been due to increased incorporation of NH_3-N, peptide, or amino acids into microbial protein production (6); however, no difference was observed in microbial protein production due to either PEH or PEM inclusion in the current study. In another study, a decrease in ammonia due to addition of *M. oleifera* aqueous methanol extract (0.75 and 1 mg/mL) has been reported (20). In contrast, in another study (22) it was reported that NH_3-N production was not altered by addition of 2.2 mg/L rumen fluid of cinnamaldehyde, eugenol, and d-carvone. A major effect of either PEH or PEM on ruminal fermentation was the reduction in total protozoa and especially the subfamily *Entodiniinae*. Another study (28) found strong antiprotozoal properties associated with the saponin component of an African multipurpose tree (*Sesbania sesban*) fed to sheep. The inclusion of either PEH or PEM has a detrimental effect on *Dasytricha* spp., *Isotricha* spp., and subfamilies *Diplodiniinae* and *Ophrioscolecinae* populations. In agreement with our result, it has been reported that tannins and secondary metabolites decreased protozoa numbers (29,30). However, no conclusive explanation could be found from comparing studies about the effect of tannins on protozoa population in the rumen, due to variation in the diet type, phenolic level, species, individual animal differences, and sampling methods (31). A decreased concentration of rumen protozoa could increase microbial protein production, benefiting the ruminant by increasing the amount amino acids available for absorption (27). In the present experiment, when microbial biomass production

Table 5. Effects of pomegranate-peel extract (mg TP/g DM) on ruminal protozoa concentration.

Time	Diet	Protozoa (log_{10}/g digesta)					
		Total	*Isotricha*	*Dasytricha*	*Entodiniinae*	*Diplodiniinae*	*Ophrioscolecinae*
3	Control	5.85[a]	0.00	2.22[a]	5.79[a]	1.62	0.55
	PEH15	5.25[c]	0.00	0.00[b]	5.22[b]	0.52	0.00
	PEH30	5.49[b]	0.52	2.68[a]	5.38[b]	2.61	0.52
	PEM15	5.21[c]	0.00	0.00[b]	5.20[b]	0.52	0.00
	PEM30	5.48[b]	0.52	2.68[a]	5.36[b]	2.61	0.52
SEM		0.056	0.330	0.664	0.060	0.716	0.440
P value		<0.0001	0.564	0.005	<0.0001	0.095	0.735
6	Control	5.38[c]	0.52	1.04	5.32[b]	0.00[b]	0.55
	PEH15	5.73[a]	0.00	2.61	5.66[a]	3.20[a]	1.04
	PEH30	5.52[b]	0.00	2.15	5.42[b]	2.66[a]	0.00
	PEM15	5.72[a]	0.00	2.61	5.63[a]	3.19[a]	1.04
	PEM30	5.50[bc]	0.00	2.15	5.39[b]	2.66[a]	0.00
SEM		0.046	0.233	0.812	0.054	0.736	0.502
P value		<0.0001	0.419	0.649	0.0001	0.020	0.379
12	Control	5.48[a]	0.00	1.04	5.45[a]	0.55	0.52
	PEH15	5.32[b]	0.00	0.52	5.28[b]	1.57	0.00
	PEH30	5.12[c]	0.00	1.04	5.04[c]	0.00	0.52
	PEM15	5.28[a]	0.00	0.52	5.23[b]	1.55	0.00
	PEM30	5.08[c]	0.00	1.04	5.00[c]	0.00	0.50
SEM		0.035	0.000	0.629	0.043	0.554	0.404
P value		<0.0001	0.000	0.933	<0.0001	0.108	0.736
24	Control	5.60[a]	3.13[a]	2.09[a]	5.60[a]	2.61[a]	1.57[a]
	PEH15	5.12[b]	0.00[b]	0.00[b]	5.08[b]	0.00[b]	0.00[b]
	PEH30	5.07[b]	0.00[b]	0.00[b]	5.07[b]	0.00[b]	0.00[b]
	PEM15	5.08[b]	0.00[b]	0.00[b]	5.04[b]	0.00[b]	0.00[b]
	PEM30	5.00[b]	0.00[b]	0.00[b]	5.00[b]	0.00[b]	0.00[b]
SEM		0.059	0.350	0.369	0.065	0.370	0.350
P value		<0.0001	<0.0001	0.0004	<0.0001	<0.0001	0.008

PEH: pomegranate-peel extract by water. PEM: pomegranate-peel extract by solvent mixture.

was calculated (11,15) there was no significant effect of either PEH or PEM on the production of microbial protein (Table 3).

In conclusion, inclusion of pomegranate peel extracts was shown to favorably manipulate rumen fermentation parameters, particularly increased propionate concentration and decreased acetate, acetate to propionate ratio, NH_3-N, and protozoa population. Water extraction was shown to be a simple, cheap, and alternative procedure to the more expensive organic solvent extraction. In vivo studies must be conducted to validate the in vitro results.

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