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The Effects of Phenolic Compounds in Iranian Propolis Extracts on *in vitro* Rumen Fermentation, Methane Production and Microbial Population

Research Article

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ABSTRACT

The objective of this study was to determine the chemical compounds of Iranian propolis (IP) extracts and to show flavonoids and phenol effects on methane production, fermentation characteristics and rumen microbial population (*in vitro*). In this study two diets with different concentrate: forage ratios as (HC: high concentrate) and (MC: middle concentrate), respectively as non-supplemented or supplemented with different Iranian propolis (IP) extracts were used. The treatments were HC (control), HC + IP 25%, HC + IP 50%, HC + IP 75%, MC (control), MC + IP 25%, MC + IP 50% and MC + IP 75%, which means 25, 50 and 75 g of propolis in 100 mL ethanol 70%, respectively. The results showed that IP significantly increased gas production in IP 75% with different concentrate: forage ratios. Adding IP caused a decrease in pH, however this decrease was not significant in all treatments. There was a significant difference between the effect of HC + IP 50% on NH_3-N compared to the HC + IP 25% and HC. Adding IP 75% significantly decreased CH_4 production compared to the other treatments. In HC diet, there was no significant difference in total populations of protozoa and *Ruminococcus albus* between the individual IP 25%, 50%, 75% treatments, however when they were considered as the group, a significant difference was observed between them and the control group. The highest decrease and the highest increase in total populations of *Prevotella bryantii* was observed in IP 75% and in IP25%, respectively. In MC diet, methanogens were significantly reduced in IP 25%, 50% and 75%, compared to control treatment. The propolis extract caused improvement in fermentation and decreased methane and nitrogen ammonia. This may help the nitrogen retain longer in ruminants.

KEY WORDS gas production, Iranian propolis extracts, methane production, microbial, rumen fermentation.

INTRODUCTION

Nutritional strategies to improve the production of ruminants have attracted the attention of nutritionists for several years. Making use of some additives such as antibiotics and probiotics in the diet leads to a remarkable reduction of methane production in ruminants (McGuffey *et al*. 2001).

The risk of residue transmission into milk and meat and the prohibition of utilizing antibiotics by the European Union in 2006 made the researchers exploit natural products to manipulate rumen fermentation (Nisbet *et al*. 2009). The plants have secondary compounds that affect ruminant's production positively (Wallace, 2004). These compounds, including phenolic, change the fermentation conditions

(pH, propionate proportion, and protein degradation) and therefore affect rumen microbial metabolism (Balcells *et al*. 2012). Propolis is a bee product of plant origin. Bee workers, over three weeks of age collect the resinous material from leaves, buds and other plant parts and mixed it with wax and beta-glucosidase enzyme secreted by them (Castaldo and Capasso, 2002; El-Bassuony, 2009; Zia *et al*. 2009). Bees use propolis to disinfect combs, to fill up the narrow openings of the hive, to strengthen the border of the combs, and embalm dead intruders to protect the hive from diseases (Marcucci, 1995; Bankova *et al*. 2000). The chemical composition of propolis is not thoroughly known as it differs according to locality. It mainly depends on the characteristic feature of the flora (Bankova *et al*. 1992). Meanwhile, raw propolis can hardly be used (Sforcin and Bankova, 2011), it must be extracted with solvents to remove the unwanted substances and to keep phenolic compounds (Cottica *et al*. 2011).

The propolis extract has numerous pharmacological properties. It has antibacterial (Velikova *et al*. 2000), antifungal (Murad *et al*. 2002), antiviral (Amoros *et al*. 1994), local-anaesthetic (Paint and Metzner, 1979), anti-inflammatory (Miyataka *et al*. 1997), antioxidant (Isla *et al*. 2001), and immunostimulating (Dimov *et al*. 1991) beneficial activities. The existence of phenolic compounds in propolis extract is known to cause the improvement of rumen fermentation, reduction of NH_3-N (Ozturk *et al*. 2010) and methane (Oskoueian *et al*. 2013). Up to now, few investigations have been made of the effect of propolis extract on rumen microbial population. The objective of this study was to determine the chemical compounds of Iranian propolis (IP) extracts and to examine phenolic effects on methane production, fermentation characteristics and *in vitro* rumen microbial population.

MATERIALS AND METHODS

Origin of propolis

The Iranian propolis (IP) was collected from northeast Iran (37˚ 37' 31.07" N,58˚ 43' 49.74" E), a mountainous region with relatively cold weather (3 ˚C and 27% humidity) in Khorasan Razavi, from Ehtesham Apiary in October 2014 from 5 hives. For this study approximately1700 g of propolis was collected. The collected samples were weighed and preserved in the refrigerator at 4 ˚C for extraction.

Preparation of propolis extracts

According to previous studies (Aguiar *et al*. 2014; Mirzoeva *et al*. 1997) three extracts of IP [25%, 50% and 75%, which means 25, 50 and 75 g of propolis in 100 mL ethanol 70%, were provided. Propolis extraction was performed according to Sforcin *et al*. (2000) with some modification. The IP was cut into small pieces (about 4-5 mm) and extracted with ethanol 70% in a shaker (GFL model 3005, Germany) with 300 rpm at room temperature for 72 h. The ethanol extract solution was then filtered through a Whatman No.41 filter paper. The ethanol was removed in a rotary evaporator (Heidolph laborota 4000, Germany) at 42 ˚C for 30 min.

Phenolic compounds measurement

Total phenolic compounds of Iranian propolis were measured by Swain and Hillis (1959); (Table 1).

Table 1 Total Phenolic compounds of Iranian propolis

Items	Total phenolic compounds
Control	0.00[a]
IP 25% (v/w)	2.23[b]
IP 50% (v/w)	6.34[c]
IP 75% (v/w)	7.79[d]
SEM	0.007
P-value	< 0.001

IP: Iranian propolis.
The means within the same row with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the means.

Experimental diets and treatments

Two diets with different concentrate: forage ratios as 80:20 (HC: high concentrate) and 60:40 (MC: middle concentrate as non-supplemented or supplemented), respectively, with different IP extracts were used. The treatments were HC (control), HC + IP 25%, HC + IP 50%, HC + IP 75%, MC (control), MC + IP 25%, MC + IP 50% and MC + IP 75%. Ingredients and chemical composition of the experimental diets are shown in Table 2.

Chemical analysis

Dry matter content of feed samples was determined by drying the oven-dried samples at 65 ˚C to a constant weight (AOAC, 2005). Ether extract (EE) (AOAC, 2005), and ash content were determined after 3 h of incineration at 550 ˚C in a muffle furnace (AOAC, 2005). Crude protein (CP) (Kjeldahl N × 6.25) was measured by the block digestion method using copper catalyst and steam distillation into boric acid solution (AOAC, 2005) on a 2100 Kjeltec distillation unit. Neutral detergent fiber (NDF) and acid detergent fiber (ADF) were analyzed by the fibertec system (1010 Heat Extractor, Tecator, Sweden) according to Van Soest *et al*. (1991) and were corrected for ash. Sodium sulfite and heat-stable α-amylase (Sigma A3306; Sigma-Aldrich, Steinheim, Germany) were used during NDF analysis.

In vitro gas production

Rumen fluid was collected from two ruminally fistulated dairy cows (580±4.5 kg, body weight) prior to offering the morning feed.

Table 2 Ingredients and chemical composition of the experimental diets

Ingredients (% DM)	MC diet	HC diet
Alfalfa	5	5
Corn silage	15	15
Wheat straw	20	0
Barley	20.4	27.2
Corn	18	24
Soybean meal	4.8	6.4
Sugar pulp meal	3	4
Cotton seed	7.2	9.6
Wheat bran	6.3	8.4
Calcium carbonate	0.18	0.24
Salt	0.12	0.16
Chemical composition (%)		
Dry matter	82	88
Crude protein	13.12	15.28
Ether extract	13.32	14.80
Neutral detergent fiber	48.30	35.10
Acid detergent fiber	22.80	16.42
Organic matter	93.04	92.24
Ash	6.96	7.76

HC: high concentrate; MC: middle concentrate and DM: dry matter.

Animals were fed 10.4 kg DM, a diet containing 50% alfalfa hay, 20% wheat straw, 15% barley grain, 14% soybean meal, and 1% mineral-vitamin premix (Ca: 195000 mg/kg; P: 90000 mg/kg; Na: 55000 mg/kg; Mg: 20000 mg/kg; vitamin A: 500000 IU; vitamin D_3: 100000 IU and vitamin E: 100 IU).

Effect of IP on gas production was assessed by incubating approximately 200 mg experimental sample (2 mg IP 25%, 50% and 75%) with 30 mL of rumen buffer mixture in 125 mL glass syringes based on Menke and Steingass (1988) procedure.

For this procedure, ruminal content was immediately blended and strained through four layers of cheesecloth to eliminate large feed particles, and transferred to the laboratory in a pre-warmed thermos. A sample of 200 mg was weighed into 125-mL serum bottles, in 3 runs and 12 replicates. The filtrate was then mixed with carbonate buffer (containing ammonium bicarbonate at 4 g/L), then sodium bicarbonate (35 g/L in N-rich incubation medium and sodium bicarbonate at 39.25 g/L in N-low medium), macro-mineral solution (5.7 g anhydrous Na_2HPO_4, 6.2 g anhydrous KH_2PO_4 and 0.6 g $MgSO_4 \cdot 7H_2O$ per liter), and deionized water in a ratio of 1:1:0.5:1.5 and 0.1 mL micro-mineral solution (13.2 g $CaCl_2 \cdot 2H_2O$, 10.0 g $MnCl_2 \cdot 4H_2O$, 1 g $CoCl_2 \cdot 6H_2O$ and 8.0 g $FeCl_3 \cdot 6H_2O$ per 100 mL) were included. The medium was reduced by the addition of 41.7 mL reducing agent (40 mL deionized water, 1 mL 1N NaOH and 1 g$Na_2S \cdot 9H_2O$) per liter. Twenty milliliters of medium was dispensed into a 125-mL glass serum bottle, the top of each was stopped with rubber and aluminum caps and placed in a 39 degree centigrade water bath for 96 h.

Blank samples, without substrate, were placed throughout the water bath and used to measure gas production from the medium alone. Rumen liquor was handled under a constant stream of CO_2, all containers used were pre-warmed at 39 degree centigrade and filled with CO_2. Gas production (mL) was recorded at 2, 4, 6, 8, 12, 16, 24, 36, 48, 72 and 96 h. Total gas values were corrected for blank with a known gas production. After subtraction of gas production from blank bottles, data were fitted to the exponential model of Ørskov and McDonald (1979).

For methane measurement, at the 24 h of the incubation period using the device of multiple gas detector (SR2-BIO System, Sewerin, Germany). Methane production was determined in four bottles of each treatment in each run.

In vitro rumen fermentation characteristics

Ammonia nitrogen (NH_3-N) concentration was measured in the supernatant using phenol-hypochlorite reaction (Weatherburn, 1967). The pH was determined with pH meter in 24th h incubation.

Determination of ruminal flora

After 24 h incubation, for each treatment 4 ruminal fluid samples were transferred to 1.5 mL microtubes containing incubation on ice between shakings for disruption of microbial cell wall and detached microbes from feed particles. Microtubes were centrifuged at 2000 × g for 5 min at 4 ˚C for the sedimentation of feed particles, and supernatants (200 µL) were transferred to fresh 1.5 mL microtubes. Extraction of DNA was performed using a genomic DNA extraction kit (AccuPrepTM, Bioneer Corporation, Daejeon,

South Korea) equipped with spin columns according to the manufacturer's instructions.

The relative abundance of protozoa, methanogens, *R. albus*, *Prevotella bryantii*, fibrolytic and amylolytic was measured by real-time PCR and the Maxima® SYBR Green/ROX qPCR Master Mix (2X) (K0221, Fermentas) according to Valizadeh *et al*. (2010). Species-specific PCR primers used in the present study to amplify partial 16S rDNA regions are shown in Table 6. The reaction was assayed in a 25 µL reaction mixture containing 12.5 µL of SYBR Green PCR Master Mix Kit, 0.5 µL of primer mixture containing 10 pmol of each primer, 1 µL of DNA template and 11 µL of deionized water. SYBR Green qPCR Master Mix contained Taq DNA polymerase, reaction buffer (KCl and $(NH_4)2SO_4$), dNTPs, $MgCl_2$ and SYBR Green. 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 obtain optimal relative expression results in real-time PCR. Amplification and detection were performed using an ABI 7300 Sequence Detection System (Applied Biosystems) 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 60 ˚C for 30 s, and extension at 72 ˚C for 30 s. Amplicon specificity was performed via dissociation curve analysis of PCR end products by enhancing the temperature from 65 to 95 ˚C at a rate of 1 ˚C every 30 s. The relative abundances of protozoa, methanogens, *R. albus*, *Prevotella bryantii*, fibrolytic and amylolytic were determined using total bacteria as reference according to the $2^{-\Delta\Delta Ct}$ method.

The fold change in protozoa and specific bacteria species DNA in treatments with different concentrations of IP compared with the control treatment was calculated by normalizing protozoa and specific bacteria species DNA to total bacterial DNA in the experimental groups and relating that ratio to that of the control. Change in protozoa and certain bacteria species are reported as fold change in genomic DNA per l µL of extracted DNA compared with control.

Statistical analysis

The trial was analyzed considering a completely randomized design by the general linear model (GLM) procedure of SAS 9.1 (SAS, 2001). Means among treatment were compared by Tukey test on following model:

$$y_{ij} = \mu + Ei + Bj + e_{ij}$$

Where:
y: depended variable.
µ: overall mean.
Ei: effect of IP extracts.
Bj: effect of diet.
eij: residual error.

The values of a (the gas production from the immediately soluble fraction), b (the gas production from the insoluble fraction), a + b (potential extent of gas production), and c (gas production rate constant for the insoluble fraction b) were estimated using the nonlinear regression (NLIN) procedure of SAS.

RESULTS AND DISCUSSION

The total phenolic compounds of propolis are shown in Table 1. As the results indicate, there were significant differences (P>0.05) between control and IP 25%, IP 25% and IP 50%, IP 50% and IP 75%.

The results of the different concentrations of IP on rumen fermentation characteristics and *in vitro* gas production are shown in Tables 3 to 5 and Figure 1 and 2, respectively.

The results of this study showed that IP led to a significant (P<0.05) increase in gas production in IP 75% with different concentrate: forage ratios however no significant (P>0.05) difference was seen in other treatments. Adding different concentrations of IP caused a decrease in pH, however this decrease was not significant (P>0.05) in all treatments.

In 60:40 (MC) diet, adding IP 25% and 50% did not statistically change NH_3-N (P>0.05) compared to the control treatment but there was a significant difference (P<0.05) between the effect of IP 75% on NH_3-N compared to the others.

In 80:20 (HC) diet, there was neither significant difference between the effect of IP 25% on $_{NH3}$-N and the control, nor was there a significant difference between the effect of IP 50% and 75%, while a significant difference (P<0.05) between IP 50% and 75% and the rest was observed.

In 60:40 (MC) ration adding IP 25% reduced (P<0.05) CH_4 production in comparison with the control and also IP 50% significantly reduced (P<0.05) it. Furthermore, IP 75% statistically decreased (P<0.05) CH_4 production compared to the other treatments. It should be noted that the highest decrease was observed in IP 75%.

In 80:20 (HC) diet, adding IP 25% did not significantly (P>0.05) affect CH_4 production compared to the control treatment. For the other treatments, the same results as 60:40 (MC) ratio was obtained.

Rumen microbial population

The results of rumen microbial population are shown in Tables 6, 7 and 8. In HC diet, there was a significant difference in the population of *R. albus* between IP 25% treatment and the other treatments.

Table 3 Cumulative gas production (96h) in experimental diets

Treatments	Cumulative gas production (96 h)					
	MC diet			HC diet		
	Volume	SEM	P-value	Volume	SEM	P-value
Control	77.30[b]	3.02	< 0.0001	47.13[b]	3.38	< 0.0001
IP 25%	71.74[b]	-	-	51.48[b]	-	-
IP 50%	79.63[b]	-	-	55.01[b]	-	-
IP 75%	89.67[a]	-	-	67.60[a]	-	-

HC: high concentrate; MC: middle concentrate and IP: Iranian propolis.
The means within the same row with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the means.

Table 4 Gas production parameters at 96 h incubation and half time of gas production (t1/2) of diets containing different IP extracts

Gas production parameters	Gas production parameters at 96 h incubation and half time of gas production (t1/2) of diets					
	HC diet			MC diet		
	b (mL/250 mg DM)	c (h^{-1})	t (1/2)	b (mL/250 mg DM)	c (h^{-1})	t (1/2)
Control	76.13	0.08	8.66	68.09	0.08	8.66
IP 25%	69.91	0.08	8.66	74.29	0.08	8.66
IP 50%	78.24	0.08	8.66	78.81	0.08	8.66
IP 75%	89.20	0.10	6.93	90.08	0.11	6.3

HC: high concentrate; MC: middle concentrate; IP: Iranian propolis and DM: dry matter.
b: insoluble but fermentable; c: rate constant and t (1/2): time.

Table 5 Effect of different concentration of IP on rumen *in vitro* characteristics with different diets

Items	Treatments					
	MC diet			HC diet		
	NH_3-N (mmol/L)	pH	CH_4 (mg)	NH_3-N (mmol/L)	pH	CH_4 (mg)
Control	19.59[a]±0.44	6.80±0.06	21.26[a]±0.57	20.78[a]±0.32	6.60±0.07	19.74[a]±0.66
IP 25%	19.17[a]±0.44	6.70±0.06	20.89[ab]±0.57	20.68[a]±0.32	6.58±0.07	16.78[a]±0.66
IP 50%	18.58[a]±0.44	6.60±0.06	18.65[b]±0.57	19.11[b]±0.32	6.51±0.07	17.67[ab]±0.66
IP 75%	17.22[b]±0.44	6.53±0.06	14.99[c]±0.57	18.73[b]±0.32	6.52±0.07	14.77[b]±0.66
SEM	1.75	0.01	0.99	0.94	0.02	1.31
P-value	< 0.0001	0.67	< 0.0001	< 0.0001	0.10	< 0.0001

HC: high concentrate; MC: middle concentrate and IP: Iranian propolis.
NH_3-N: nitrogen ammonia and CH_4: methane gas.
The means within the same row with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the means.

In addition, there was no significant difference in total populations of protozoa between the individual IP 25%, 50%, 75% treatments, however when they were considered as a group, a significant difference was observed between them and the control group. There was no significant difference in total populations of *Prevotella bryantii* between the individual control, IP50%, 75% treatments, but when they were considered as a group, a significant difference was observed between them and the IP 25% group. The highest decrease and the highest increase in total populations of *Prevotella bryantii* was seen in IP 75%, and in IP25%, respectively. There was no significant difference in total populations of methanogens, fibrolytic and amylolytic between IP 75% and 50%, however a significant difference between both of them and IP 25% and control was seen. It should be noted that there was a significant difference between IP 25% and control. In MC diet, there was a significant (P<0.05) decrease in the population of protozoa in IP 75% compared to the others.

The population of *R. albus* was not significantly affected in IP 50% compared to control, but a significant decrease was observed in IP 25% compared to control. It should be noted that the highest decrease in the population of *R. albus* was observed in IP 75%. The population of *Prevotella bryantii* was statistically reduced in IP 25%, 50%, and 75% compared to the control. Fibrolytic microorganisms significantly decreased in IP 25%, 50% and 75% compared to control.

Protozoa and amylolytic populations were not statistically affected in IP 25%, 50% compared to control, however they were statistically affected in IP 75% compared to control. Methanogens were significantly (P<0.05) reduced in IP 25%, 50%, and 75% compared to control treatment. The results of the recent experiment indicated that the adding of IP 75% led to the increase of gas production (Figures 1 and 2) which can probably the cause of propionate decrease and butyrate increase (Ozturk *et al*. 2010; Oskoueian *et al*. 2013).

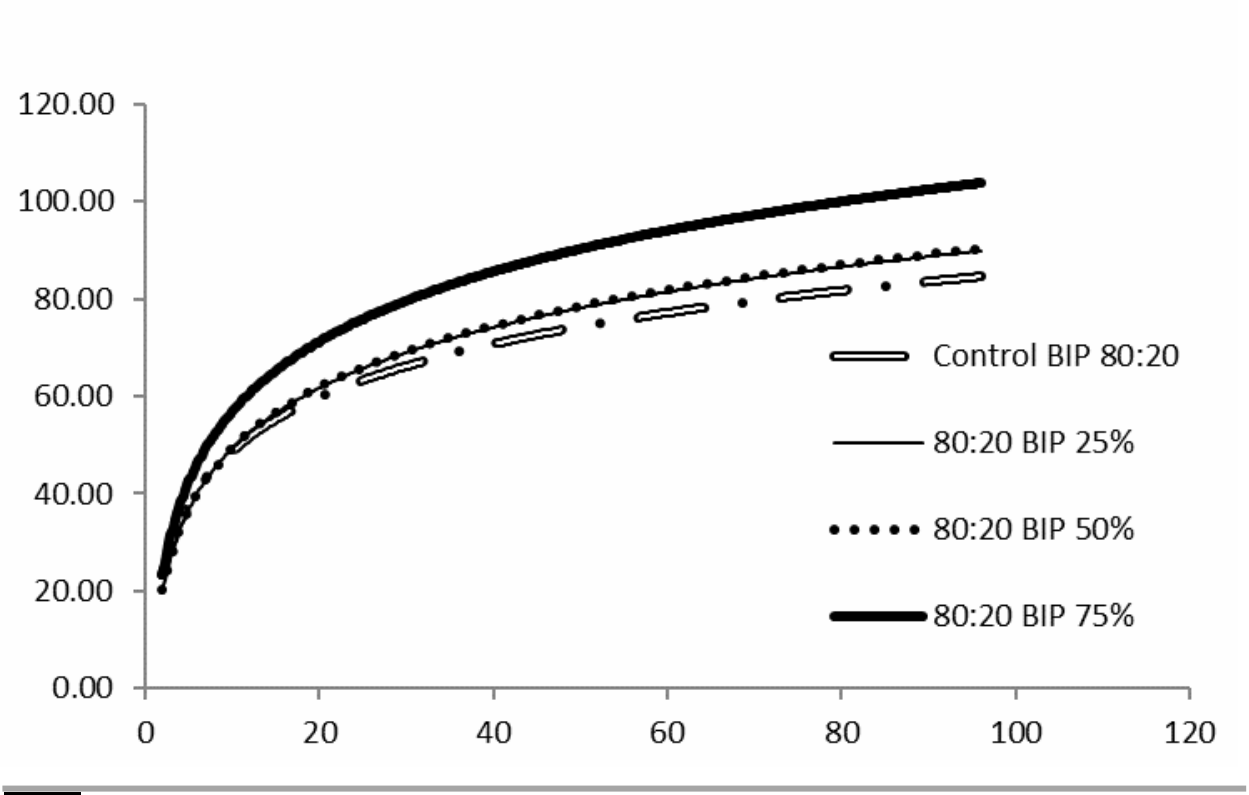

Figure 1 Cumulative gas production (96 h) in high concentrate diet
BIP: Iranian propolis

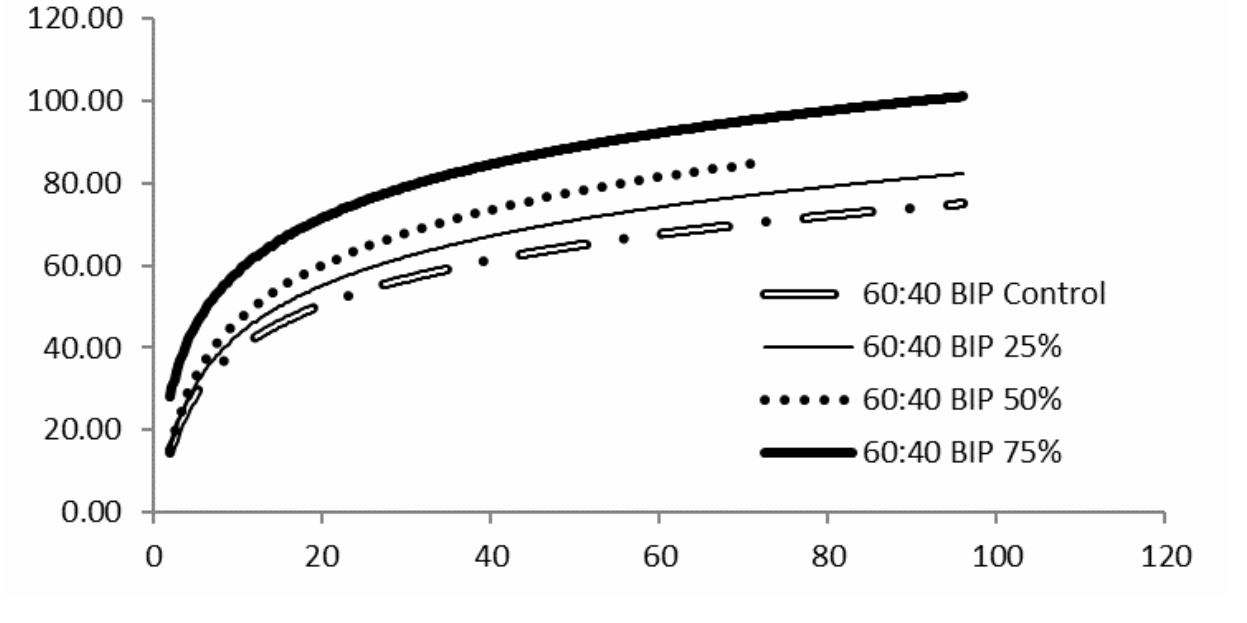

Figure 2 Cumulative gas production (96 h) in middle concentrate diet
BIP: Iranian propolis

Additionally the increase of gas production and propionate decrease and butyrate increase means that propolis can improve the rumen fermentation. Finally, it can be concluded that the increase in gas production is a sign of better fermentation. The present study signified that adding various levels of IP in rumen fluid caused the NH_3-N concentration to be statistically reduced ($P<0.05$) compared with control treatment.

Table 6 PCR primer sets for real-time PCR assay

Target species	Forward/reverse	Primer sequence	References
Total bacteria	F	GTGSTGCAYGGYTGTCGTCA	Maeda *et al.* (2003)
	R	ACGTCRTCCMCACCTTCCTC	
R. albus	F	CCCTAAAAGCAGTCTTAGTTCG	Koike and Kobayashi (2001)
	R	CCTCCTTGCGGTTAGAACA	
Prevotella bryantii	F	ACCTTACGGTGGCAGTGTCTC	
	R	ACTGCAGCGCGAACTGTCAGA	
Fibrolytic	F	GTTCGGAATTACTGGGCGTAAA	Denman and McSweeney (2006)
	R	CGCCTGCCCCTGAACTATC	
Amylolytic	F	ATGCAAGTCGAACGGTAACAGCAG	
	R	GCACCCGTTTCCAGGTGTTGTCC	
Methanogens	F	TTCGGTGGATCDCARAGRGC	Denman *et al.* (2007)
	R	GBARGTCGWAWCCGTAGAATCC	
Protozoa	F	GCTTTCGWTGGTAGTGTATT	Denman *et al.* (2007)
	R	CTTGCCCTCYAATCGTWCT	

Table 7 Effects of different concentration of Iranian propolis (IP) on ruminal microorganism population in high concentrate (HC) diet*

Items	Control	IP 25%	IP 50%	IP 75%	SEM	P-value
R. albus	1^{a}	4196.92^{b}	-2.96^{a}	0.53^{a}	633.55	0.0100
Prevotella bryantii	1^{b}	941.90^{a}	-31.80^{b}	-41.48^{b}	46.02	< 0.0001
Fibrolytic	1^{ab}	124.19^{a}	-7.25^{b}	-116.97^{b}	33.69	0.0100
Amylolytic	1^{ab}	569.81^{a}	-1.38^{b}	-3.67^{b}	144.80	0.1100
Methanogens	1^{ab}	2118.62^{a}	-4.66^{b}	-1.59^{b}	513.75	0.0900
Protozoa	1^{a}	218.35^{b}	-11.79^{b}	-11.31^{b}	24.07	0.0015

* Fold change compared to control.
The means within the same row with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the means.

Table 8 Effects of different concentration of Iranian propolis (IP) on ruminal microorganism population in middle concentrate (MC) diet*

Items	Control	IP 25%	IP 50%	IP 75%	SEM	P-value
R. albus	1^{a}	-3.75^{b}	-1.26^{a}	-6.61^{c}	0.45	< 0.0001
Prevotella bryantii	1^{b}	-9.38^{ab}	-6.69^{a}	-15.14^{c}	2.33	0.04
Fibrolytic	1^{a}	-74.12^{b}	-2612.68^{c}	-1523.20^{d}	605.03	0.0600
Amylolytic	1^{ab}	-1.92^{ab}	-10.58^{ab}	-50.79^{b}	12.85	0.0800
Methanogens	1^{a}	-3.57^{b}	-16.04^{c}	-7.12^{d}	2.13	0.0075
Protozoa	1^{a}	-5.76^{a}	-6.79^{a}	-47.04^{b}	6.82	0.0051

* Fold change compared to control.
The means within the same row with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the means.

The outcome of the experiment coincided with published data (Ozturk *et al.* 2010; Oliveira *et al.* 2004; Oliveira *et al.* 2006). The reason for the reduction of NH_3-N concentration by addition of IP can be related to the decrease of deamination of amino acids and/or reduced growth rate of amino acid- fermenting bacteria. The research concluded that the IP in two experimental ratios led to the significant decrease of CH_4 compared with that in control group. The higher decrease of Gram-positive bacteria in proportion to gram-negative bacteria (Mirzoeva *et al.* 1997; Padmavati *et al.* 1997) can be the possible cause of adding IP to the ratios. Furthermore, there is a possibility that the antiprotozoal effect of IP (Rispoli *et al.* 2009; Kreuzer *et al.* 1986; Santos *et al.* 2016) causes the decrease of protozoa population and subsequently hinders the production of CH_4 by changing the reducing equivalents of CH_4 to propionate synthesis in the rumen. The above mentioned conclusions verified the findings of by Patra *et al.* (2006) and Tavendale *et al.* (2005). Finally, it should be noted that decreasing the methane production will be beneficial to the environment and will also decrease wasting of digestion energy in ruminants (Makkar and Harinder, 2007). Up to now few studies have been made on the effect of propolis on the population of rumen micro-organisms and what has been conducted is generally obtained in pure culture. The goal of this study was to find the effect of IP on the rumen characteristics with various ratios and also the reciprocal effect of rumen micro-organisms in the presence of propolis. The result of this study indicated that in HC diet the population of *Prevotella bryantii* in IP 75% did not statistically change. No similarity was observed between the results of this study on *R. albus* with the findings of Aguiar *et al.* (2013).

A reason for this dissimilarity can probably be the reciprocal effect of other bacteria on micro-organisms or the feed used in the experiment. There is remarkable similarity between the concluded result of MC diet and that obtained by Ware *et al*. (1989) and Krause *et al*. (1999). Regarding the difference existing between genotypes and physiological properties of *R. albus* strains the reason for the similarity can be found either in their cellulatic system, fiber digestion capabilities or in their ability to produce H_2. The study concluded that the addition of IP to the rumen fluid makes the population of methanogens and protozoa in both experimental diets decrease.

This is quite similar to what attained by Oskoueian *et al*. (2013). A reason for the decrease can possibly be the presence of phenolic compounds (Bodas *et al*. 2012) in propolis extract and their antiprotozoal effect (Rispoli *et al*. 2009; Kreuzer *et al*. 1986). Propolis has bacteriostatic activity against Gram-positive and some Gram-negative bacteria (Mirzoeva *et al*. 1997). The function of propolis depends on the change in bio energetic condition of the bacterial membrane that hinders bacterial motility. Future research can be done on the effects of propolis on the *in vivo* rumen microbiota.

CONCLUSION

The propolis extract caused improved fermentation and decreased methane and nitrogen ammonia. This may help the nitrogen retain longer in ruminants.

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REFERENCES

Aguiar S.C., Cottica S.M., Boeing J.S., Samensari R.B., Santos G.T., Visentainer J.V. and Zeoula L.M. (2014). Effect of feeding phenolic compounds from propolis extracts to dairy cows on milk production, milk fatty acid composition, and the antioxidant capacity of milk. *Anim. Feed Sci. Technol.* **193,** 148-154.

Aguiar S.C., Zeoula L.M., Franco S.L., Peres L.P., Arcuri P.B. and Forano E. (2013). Antimicrobial activity of Brazilian propolis extracts against rumen bacteria *in vitro*. *World J. Microbiol. Biotechnol*. **29,** 1951-1959.

Amoros M., Lurton E., Boustie J., Girre L., Sauvager F. and Cormier M. (1994). Comparison of the anti-herpes simplex virus activities of propolis and 3-methyl-but-2-enyl caffeate. *J. Nat. Prod*. **57,** 644-647.

AOAC. (2005). Official Methods of Analysis. Vol. I. 18th Ed. Association of Official Analytical Chemists, Arlington, VA, USA.

Balcells J., Aris A., Serrano A., Seradj A.R., Crespo J. and Devant M. (2012). Effects of an extract of plant flavonoids (Bioflavex) on rumen fermentation and performance in heifers fed high-concentrate diets. *J. Anim. Sci*. **90,** 4975-4984.

Bankova V., Castro S.L. and Marcucci M.C. (2000). Propolis: recent advances in chemistry and plant origin. *Apidologie*. **31,** 3-15.

Bankova V., Dyulgerov A., Popov S. and Marekov V. (1992). Propolis produced in Bulgaria and Mongolia: Phenolic compounds and plant origin. *Apidologie*. **23,** 79-85.

Bodas R., Prieto N., García-González R., Andrés S., Giráldez F.J. and López S. (2012). Manipulation of rumen fermentation and methane production with plant secondary metabolites. *Anim. Feed Sci. Technol*. **176,** 78-93.

Castaldo S. and Capasso F. (2002). Propolis, an old remedy used in modern medicine. *Fitoterapia*. **73(1),** 1-6.

Cottica S.M., Sawaya A.C.H.E., Eberlin M.N., Franco S.L., Zeoula L.M. and Visentainer J.V. (2011). Antioxidant activity and composition of propolis obtained by different methods of extraction. *J. Brazilian Chem. Soc*. **22,** 929-935.

Denman S.E. and McSweeney C.S. (2006). Development of a real-time PCR assay for monitoring anaerobic fungal and cellulolytic bacterial populations within the rumen. *FEMS Microbiol. Ecol*. **58,** 572-582.

Denman S.E., Tomkins N.W. and McSweeney C.S. (2007). Quantitation and diversity analysis of ruminal methanogenic populations in response to the antimethanogenic compound bromochloromethane. *FEMS Microbiol. Ecol*. **62,** 313-322.

Dimov V., Ivanovska N., Manolova N., Bankova V., Nikolov N. and Popov S. (1991). Immunomodulatory action of propolis: Influence on anti-infectious protection and macrophage function. *Pidologie*. **22,** 155-162.

El-Bassuony A.A. (2009). New prenilated compound from Egyptian propolis with antimicrobial activity. *Rev. Latinoam. Quím*. **39,** 85-90.

Isla M.I., Nieva Moreno M.I., Sampietro A.R. and Vattuone M.A. (2001). Antioxidant activity of Argentine propolis extracts. *J. Ethnopharmacol*. **76,** 165-170.

Koike S. and Kobayashi Y. (2001). Development and use of competitive PCR assays for the rumen cellulolytic bacteria: *Fibrobacter succinogenes*, *Ruminococcus albus* and *Ruminococcus flavefaciens*. *FEMS Microbiol. Lett*. **204,** 361-366.

Krause D.O., Bunch B.R., Smith W.J.M. and McSweeney C.S. (1999). Diversity of *Ruminococcus* strains: A survey of genetic polymorphisms and plant digestibility. *J. Appl. Microbiol*. **86,** 487-495.

Kreuzer M., Kirchgessner M. and Müller H.L. (1986). Effect of defaunation on the loss of energy in wethers fed different quantities of cellulose and normal or steamflaked maize starch. *Anim. Feed Sci. Technol*. **16,** 233-241.

Maeda H., Fujimoto C., Haruki Y., Maeda T., Kokeguchi S., Petelin M., Arai H., Tanimoto I., Nishimura F. and Takashiba S. (2003). Quantitative real-time PCR using TaqMan and SYBR green for *Actinobacillus actinomycetemcomitans*, *Porphyro-*

monas gingivalis, *Prevotella intermedia*, *tetQ* gene and total bacteria. *FEMS Immunol. Med. Microbiol*. **39,** 81-86.

Makkar H.P.S. and Vercoe P.E. (2007). Measuring Methane Production from Ruminants. Publications of Springer, Berlin, Germany.

Marcucci M.C. (1995). Propolis: Chemical composition, biological properties and therapeutic activity. *Apidologie*. **26,** 83-89.

McGuffey R.K., Richardson L.F. and Wilkinson J.I.D. (2001). Ionophores for dairy cattle: Current status and future outlook. *J. Dairy Sci*. **84,** 194-203.

Menke H.H. and Steingass H. (1988). Estimation of the energetic feed value obtained from chemical analysis and *in vitro* gas production using rumen fluid. *Anim. Res. Dev*. **28,** 7-55.

Mirzoeva O.K., Grishanin R.N. and Calder P.C. (1997). Antimicrobial action of propolis and some of its components: The effects on growth, membrane potential and motility of bacteria. *Microbiol. Res*. **152,** 239-246.

Miyataka H., Nishiki M., Matsumoto H., Fujimoto T., Matsuka M. and Satoh T. (1997). Evaluation of propolis: Evaluation of Brazilian and Chinese propolis by enzymatic and physicochemical methods. *Biol. Pharm. Bull*. **20,** 496-501.

Murad J.M., Calvi S.A., Soares A.M.V.C., Bankova V. and Sforcin J.M. (2002). Effects of propolis from Brazil and Bulgaria on fungicidal activity of macrophages against *Paracoccidioides brasiliensis*. *J. Ethnopharmacol*. **79,** 331-334.

Nisbet D.J., Edrington T.S., Anderson R.C. and Krueger N. (2009). Effects of the dicarboxylic acids malate and fumarate on *E. coli* O157:H7 and *Salmonella enteric typhimurium* populations in pure culture and in mixed ruminal microorganism fermentations. *Curr. Microbiol*. **58,** 488-492.

Oliveira J.S., Borges L.R., Queiroz A.C. and Almeida I.C.C. (2004). Effect of monensin and propolis extract on ammonia production and *in vitro* degradability of crude protein of different nitrogen sources. *Rev. Brasileira Zootec*. **33,** 504-510.

Oliveira J.S., Queiroz A.C., Lana R.P., Mantovani H.C. and Generoso R.A.R. (2006). Effect of monensin and bee propolis on *in vitro* fermentation of amino acids by mixed ruminal bacteria. *Rev. Brasileira Zootec*. **35,** 275-281.

Orskov E.R. and McDonald I. (1979). The estimation of protein degradability in the rumen from incubation measurements weighed according to rate of passage. *J. Agric. Sci*. **92,** 499-503.

Oskoueian E., Abdullah N. and Oskoueian A. (2013). Effects of flavonoids on rumen fermentation activity, methane production, and microbial population. *BioMed Res. Int*. **2013,** 1-8.

Ozturk H., Pekcan M., Sireli M. and Fidanci U.R. (2010). Effects of propolis on *in vitro* rumen microbial fermentation. *Ankara Üniv. Vet. Fak. Derg*. **57,** 217-221.

Padmavati M., Sakthivel N., Thara K.V. and Reddy A.R. (1997). Differential sensitivity of rice pathogens to growth inhibition by flavonoids. *Phytochemistry*. **46,** 499-502.

Paint Z.M. and Metzner J. (1979). On the local anaesthetic action of propolis and some of its constituents. *Biol. Pharm. Bull*. **34,** 839.

Patra A.K., Kamra D.N. and Agarwal N. (2006). Effect of plant extracts on *in vitro* methanogenesis, enzyme activities and fermentation of feed in rumen liquor of buffalo. *Anim. Feed Sci. Technol*. **128,** 276-291.

Rispoli T.B., Rodrigues I.L., Martins Neto R.G., Kazama R., Prado O.P.P., Zeoula L.M. and Arcuri P.B. (2009). Ruminal ciliate protozoa of cattle and buffalo fed on diet supplemented with monensin or extracts from propolis. *Pes. Agrop. Brasileira*. **44,** 92-97.

Santos N.W., Yoshimura E.H., Machado E., Matumoto-Pintro P.T., Montanher P.F., Visentainer J.V., dos Santos G.T. and Zeoula L.M. (2016). Antioxidant effects of a propolis extract and vitamin E in blood and milk of dairy cows fed diet containing flaxseed oil. *Livest. Sci*. **191,** 132-138.

SAS Institute. (2001). SAS®/STAT Software, Release 9.1. SAS Institute, Inc., Cary, NC. USA.

Sforcin J.M. and Bankova V. (2011). Propolis: Is there a potential for the development of new drugs? *J. Ethnopharmacol*. **133,** 253-260.

Sforcin J.M., Fernandes Jr A., Lopes C.A.M., Bankova V. and Funari S.R.C. (2000). Seasonal effect on Brazilian propolis antibacterial activity. *J. Ethnopharmacol*. **73,** 243-249.

Swain T. and Hillis W.E. (1959). The phenolic constituents of Prunus domestica. I.-The quantitative analysis of phenolic constituents. *J. Sci. Food. Agric*. **10(1),** 63-68.

Tavendale M.H., Meagher L.P., Pacheco D., Walker N., Attwood G.T. and Sivakumaran S. (2005). Methane production from *in vitro* rumen incubations with *Lotus pedunculatus* and *Medicago sativa*, and effects of extractable condensed tannin fractions on methanogenesis. *Anim. Feed Sci. Technol*. **123(1),** 403-419.

Valizadeh R., Behgar M., Mirzaee M., Naserian A.A., Vakili A.R. and Ghovvati S. (2010). The effect of physically effective fiber and soy hull on the ruminal cellulolytic bacteria population and milk production of dairy cows. *Asian-Australasian J. Anim. Sci*. **23,** 1325-1332.

Van Soest P.J., Robertson J.B. and Lewis B.A. (1991). Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. *J. Dairy Sci*. **74,** 3583-3597.

Velikova M., Bankova V., Tsvetkova I., Kujumgiev A. and Marcucci M.C. (2000). Antibacterial ent-kaurene from Brazilian propolis of native stingless bees. *Fitoterapia*. **71,** 693-696.

Wallace R.J. (2004). Antimicrobial properties of plant secondary metabolites. *Proc. Nutr. Soc*. **63,** 621-629.

Ware C.E., Banehop T. and Gregg K. (1989). The isolation and comparison of ellulose genes from two strains of *Ruminococcus albus*. *J. Gen. Microbiol*. **135,** 921-930.

Weatherburn M.W. (1967). Phenol-hypochlorite reaction for determination of ammonia. *Anal. Chem*. **39,** 971-974.

Zia M., Mannani R., Mahmoodi M., Bayat M. and Mohaghegh F. (2009). The Effects of alcoholic extract of propolis obtained from Iran bee hives on the growth of trichophyton mentagrophytis, trichophyton rubrum and trichophyton verrucosum. *J. Isfahan Med. Sch*. **27,** 232-241.