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The Effects of Feeding Iranian Propolis Extracts to Holstein Dairy Cows on Blood Metabolites, Milk Composition and Rumen Microbial Population

Research Article

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ABSTRACT

The purpose of this study was to investigate the effect of feeding Iranian propolis extract on performance of Holstein dairy cows. In this study 18 dairy postpartum Holstein cows (120 days in milk (DIM) and body weight of 577±37.54 kg) were used. The experiment was carried out in a period of 29 d (10 d adaptation and 19 d sample collection). The data was analyzed considering a completely randomized design by the GLM procedure of SAS 9.1. In this experiment 3 treatments [A: TMR diet, B: TMR diet + Iranian propolis extract (IPE) 50% (67.50 g/day/animal) and C: TMR diet + IPE 75% (67.50 g/day/animal)] were used. The results of this study showed that adding different concentrations of IPE to dairy cow ration did not have any significant effect ($P>0.05$) on ruminal pH, but significant decrease ($P<0.05$) in NH_3-N was observed in IPE 75% treatment in comparison with the others. The total population of *Ruminococcus albus*, *Prevotella bryantii*, fibrolytic, amylolytic, methanogens and protozoa were counted and a significant difference ($P<0.05$) was observed in total rumen microbial population. Furthermore, adding IPE to the cow ration did not significantly ($P>0.05$) change some the blood parameters while statistically changed ($P<0.05$) the blood concentrations of aspartate aminotransferase, albumin, glucose and protein. The phenolic compounds in propolis changed rumen nitrogen ammonia, rumen short chain fatty acids, rumen microbial population, some blood parameters and fatty acid composition in the milk. These changes improve fermentation process and consequently affect significantly fatty acid composition in milk.

KEY WORDS Iranian propolis extracts, blood metabolites, Holstein dairy cows, milk composition, rumen microbial population.

INTRODUCTION

In recent years consumers, producers and researchers have been concerned with diets that help people improve their health care (Nagai and Inoue, 2004). The relation between diet and health care is considered a key factor to prevent diseases. In some countries antibiotics are added to the dairy cow's diet while in others it is prohibited (Mills *et al.* 2009). The prohibition of antibiotics in 2006 caused the researchers to spend a lot of time on studying and researching the secondary metabolites and extracts of herbal plants (Mills *et al.* 2011). Some plants may produce compounds which affect ruminant production (Wallace, 2004). The Compounds like phenylpropanoids and flavonoids have an effect on rumen microbial metabolism by changing the fermentation process (Broudiscou *et al.* 2002; Balcells *et al.* 2012). Propolis is made by bees by collecting some substances from buds and flowers and mixing them with their saliva, wax and pollen (Bankova, 2005). The function of propolis is to seal and protect the hives against intruders

and microbes (Ghisalberti, 1979). Iran has 5600000 honey bee colonies (Omidi-Arjenaki *et al*. 2016) and the mean product of propolis has about 1100 tones and it have a good potential to use it for animal feed. The chemical composition of the propolis is different and depends on the characteristics of the regional flora (Bankova, 2005). The diet of dairy cows significantly affects their milk fat and fatty acid composition, and this has an effect on human health, therefore, propolis can be a good alternative to improve the milk quality by changing lipid metabolism and adjusting the fatty acid composition (Lock *et al.* 2004). The purpose of this study was to investigate the effects of feeding Iranian propolis extracts (IPE) on milk production, milk fatty acid composition, blood metabolites and rumen microbial population of Holstein dairy cows.

MATERIALS AND METHODS

Propolis origin and extracts

Iranian propolis extract was obtained from Ehtesham Apiary, Hezar Masjed Mountains, Khorasan Razavi province (37° 37' 31.07" N, 58° 43' 49.74" E) which has a relatively warm weather in October 2022 from 180 hives. According to a previous study (Ehtesham *et al*. 2018), two extracts of IPE 50% and 75% [(it means 50 and 75 grams of propolis in 100 mL ethanol 70%)] were used. Small pieces of propolis (about 4-5 mm) were mixed with ethanol 70% and distilled water in a shaker (GFL model 3005, Germany) with 300 rpm at room temperature for 72 h. Then, the ethanol extract was filtered with a Whatman No.41 filter paper. To eliminate the ethanol, a rotary evaporator (Heidolph laborota 4000, Germany) at 42 ˚C for 30 min was used. Total phenolic compounds of IPE were measured by Swain and Hillis (1959) (Table 1).

Table 1 Total phenolic compounds of Iranian propolis extracts (IPE)

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

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

GC-MS analysis of Iranian propolis extracts

The GC–MS analysis was performed after silylation of the samples with a Hewlett Packard gas chromatograph 5890 Series II Plus linked to a Hewlett Packard 5972 mass spectrometer system, equipped with a 23 m long, 0.25 mm i.d. and 0.5 mm film thickness HP-5 capillary column. The temperature was programmed from 100 ˚C to 310 ˚C at a rate of 5 ˚C min 1. Helium was used as a carrier gas, at flow rate 0.7 mL min 1, split ratio 1:80, injector temperature 280 ˚C and ionization voltage 70 eV. For silylation, about 5 mg of the sample was mixed with 75 mL bis (trimethylsilyl) trifluoroacetamide and 50 mL of dry pyridine, heated at 80 ˚C for 20 min and analyzed by GC–MS. The composition of the propolis extracts were shown in Table 2.

Table 2 Composition[1] of Iranian propolis extracts (IPE; GC-MS after silylation)

Phenolic compounds (g/kg of IPE)	IPE 50%	IPE 75%
Chrysin	2.2	2.5
Galangin	3.1	3.0
Pinocembrin	1.6	1.8
CAPE	2.5	2.6
p-Coumaric acid	0.3	0.6
Caffeic acid	0.6	1.6
Hydroxybenzoic acid	0.2	0.3
Ferulic acic	0.5	1.5
Dimethoxycinnamic acid	0.3	1.3
Pentenyl caffeate (cis-isomere)	0.1	0.6
Bornyl/isobornyl vanilate	3.7	1.5
Communic acid	2.6	1.7
Pentyl caffeate	-	-
Dimethylallyl caffeate	5.3	3.7
Abietic acid	1.2	0.7
Pinostrobin chalcone	2.4	2.3
Pinocembrin chalcone	3.3	2.8
Pinostrobin	1.0	0.7
Pinobanksin chalcone	1.0	0.8
Pinobanksin	3.3	2.2
Benzyl caffeate	1.2	0.7

[1] The identification of the compounds was performed using commercial libraries and comparison of mass spectra and retention times of reference compounds. The semi-quantification of the main compounds was carried out by internal normalization with the area of each compound (percentage of the total ion current). The addition of individual areas of the compounds corresponds to 100% area.

Animals, diet and sample collection

In this study 18 dairy postpartum Holstein cows (120 DIM, body weight of 577±37.54 kg) were used. In this experiment 3 treatments [A: TMR (total mixed ration) diet, B: TMR diet + IPE50% (67.50 g/day/animal) and C: TMR diet + IPE 75% (67.50 g/day/animal)] were used. The cows were milked individually 3 times a day at 06:00, 14:00 and 20:00 h. The experiment was carried out in a period of 29 d (10 d adaptation and 19 d sample collection). The cows were fed *ad libitum* (total mixed ratio) individually at 08:00 and 16:00. Ingredient and chemical composition of the TMR diet was shown in Table 3. Feed intake and milk yield were measured from d 19 to 29 and the average of the data was calculated. Feed refusals were collected and weighed daily before the morning feeding. Daily DMI was calculated as difference between feed offered and refusals. An aliquot was collected in a sealed tube with preservative (potassium dichromate) and stored at 4 ˚C for milk component analysis.

Table 3 Ingredients and chemical composition of the experimental diet

Ingredients (% DM)	Total mixed ration diet (%)
Alfalfa	15
Corn silage	25
Canola	10.68
Barley	5.40
Corn	23.40
Meat meal	3.60
Rice bran	6.90
Wheat bran	6.90
Calcium carbonate	0.60
Salt	0.18
Urea	0.12
Bentonite	0.60
Sodium bicarbonate	0.48
Magnesium oxide	0.18
Di calcium phosphate	0.12
Vitamin and mineral premix[1]	0.84
Chemical composition (%)	
Dry matter	54.32
Crude protein	18.28
Ether extract	3.50
Neutral detergent fiber	31.95
Acid detergent fiber	19.77
Organic matter	91.46
Ash	5.64

[1] Each kg of the vitamin–mineral premix contained (DM basis): vitamin A: 50000 IU; vitamin D_3: 10000 IU; vitamin E: 0.1 g; Calcium: 196 g; Phosphorus: 96 g; Sodium: 71 g; Magnesium: 19 g; Iron: 3 g; Copper: 0.3 g; Manganese: 2 g; Zinc: 3g; Cobalt: 0.1 g; Iodine: 0.1 g and Selenium: 0.001 g.

The second aliquot was stored without preservative at -20 ˚C until analysis for FA profile. On day 29 of period, ruminal fluid samples were taken from experimental cows by 2 h after the morning feeding and the samples were controlled to have no saliva contamination (Voelker and Allen, 2003). Ruminal pH was immediately measured with a portable pH meter (Metrohm744, Herisau, Switzerland). The ruminal fluid samples were strained through 4 layers of cheesecloth and prepared for subsequent analyses of NH_3-N (5 mL of rumen fluid was acidified with 5 mL of 0.2 N HCl) and VFA (5 mL of rumen fluid was mixed with 1 mL of 250 g/L meta-phosphoric acid) and kept at -20 ˚C until laboratory analyses. Ruminal fluids were transferred to 1.5 mL microtubes and were kept at -20 ˚C until the time of counting micro-organisms. Blood samples of all cows were taken from the jugular vein on day 15 and 29 two h after morning feeding (10 mL into sterile tubes containing EDTA solution). Blood samples were then centrifuged at 3000 × g for 15 min, and plasma was collected and stored at -20 ˚C until analysis.

Laboratory analysis

The dry matter, ether extract (EE), ash and crude protein (CP) of the diet were determined according to AOAC (2005) using the following methods respectively: 934.01, 920.39, 942.05 and 2001.11. 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). Sodium sulfite and heat-stable α-amylase (Sigma A3306; Sigma-Aldrich, Steinheim, Germany) were used during NDF analysis.

Milk composition and Milk Fatty acid analysis

To determine fat, protein and lactose concentrations of individual milk samples, Milko-Scan 605 analyzer (Foss Electric, Hillerød, Denmark) was used. Milk urea nitrogen (MUN) was measured by Auto Analyzer (A15, Biosystem S.A. Barcelona, Spain). Using a centrifugation technique, milk fat was extracted according to Lana *et al*. (2005). Next, about 100 mg of the milk fat was mixed with 2 mL of 1 M KOH, and then 5 mL of 14% boron trifluoride in ethanol was added. The sample was methylated at 100 ˚C for 60 min and then extracted with 5 mL of hexane. The FA methyl esters (FAME) were analyzed by a gas chromatograph (3400 Varian Star; Varian Inc., Palo Alto, CA) equipped with CP-SIL-88 capillary column (Chrompack, 60 m × 0.25 mm, Varian, Palo Alto, CA) and helium was used as carrier gas. Column temperature was initially at 50 ˚C for 1 min, and increased by 10 ˚C/min to 190 ˚C which lasted for another 130 min. The temperature of the injector was 270 ˚C, and that of the detector was 300 ˚C. Peaks of FAME were identified by comparing their retention times with those of the standard mixture 37 Component FAME Mix (Supelco, Bellefonte, PA).Quantification of FA was based on tridecanoic acid (13:0, Sigma, St. Louis, MO) as an internal standard.

NH_3-N of Rumen Fluid

To measure ammonia nitrogen (NH_3-N) concentration, phenol- hypochlorite reaction was used (Weatherburn, 1967).

DNA extraction and real-time polymerase chain reaction (Real-Time PCR)

In order to get the sedimentation of feed particles microtubes at 4 ˚C were centrifuged at 2000 × g for 5 min and 200 µL of supernatants were added to fresh 1.5 mL microtubes. The extraction of DNA was carried out by utilizing a genomic DNA extraction kit (AccuPrepTM, Bioneer Corporation, Daejeon, South Korea) as it was instructed by manufactured. Real –time PCR and the Maxima® SYBR Green/ROX qPCR Master Mix (2X) (K0221, Fermentas) was used to measure the relative abundance of protozoa, methanogens, *Ruminococcus albus*, *Prevotella bryantii*, fibrolytic and amylolytic. It was tried to make used of species-specific PCR primers to show partial 16S rDNA regions that can be seen in Table 4.

Table 4 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	AGCGCAGGCCGTTTGG	Stevenson and Weimer (2007)
	R	GCTTCCTGTGCACTCAAGTCTGAC	
Fibrolytic	F	GTTCGGAATTACTGGGCGTAAA	Denman *et al*. (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	

The reaction mixture totally contained 25 µL of which 12.5 µL of SYBR Green PCR Master Mix Kit (contained Taq DNA polymerase, reaction buffer (KCl and (NH4)2SO4), dNTPs, $MgCl_2$ and SYBR Green), 0.5 µL of primer mixture containing 10 pmol of each primer, 1 µL of DNA template and 11 µL of deionized water. The samples of DNA performed by us was not adjusted for differences in DNA concentrations, but all the measurement was figured out on the basis of constant volume of DNA-extract to conclude 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. Total bacteria as reference was used to determine The relative abundances of protozoa, methanogens, *R. albus*, *Prevotella bryantii*, fibrolytic and amylolytic described by (Livak and Schmittgen, 2001) $2^{-\Delta\Delta Ct}$ method. The change in protozoa as well as the change in bacteria species are reported as fold change in genomic DNA per l µL of extracted DNA compared with control. The calculation of fold change in protozoa and specific bacteria species DNA in treatments with different concentrations of IPE compared with the control treatment was done 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.

Blood metabolites

The concentrations of aspartate aminotransferase (AST), aminotransferase (ALT), blood urea nitrogen (BUN), albumin (ALB), triglyceride (TG), cholesterol (CHL), calcium (Ca), phosphorus (P), glucose (GLU) was measured by Auto Analyzer (A15, Biosystem S.A. Barcelona, Spain) using trade kits (Biosystem S.A. Costa Brava 30, Barcelona, Spain).

Statistical analysis

The trial was analyzed considering a completely randomized design by the GLM procedure of SAS (2003). Means among treatment were compared by Tukey test on following model:

$$Y_{ij} = \mu + E_i + B_j + e_{ij}$$

Where:
Y: dependent variable.
µ: overall mean.
E_i: effect of IPE extract
B_j: effect of diet
e_{ij}: residual error

RESULTS AND DISCUSSION

The composition of IPE and total phenolic compounds of IPE are in Table 1 and Table 2 respectively. As the results indicate, there were significant differences ($P<0.05$) between total phenolic compounds in control, IPE 50% and IPE 75% treatments. Feeding the cows with different concentrations of IPE did not statistically ($P>0.05$) change dry matter intake (DMI), feed conversion efficiency (FCE), milk production, milk protein and milk urea nitrogen, but milk fat and lactose significantly($P<0.05$) changed (Table 5).

The results of this study showed that adding different concentrations of IPE to dairy cow ration did not have any significant effect ($P>0.05$) on the ruminal pH, but significant decrease ($P<0.05$) in NH_3-N was observed in IPE 75% in comparison to control and IPE 50% (Table 6).

Table 5 Effect of Iranian propolis extract on dry matter intake, feed conversion efficiency, milk production and milk composition of experimental treatments

Item	Treatments			SEM	P-value
	Control	IP 50%	IP 75%		
Dry matter intake (kg/d)	20.63±0.52	20.34±0.68	20.48±0.32	0.40	0.073
Feed conversion efficiency (kg of milk/kg of ingested DM)	1.80±0.01	1.88±0.01	1.79±0.01	0.09	0.423
Milk production (kg/d)	37.16±2.10	38.26±2.56	36.85±2.23	7.50	0.6509
Fat (%)	3.70±0.26^{a}	3.76±0.21^{b}	3.81±0.24^{c}	0.05	0.0251
Protein (%)	3.10±0.02	3.11±0.03	3.11±0.03	0.20	0.3300
Lactose (%)	4.67±0.02^{a}	4.75±0.02^{b}	4.87±0.02^{c}	0.07	0.0025
Milk Urea Nitrogen (mg/dL)	12.77±3.51	12.31±2.94	12.80±3.23	1.72	0.0643

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 6 Effect of Iranian propolis extract on rumen pH and ammonia concentration

Treatments	NH_3-N (mmol/L)	pH
Control	19.47±0.44^{a}	6.64±0.03
IP 50%	18.43±0.50^{a}	6.57±0.02
IP 75%	17.02±0.62^{b}	6.57±0.02
SEM	1.40	0.14
P-value	< 0.0001	< 0.0001

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

The reason why ruminal pH did not change can be the fact that the concentrations of total volatile fatty acids (VFA) did not change (Geraci *et al*. 2012). The existence of phenolic compounds in IPE in the fermentation environment has caused a decrease in NH_3-N either by decreasing the activity of urease enzyme (Hussain and Cheeke, 1995) or by reduction growth rate of amino acid-fermenting bacteria (McIntosh *et al*. 2003). Protozoa, due to their proteolytic activity also play a role in producing NH_3-N (Benchaar *et al*. 2008). This group of rumen micro-organisms is sensitive to phenolic compounds existing in plants and by changing the protozoa cell membrane causes their destruction and death. In addition, reduction of NH_3-N concentration can increase the production of microbial protein and nitrogen has probably been used in the production of microbial protein (McIntosh *et al*. 2003). NH_3-N decrease has also been reported by Ozturk *et al*. (2010); Oliveira *et al*. (2004) and Oliveira *et al*. (2006).

The total population of *R. albus*, *Prevotella bryantii*, fibrolytic, amylolytic, methanogens and protozoa are shown in Table 7. In addition, a significant difference (P<0.05) was observed in total population of micro-organisms between IPE 50% and control treatments and also between IPE 75% and both control and IPE 50% groups. Addition of IPE 50% to the diet of dairy cows led to the highest decrease in total population of *R. albus*, methanogens and protozoa.

In IPE 75% the highest decrease in total population of fibrolytic and amylolytic, and the highest increase in total population of *Prevotella bryantii* was observed. What is concluded in the present research is similar to the findings of Ware *et al*. (1989) and Krause *et al*. (1999). Despite the difference existing between genotypes and physiological properties of *R. albus* strains, a similarity exists in their cellulatic system and their ability to produce H2. This indicates that the addition of IPE to the cows diet can be the probable cause of higher decrease of Gram-positive bacteria in proportion to Gram-negative bacteria (Mirzoeva *et al*. 1997; Padmavati *et al*. 1997). Furthermore, the anti-protozoal effect of IPE can be the reason of population decrease of protozoa reported by other researchers (Kreuzer *et al*. 1986; Rispoli *et al*. 2009; Santos *et al*. 2016).

The reduction of protozoa population may cause the decrease methane production which in turn reduces equivalents of CH_4 to propionate synthesis in the rumen. The reduction of protozoa population and methanogenes was reported by Oskoueian and Oskoueian (2013), quite similar to our findings.

The function of propolis depends on the change bio-energetic condition of the bacterial membrane impends bacterial motility. There is a remarkable similarity between concluded results of this research and the effect of IPE on rumen micro-organism (*in vitro*) reported by Ehtesham *et al*. (2018).

Table 7 Effect of Iranian propolis extract on ruminal *(in vivo)* microorganism population

Items	Treatments*			SEM	P-value
	Control	IP 50%	IP 75%		
R. albus	1[a]	-148.30[c]	-42.38[b]	0.025	< 0.0001
Prevotella bryantii	1[b]	0.80[c]	1.43[a]	0.093	< 0.0001
Fibrolytic	1[a]	0.10[b]	-1.09[c]	0.086	< 0.0001
Amylolytic	1[a]	-0.67[b]	-1.48[c]	0.072	< 0.0001
Methanogens	1[a]	-10.57[c]	-3.56[b]	0.056	< 0.0001
Protozoa	1[a]	-3.02[c]	-1.21[b]	0.062	< 0.0001

* Fold change compared to control.
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.

So far, few researchers have been carried out into function of propolis extract on the population of rumen microorganisms. There are still a lot of be learned in order to get more information in this field.

The results of this study showed that increasing the concentration of IPE did not significantly (P>0.05) affect rumen acetic acid, but statistically (P<0.05) increased butyric and propionic and decreased total VFA (Table 8). The same results were found by Lana *et al*. (2005); Lana *et al*. (2007); Ozturk *et al*. (2010) and Aguiar *et al*. (2013). Adding propolis extract to rumen fluid (*in vitro*) increased propionate concentrations (Broudiscou *et al*. 2000) which is quite similar to the results of our experiment, but disagrees with the findings of Lana *et al*. (2005) and Lana *et al*. (2007). The reason for this difference can be related to the propolis origin and livestock used in the experiment. Furthermore, adding IPE to the cows' ration did not significantly (P>0.05) change the following blood parameters: alanine aminotransferase (ALT), calcium (Ca), phosphorus (P), cholesterol (CHL), triglyceride (TG) and blood urea nitrogen (BUN) but statistically changed (P<0.05) the blood concentrations of aspartate aminotransferase (AST), albumin, glucose and protein (Table 9). There is a remarkable similarity between the results of our research with the findings of Eraslan *et al*. (2007) and Cetin *et al*. (2010). In this paper adding IPE 75% to cows' diet increased blood protein, therefore it can be concluded that propolis can modulate protein metabolism. The results of this study demonstrated that adding IPE did not significantly change triglyceride (TG) that is contrary to the results of the experiments of Kolankaya *et al*. (2002); Fuliang *et al*. (2005) and Cetin *et al*. (2010). The reasons for this disagreement are that the above-mentioned studies have been carried out on rats, while we conducted our research on dairy cows. Another reason may be the fact that the lipids metabolism takes place in rumen and this causes triglyceride (TG) not to change. Finally, the origins of the propolis used in studies are different.

Lipid metabolism and consequently rumen fermentation may increase FA composition, that is, these changes may be caused by phenolic compounds existing in propolis which manipulate the bacterial population of the rumen and finally affect lipid metabolism. Up to now, few studies have been carried out on the effect of propolis extract on ruminant performance. The results of the present study are not similar to the results obtained by Stelzer *et al*. (2009). This difference may be due to the different concentrations of propolis used and their plant origins. In their study, Lana *et al*. (2005) supplemented Alpine dairy goats with 10 mL of propolis extract and reported no significant differences in milk production, fat, protein and lactose percentages. The results of our study are consistent with Lana *et al*. (2005) findings in terms of milk production and protein percentage, but contradict in terms of fat and lactose percentages. This difference may be related to the kind of livestock, the different concentrations of propolis used and their plant origins. Finally, our results are quite similar with the results of study done by Aguiar *et al*. (2014) in terms of milk production, fat, protein and lactose percentages.

There was a significant increase (P<0.05) in milk short chain fatty acid composition (C4:0) in IPE 75% treatment compared to IPE 50%, and also in IPE 50% compared to control treatment. The increase in milk short chain fatty acid composition (C6:0) was not significant (P>0.05) between IPE 50% and 75%, however there was a significant increase (P<0.05) in IPE 50% and 75% compared to the control. No significant increase (P>0.05) was observed in milk short chain fatty acid composition (C8:0). The increase in milk medium chain fatty acid composition (C10:0, C12:0 and C14:0) was not significant (P>0.05) between IPE 50% and 75%, however there was a significant increase (P<0.05) in IPE 50% and 75% compared to the control. No significant increase (P>0.05) was observed in milk medium and odd chain fatty acid composition (C11:0, C 13:0). No statistical changes (P>0.05) were seen in milk medium and odd chain fatty acid composition between Cis 9-14:1, C15:0, Cis 9-15:1, C16:0, Cis 9-16:1, C17:0, Cis 9-17:1, C18:0, Cis 9-C18:1, Trans 9-12 C18:2, Cis 9-12 C18:2 and C18:3. The same results were obtained (P>0.05) for milk long chain fatty acid composition (C20:0, C20:1, C22:0 and C22:1).

Table 8 Effect of Iranian propolis extract on rumen short chain fatty acids (SCFA)

Short chain fatty acids	Treatments[1]			SEM	P-value
	Control	IP 50%	IP 75%		
Total VFA (mmol/L)	71.21±0.91[a]	68.72±0.87[b]	65.28±0.97[b]	2.89	0.0001
Acetic acid (mmol/L)	27.18±2.76	27.01±1.15	26.98±2.37	2.97	0.0800
Butyric acid (mmol/L)	2.98±1.03[a]	3.57±1.28[b]	3.59±1.42[b]	0.51	0. 0400
Propionic acid (mmol/L)	10.98±1.38[a]	11.67±1.02[b]	11.97±1.54[b]	0.39	0. 0100

IP: Iranian propolis and VFA: volatile fatty acids.
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 9 Effect of Iranian propolis extract on the blood parameters of Holstein dairy cows

Parameter	Treatments			SEM	P-value
	Control	IP 50%	IP 75%		
Albumin (g/L)	32.86±0.58[a]	28.04±0.58[b]	28.06±0.58[b]	0.68	0.0157
ALT (u/L)	7.71±4.11	7.73±4.11	7.02±4.11	9.94	0.4371
AST (u/L)	60.75±1.77[a]	73.51±1.77[b]	73.20±1.77[b]	6.28	<0001
Ca (mg/dL)	9.40±0.22	9.22±0.22	9.05±0.22	1.20	0.0700
P (mg/dL)	6.20±0.46	6.76±0.46	6.17±0.46	1.30	0.4000
Glucose (mg/dL)	40.90±2.53[b]	53.13±2.53[a]	56.99±2.53[a]	12.80	0.0218
CHL (mg/dL)	220.86±23.12	221.03±23.12	223.49±23.12	4.43	0.3850
Protein (g/dL)	6.95±1.61[b]	6.98±1.61[b]	7.07±1.61[a]	0.07	0.0176
TG (mg/dL)	138.61±1.18	139.83±1.18	142.32±1.18	4.79	0.2252
BUN (mg/dL)	10.73±0.15	10.25±0.15	10.40±0.15	0.78	0.0816

IP: Iranian propolis; ALT: alanine aminotransferase; AST: aspartate aminotransferase; Ca: calcium; P: phosphorus; CHL: cholesterol; TG: triglyceride and BUN: blood urea nitrogen.
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 10 Effect of Iranian propolis extract on fatty acid composition in milk (% of total lipids) of Holstein cows fed no phenolic compounds from propolis (control), 6.34 v/w of phenolic compounds/kg of DM intake (IP 50%), 7.79 mg of phenolic compounds/kg of DM intake (IP 75%)

Fatty acid[2]	Treatment[1]			SEM	P-value
	control	IP 50%	IP 75%		
C4:0 (%)	1.90[c]	2.02[b]	2.09[a]	0.03	< 0.0001
C6:0 (%)	1.82[b]	1.88[a]	1.89[a]	0.05	0.0400
C8:0 (%)	1.53	1.55	1.59	0.08	0.7800
C10:0 (%)	2.95[b]	3.12[a]	3.13[a]	0.04	0.0216
C11:0 (%)	0.43	0.44	0.45	0.02	0.4300
C12:0 (%)	3.33[b]	3.87[a]	3.96[a]	0.17	0.0421
C13:0 (%)	0.37	0.35	0.38	0.70	0.2410
C14:0 (%)	11.16[b]	12.38[a]	12.42[a]	0.90	0.0326
Cis9-14:1 (%)	0.73	0.76	0.79	1.23	0.7896
C15:0 (%)	1.08	0.91	1.07	0.20	0.3200
Cis9-15:1 (%)	0.45	0.53	0.57	0.18	0.4250
C16:0 (%)	29.68	30.02	30.09	0.73	0.6520
Cis9-16:1 (%)	2.15	2.24	2.26	0.12	0.2100
C17:0 (%)	0.67	0.67	0.69	0.30	0.8200
Cis9-17:1 (%)	0.38	0.38	0.39	0.02	0.8000
C18:0 (%)	12.78	12.85	12.93	0.47	0.7652
Cis9-C18:1 (%)	2.90	2.91	2.91	0.41	0.8931
Trans9-12 C18:2 (%)	1.08	1.08	1.09	0.13	0.6751
cis 9-12 C18:2 (%)	3.25	3.25	3.25	0.20	0.7963
C18:3 (%)	0.87	0.93	0.93	0.12	0.3651
C20:0 (%)	0.61	0.62	0.62	0.02	0.2700
C20:1 (%)	0.49	0.49	0.50	0.04	0.8300
C22:0 (%)	0.33	0.33	0.33	0.01	0.8725
C22:1 (%)	0.31	0.31	0.31	0.01	0.3221
SFA (g/100g)	66.36[b]	67.93[a]	67.97[a]	0.57	< 0.0001
MUFA (g/100g)	33.15	32.76	32.69	0.58	0.1495
PUFA (g/100g)	45.42	43.42	43.23	3.74	0.2851

IP: Iranian propolis; SFA: saturated fatty acids; PUFA: polyunsaturated fatty acids and MUFA: monounsaturated fatty acids.
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, a significant changes was observed (P<0.05) in saturated fatty acids (SFA), but no significant difference was seen (P>0.05) in polyunsaturated fatty acids (PUFA) and monounsaturated fatty acids (MUFA). The above-mentioned results can be seen in Table 10. Milk fatty acids are almost equally derived from food and rumen microbial activity (Parodi, 2004). The reason for the significant increase in milk short and medium chain fatty acids can be the increase of rumen butyric acid and its conversion to ß-hydroxybutyrate during its absorption from the rumen epithelium (German and Dillard, 2006). The significant increase in rumen butyric acid and milk short and medium chain fatty acids can be seen respectively in Tables 7 and 10. The anti-microbial property of propolis can be the probable cause of the changes in the population of rumen microorganisms and consequently the increase in rumen butyric acid and milk short and medium chain fatty acids. The results of the present study are not consistent with the results of the study done by Aguiar *et al*. (2014). The reason can be the fact that Aguiar *et al*. (2014) carried out their study using different kinds of propolis obtained from different regions.

CONCLUSION

The propolis extract changed rumen nitrogen ammonia, rumen short chain fatty acids (SCFA), rumen microbial population, some blood parameters and fatty acid composition in milk. These changes improve fermentation process and consequently affect milk quality.

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