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Nitrogen balance, blood metabolites and milk fatty acid composition of dairy cows fed pomegranate-peel extract

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ABSTRACT

An experiment was carried out to determine the effect of pomegranate peel extract (PPE) on nitrogen balance, blood parameters and the milk fatty acid profile of dairy cows. Four Holstein cows were used in a 4×4 Latin square design with 28-d periods and 4 treatments: PPE0 (no extract), PPE400 (400 ml PPE/cow/d), PPE800 (800 ml PPE/cow/d) and PPE1200 (1200 ml PE/cow/d). Nitrogen balance, blood parameters and the milk fatty acid profile were measured. Determination of secondary metabolites showed its high content of aqueous fraction, total phenolics and total tannin with reasonable content of saponins. Outputs of N in milk were quadratically increased ($P=0.044$) by inclusion of PPE at 400 and 800 ml PPE/cow/d in the diet. Blood cholesterol (quadratic effect, $P=0.043$), blood urea nitrogen (linear effect, $P=0.047$) and milk urea nitrogen (quadratic effect, $P=0.0008$) concentrations decreased with adding PPE. Supplementation with PPE quadratically lowered total saturated fatty acid ($P=0.005$), proportions of C12:0 (linear effect $=0.040$), C16:1*c*9 (quadratic effect, $P=0.011$), C18:0 (linear effect, $P=0.083$, quadratic effect, $P=0.011$) and ω6/ω3 (quadratic effect, $P=0.007$). Amount of C18:3c (n-3) (linear effect, $P=0.046$), DHA (C22:6) (quadratic effect, $P=0.009$) and EPA (C20:5) (quadratic effect, $P=0.012$) were increased by inclusion of PPE. Adding PPE lowered blood cholesterol, blood urea nitrogen and milk urea nitrogen. Milk from cows fed PE had significantly lower total saturated fatty acid, desirable ω6/ω3 ratio and higher content of EPA and DHA.

1. Introduction

In livestock production systems, different approaches such as antibiotics and ionophores have been used to improve feed efficiency and fatty acids profile in milk and maximize the performance of dairy cows. However, due to the public concern for adding antibiotics in livestock ration, great work has been devoted towards developing replacements to such additives. Plant secondary metabolites offer a chance in this regard (Benchaar et al., 2008). Plant secondary metabolites in tree leaves such as *Salix babylonica*, *Leucaena leucocephala*, and grape pomace

Abbreviations: ADF, ash-free acid detergent fiber; BUN, blood urea nitrogen; CP, crude protein; DHA, Docosahexaenoic acid; DM, dry matter; DMI, Dry matter intake; EPA, Eicosapentaenoic acid; MUFA, monounsaturated fatty acid; MUN, milk urea nitrogen; NE_L, net energy for lactation; NDF, ash-free neutral detergent fiber; OM, organic matter; PP, pomegranate peel; PPE, pomegranate peel extract; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid; TMR, total mix ration; ω6, omega-6 fatty acids; ω3, omega-3 fatty acids.

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extracts (Alipour and Rouzbehan, 2010; Salem et al., 2011, 2014a; Jiménez-Peralta et al., 2011) were found to improve the ruminal fermentation parameters and to enhance amino acid flow to the duodenum (Mueller-Harvey, 2006). This could lead to more muscle deposition and greater milk production (Vasta et al., 2008, Salem et al., 2014b). Also, plant secondary metabolites have been suggested as potential resources to manipulate bacterial populations involved in ruminal biohydrogenation to alter the fatty acid profile of ruminant-derived food products such as milk and meat. For example, Durmic et al. (2008) observed that ethanolic extracts and essential oils from some Australian plants increased the concentrations of unsaturated fatty acids in rumen fluid. In other study, Cabbidu et al. (2009) showed that in sheep fed *Sulla* (contain 25–27 g condensed tannin per kg DM), total trans fatty acids and ω6/ω3 ratio content was lower comparing to sheep fed Sulla and polyethylene glycol, but linoleic and linolenic acid were higher.

Pomegranate peel (PP) is a by-product of extracting the juice from pomegranates, with annual production of more than 120,000 t in Iran (Mirzaei-Aghsaghali et al., 2011). The PP contains secondary metabolites such as saponin, polyphenolic compounds, primarily punicalagin and ellagitannins, which have been shown to possess antimicrobial, antioxidant, anti-inflammatory, antimitotic, and immune modulatory properties (Adams et al., 2006; Oliveira et al., 2010). In conventional processes, plant secondary metabolites are extracted from PP by using organic solvents (methanol, ethanol and acetone) which are costly techniques. It was verified that the extraction efficiencies of the secondary metabolites using organic solvents are comparable with that of water, which are less expensive and polluting (Abarghuei et al., 2013, 2014). Therefore, we hypothesized that inclusion of PPE to the diet would improve protein metabolism and milk fatty acids content. Hence, this experiment was carried out to assess the influence of administering three concentrations of water PPE, on nitrogen balance, blood metabolites and milk fatty acid profile in dairy cows.

2. Materials and methods

2.1. Animal care

The experiment was carried out according to The Care and Use of Agricultural Animals in Research and Teaching (FASS, 2010) guidelines. All procedures and guidelines involving animals were approved by the Animal Experiment Committee at Tarbiat Modares University, Tehran, Iran.

2.2. Pomegranate peel extract

Pomegranate peel was obtained from two main factories in Saveh city, Iran, using similar pomegranate varieties and processing methods. Sun-dried peel was extracted at 1 g PP/ml of water. The peel was soaked in tap water at 40 °C for 72 h in a closed tank. To maximize the extraction of plant secondary metabolites from the PP, the tank was incubated in a water bath at 40 °C for one hour. The contents, then, were immediately filtered by metal mesh extraction and the filtrate was stored at 4 °C for further use.

2.3. Experimental design, cows and treatments

The experiment was designed as a balanced 4×4 Latin squares for carryover effects, using 4 dairy cows with four 28-d periods. The cows were enrolled in the experiment with average days in milk of 87 ± 29 and mean body weight of 616 ± 53 kg. Cows were housed in individual tie stalls and had free access to water during the experiment. A total mix ration (TMR, Table 1) was fed for ad libitum intake (5–10% orts, on as-fed basis). The animals were randomly assigned to 1 of 4 treatments: (1) PPE0 (control, no PPE added), (2) PPE400 (400 ml PPE/cow per day), (3) PPE800 (800 ml PPE/cow per day), and (4) PPE1200 (1200 ml PPE/cow per day). The PPE was extracted daily and mixed into the feed mixture. Each experimental period lasted 28 d with 21 d for adaptation to the diet, and 7 d for sampling and data collection. All diets were formulated to have similar concentrations of CP and net energy for lactation (NE_L) (NRC, 2001). Diets were offered in equal amounts 3 times daily (0600 h, 1400 h and, 2200 h).

2.4. Nitrogen balance

Feed consumption was recorded daily by weighing feeds offered to and refused by the cows. Samples of the TMR, feed ingredients, and orts were collected daily and kept frozen. Samples were composited by period, dried at 55 °C for 48 h, ground through a 1 mm screen Wiley mill (standard model 4; Arthur M. Thomas, Philadelphia, PA,

Table 1
Ingredients and chemical composition (means ± SD) of the TMR fed to lactating cows (n=4).

Ingredients (g/kg DM)	
Alfalfa hay	229.0
Corn silage	211.9
Barley, rolled	134.5
Corn grain, ground, dry	81.9
Wheat bran	99.4
Wheat grain, rolled	27.7
Soybean meal	66.3
Canola meal	28.0
Cottonseed meal	39.3
Vegetable oil	12.4
Limestone	6.2
Mineral+vitamin premix[a]	12.4
Salt	6.2
Molasses, beet sugar	14.5
Sodium bicarbonate	12.4
Fish meal	17.0
Chemical composition (g/kg DM)	
DM	603 ± 3.5
OM	926 ± 4.6
CP	160 ± 7.6
NDFom	340 ± 2.5
ADFom	201 ± 1.2
NE_L[b], (Mcal/kg)	1.57 ± 0.009

[a] Contained 196 g Ca, 96 g P, 71 g Na, 19 g Mg, 3 g Fe, 0.3 g Cu, 2 g Mn, 3 g Zn (per kg); 100 ppm Co, 100 ppm I, 0.1 ppm Se; and 50×10^5 IU vitamin A, 10×10^5 IU vitamin D and 0.1 g vitamin E/kg.
[b] Estimated from NRC (2001).

US). Fecal grab samples were collected from all cows about 4 h pre-feeding (a.m. sampling) and 4 h post-feeding (p.m. sampling) on d 21 to 28. Fecal samples were transferred to aluminum pans and held at 60 °C in a forced-air oven until completely dry. Fecal samples were then ground to pass a 1-mm Wiley mill screen, and a single composite was prepared for each cow by mixing equal DM from both samples. Samples of the TMR, orts and fecal samples were analyzed for DM, OM, ash-free neutral detergent fiber (NDF), ash-free acid detergent fiber (ADF) and total N. At the times of fecal sampling, spot urine samples were obtained from all cows by stimulation of the vulva. After collection, 15 ml of urine was pipetted into specimen containers holding 60 ml of 0.072 N H_2SO_4 and stored at −20 °C until analysis.

2.5. Blood sampling and analyses

On d 27 of each period and at 11:30 h, blood was collected from the jugular vein into tubes containing Na heparin. Blood was centrifuged at 14,000*g* for 15 min at 10 °C to obtain serum. Glucose, albumin, total protein, triglycerides, cholesterol and urea nitrogen (BUN), were measured using enzymatic procedures and commercial kits (Pars Azmon Co., Tehran, Iran).

2.6. Milking and milk fatty acids composition

Cows were milked 3 times daily (0500 h, 1300 h and 2100 h) and the amount of milk produced for each cow at each milking was recorded using special graduated jars (Agri and SD Co., Frankfurt, Germany). Before each milking, cows were monitored for udder inflammation and presence of milk clots in the teats to ensure that milk yield and composition were not affected by mastitis. During the last week of each 28-d period, milk samples were taken from each cow at each milking and stored at −80 °C until analyzed for fatty acid composition.

2.7. Analytical methods

Nitrogen content in feed, feces and urine was determined by the Kjeldahl method (AOAC, 1990; method 954.01). Ash-free neutral detergent fiber (NDF) and ash-free acid detergent fiber (ADF) content were analyzed according to Van Soest et al. (1991) using the ANKOM F-57 filter bags in an Ankom^{200} Fiber Analyzer unit (Ankom Technolgy, Macedon, N.Y. USA). For NDF analysis, samples were treated with α-amylase (Sigma A-3403 Sigma-Aldrich® Co., Louis MO, USA), and the neutral detergent solution contained sodium sulfite and the residues were not corrected for residual ash. Daily urine volume was estimated from urinary creatinine concentration and body weight (Valadares et al., 1999). After thawing at room temperature, the urine sample was filtered through Whatman no. 1 filter paper; the filtrate was analyzed for creatinine. The mean daily creatinine excretion rate (29.0 mg/kg of body weight per d) was computed using the data from all cows in the trial. Urine volume was used to compute daily excretion of urea, and allantoin, and uric acid from spot urine samples were estimated: body weight × 29/creatinine concentration (mg/l) (Valadares et al., 1999).

For analysis of milk fatty acids, fatty acids methyl esters were ready by base-catalyzed transmethylation (Chouinard et al., 1997). Composition of fatty acids in TMR samples was determined according to the method of Sukhija and Palmquist (1988). Fatty acid analyses were carried out with a gas chromatograph (HP 5890A Series II, Hewlett Packard, Palo Alto, CA) equipped with a 100-m CP-Sil 88 capillary column (i.d., 0.25 mm; film thickness, 0.20 μm; Chrompack, Middelburg, the Netherlands) and a flameionization detector (Farnworth et al., 2007).

Fatty acids were expressed as the proportion of each individual fatty acid to the total of all fatty acids present in the sample. The following fatty acid combinations were calculated: omega-3 (ω3) fatty acids, omega-6 (ω6) fatty acids, total saturated fatty acids (SFA), total monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), total unsaturated fatty acids (UFA) and ω6/ω3 ratio.

Total phenolics were measured using the Folin–Ciocalteau method (Makkar, 2000). Extract (200 mg) dissolved in acetone: water (10 ml; 70:30, v/v) in an ultrasonic bath for 20 min. The contents were centrifuged (4 °C, 10 min, 3000*g*) and the supernatant was kept on ice until analysis. Non tannin phenolics were determined using absorption to insoluble polyvinylpyrrolidone (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). The insoluble polyvinylpyrrolidone (100 mg) was weighed into 100 mm × 12 mm test tubes. Distilled water, 1 ml, and then 1 ml tannin containing extract were added and vortexed. The tube was kept at 4 °C for 15 min, vortexed again, and centrifuged (3000*g*) for 10 min and the supernatant collected. Phenolic content in the supernatant was measured by the Folin–Ciocalteau reaction and this was accepted as the non-tannin phenol (Makkar, 2000). Total tannins were calculated as the difference between total phenols and non-tannin phenol. Tannic acid (Merck GmbH, Darmstadt, Germany) was used as the standard to express the amount of total phenols and total tannins. Condensed tannins were measured by the HCl–butanol method (Makkar, 2000). An aliquot from the above acetone:water extract (0.5 ml) plus HCl–butanol (3 ml) and ferric ammonium sulfate (0.1 ml) reagents were heated in a boiling water bath for 60 min. Absorbance was read at 550 nm. Hydrolysable tannins were analyzed using rhodanine assay according to Makkar (2000). The results were expressed as gallotannin.

Ten milliliters of the extract were prepared after total phenols separation; a double volume of n-butanol was added to fractionate saponins (Makkar et al., 1998). The remaining solution was considered to be the aqueous fraction containing other secondary compounds such lectins, polypeptides and starch (Cowan, 1999). Dihydromaltol, butanoic acid, 3-methyl-hexyl ester and thymol were measured by gas chromatography/mass spectrometry (GC/MS).

2.8. Statistical analysis

Data were analyzed as a 4 × 4 Latin square, simple changeover, design using the MIXED procedure (SAS Inst. Inc., Cary, NC), using the statistical model:

$$Y_{ijk} = \mu + A_i + P_j + T_k + e_{ijk}$$

where Y_{ijk} is a dependent variable, μ is the overall mean, A_i is the effect of cow i, P_j is the effect of period j, T_k is the fixed effect of dose k, and e_{ijk} is the residual (random error). The contrast statement was used to determine the linear and quadratic cow response to increasing concentrations of the extract in the diet. Differences between treatments were declared significant at $P \leq 0.05$ using the Tukey correction for multiple comparisons, and trends were discussed at $P \leq 0.10$ unless otherwise stated.

3. Results

3.1. Active secondary metabolites of PPE and composition of the TMR

The content of active secondary metabolites of PPE of the basic TMR which was analyzed on the mix fed to cows is shown in Table 2. PPE extract contained a considerable amount of total phenolics and total tannins with reasonable amounts of saponins. It also contained a large content of aqueous fraction (Table 2).

3.2. Nitrogen balance

There were no significant effects ($P > 0.05$) of the extract on Intake of N, outputs of N in feces and urine and N balance but, outputs of N in milk were quadratically increased ($P=0.044$) by inclusion of PE400 and PE800 in the diet (Table 3).

3.3. Blood metabolites

Blood glucose, albumin and triglyceride concentrations were not changed by feeding PPE, although blood cholesterol (quadratic effect, $P=0.043$) and BUN (linear effect, $P=0.047$) were decreased with adding PPE (Table 4). Addition of PPE quadratically decreased ($P=0.0008$) MUN (Table 4).

3.4. Milk fatty acid composition

Addition of PPE leaded to in some modifications of milk fatty acid profile as suggested by the lowered SFA (quadratic

Table 2
Secondary metabolites levels (g/kg DM diet) of PPE.

Secondary compounds	mg/ml extract	Treatment[a]			
		PPE0 g/kg diet	PPE400	PPE800	PPE1200
Total phenolics	65	3.50	4.56	5.58	6.6
Total tannins	56	1.00	1.92	2.80	3.66
Condensed tannins	0.08	–	0.0014	0.0026	0.0038
Hydrolyzable tannins	6.3	–	0.11	0.22	0.33
Saponins	35.5	11.40	11.98	12.54	13.09
Aqueous fraction[b]	227.9	–	3.73	7.30	10.85
Dihydromaltol	0.005	–	0.00008	0.00016	0.00024
Butanoic acid, 3-methyl-hexyl ester	0.002	–	0.00084	0.00168	0.00252
Thymol	0.003	–	0.00004	0.00009	0.00013

The content of active secondary metabolites of PPE of the basic TMR was analyzed on the mix fed to cows.
[a] Treatments: PPE0=control, no additive; PPE400=400 ml pomegranate peel extract/cow/d; PPE800=800 ml pomegranate peel extract /cow/d; PPE1200=1200 ml pomegranate peel extract/cow/d.
[b] Aqueous fraction (lectins, polypeptides, starch) (Cowan, 1999).

Table 3
Nitrogen balance in lactating cows fed PPE- supplemented diets.

Treatment[1]	Parameters (g/d)					
	DMI	Intake N	Urine N	Fecal N	Milk N	Balanced N
PPE0	26.2	670	174	272	157[b]	66.2
PPE400	24.4	626	174	241	172[a]	39
PPE800	23.8	609	171	242	173[a]	23
PPE1200	25.3	646	173	254	164[ab]	55
SEM[2]	0.74	18.9	17.7	9.0	4.9	22.2
P-value						
Linear	0.667	0.350	0.940	0.237	0.369	0.645
Quadratic	0.391	0.076	0.963	0.054	0.044	0.226

[a,b]Means within a column with different superscripts differ ($P < 0.05$) using *t*-test for pairwise comparison.
[1] Treatments: PPE0=control, no additive; PPE400=400 ml pomegranate peel extract/cow/d; PPE800=800 ml pomegranate peel extract/cow/d; PPE1200=1200 ml pomegranate peel extract/cow/d.
[2] SEM=Standard error of the mean. DMI: Dry matter intake (kg/d).

Table 4
Blood metabolites in lactating dairy cows fed PPE-supplemented diets.

Treatment[1]	Parameters						
	Glucose (mg/dl)	Albumin (g/dl)	Total protein (g/dl)	Triglyceride (mmol/dl)	Cholesterol (g/dl)	BUN (g/dl)	MUN (g/dl)
PPE0	66.7	3.5	7.1	0.195	210[a]	24.4[a]	14.5[a]
PPE400	68.6	3.4	7.6	0.198	162[b]	18.8[b]	12.1[b]
PPE800	75.9	3.5	7.8	0.197	160[b]	18[b]	11.5[b]
PPE1200	72.3	3.4	7.5	0.192	215[a]	19.2[b]	13.9[b]
SEM[2]	3.26	0.09	0.23	0.003	19.8	1.48	0.38
P-value							
Linear	0.149	0.411	0.235	0.295	0.875	0.047	0.186
Quadratic	0.426	0.828	0.191	0.584	0.043	0.059	0.001

[a,b]Means within a column with different superscripts differ ($P < 0.05$) using *t*-test for pairwise comparison.
[1] Treatments: PPE0=control, no additive; PPE400=400 ml pomegranate peel extract/cow/d; PPE800=800 ml pomegranate peel extract /cow/d; PPE1200=1200 ml pomegranate peel extract /cow/d.
[2] SEM=Standard error of the mean.

Table 5
Milk fatty acid profile (g/100 g of total fatty acids) of cows fed PPE.

Fatty acid	Treatments[1]				SEM[2]	*P*-value	
	PPE0	PPE400	PPE800	PPE1200		Linear	Quadratic
C10:0	0.78	0.67	0.64	0.54	0.097	0.133	0.949
C12:0	1.08[a]	0.83[ab]	0.87[ab]	0.68[b]	0.099	0.040	0.824
C14:0	3.65	2.93	3.39	2.84	0.308	0.197	0.798
C14:1 *c*9	0.23	0.21	0.37	0.24	0.033	0.313	0.156
C16:0	12.60	10.17	12.29	11.76	0.911	0.921	0.337
C16:1 *c*9	0.46	0.47	0.59	0.46	0.018	0.260	0.011
C17:0	0.19	0.14	0.18	0.16	0.020	0.575	0.550
C17:1	0.18	0.15	0.19	0.18	0.011	0.385	0.340
C18:0	3.46[a]	2.41[b]	2.29[b]	2.83[ab]	0.219	0.083	0.011
∑C18:1	0.08	0.11	0.50	0.09	0.209	0.684	0.338
∑C18:1 *c*	6.73	5.14	6.17	6.30	0.463	0.905	0.112
C18:2 *c* (n-6)	0.74	0.60	0.77	0.81	0.068	0.258	0.232
C18:3 *c* (n-3)	0.11[cb]	0.10[b]	0.13[a]	0.11[b]	0.004	0.046	0.274
C20:0	0.15	0.07	0.05	0.11	0.029	0.426	0.055
C20:1	0.03	0.02	0.04	0.02	0.003	0.584	0.168
C20:3	0.03[a]	0.007[b]	0.03[a]	0.02[a]	0.003	0.764	0.023
C20:4	0.05[a]	0.02[b]	0.04[a]	0.05[a]	0.004	0.067	0.011
C22:0	0.01[a]	0.00[b]	0.01[a]	0.01[a]	0.002	0.012	0.014
C22:1	0.006	0.00	0.008	0.01	0.003	0.163	0.206
DHA (C22:6)	0.006[b]	0.06[a]	0.04[a]	0.01[b]	0.008	0.575	0.009
EPA (C20:5)	0.001[b]	0.05[a]	0.03[ab]	0.01[b]	0.007	0.707	0.012
ω6/ω3	7.00[a]	2.98[b]	4.20[b]	6.88[a]	0.559	0.872	0.001

[a,b,c]Means within a row with different superscripts differ ($P < 0.05$) using *t*-test for pairwise comparison.
[1] Treatments: PPE0=control, no additive; PPE400=400 ml pomegranate peel extract/cow/d; PPE800=800 ml pomegranate peel extract /cow/d; PPE1200=1200 ml pomegranate peel extract/cow/d. DHA: Docosahexaenoic acid; EPA: Eicosapentaenoic acid; ω6: omega-6 fatty acids (i.e., C18:2 *c* (n-6)+C20:3+C20:4); ω3: omega-3 fatty acids (i.e., C18:3 *c* (n-3)+DHA+EPA).
[2] SEM=Standard error of the mean.

effect, $P=0.005$), proportions of C12:0 (linear effect, $P=0.040$), C16:1 *c*9 (quadratic effect, $P=0.011$), C18:0 (linear effect, $P=0.083$, quadratic effect, $P=0.011$) and ω6/ω3 (quadratic effect, $P=0.007$). Amount of C18:3 *c* (n-3) (linear effect, $P=0.046$), DHA (quadratic effect, $P=0.009$) and EPA (quadratic effect, $P=0.012$) were increased by inclusion of PPE. Concentrations of MUFA and PUFA were not affected by addition of PPE in the diet (Table 5).

4. Discussion

4.1. Nitrogen balance

Adding PPE had no effect on the N balance which is due to the lack of any influence on protein digestibility (Abarghuei et al., 2013). Similarly, Vaithiyanathan et al. (2007) found that adding secondary metabolite (69 g

tannins/kg DM) had no effect on N balance of sheep. However, Woodward and Reed (1997) noted an improvement in the N balance in sheep fed 45 g condensed tannins/d. They illustrated that the improvement in the N balance in those sheep is due to the compensation for some fecal nitrogen loss by recycling nitrogen from blood to the rumen as a result of tannin presence in the fed diets. Inconsistently, Abarghuei et al. (2010) found that sheep fed diets contained 70.5 g total penolics/kg DM and 49.7 g total tannin/kg DM have decreased nitrogen balance in comparison to those without tannin.

However, inclusion of PPE400 and PPE800 in the diet was increased milk N content. The increased daily milk N in cows may be due to decline in degradability of protein in rumen and an increase in the flow of microbial protein to the intestine, benefiting the cows by increasing the amount of amino acids available for absorption in small intestine (Makkar, 2003). In contrast to this study, Benchaar et al. (2007) reported that using 750 mg mix essential oils/d in dairy cows diet had no influence on milk N content.

4.2. Blood metabolites

The lack of differences in blood concentrations of glucose and triglyceride with PPE supplementation was consistent with its lack of effect on DM intake. Similar to our result, Yang et al. (2010) observed no effects on glucose and triglyceride in blood of growing beef fed with 400, 800 and 1600 mg eugenol/d supplementation. This observation is in contrast to other study of Makkar et al. (1995) who demonstrated that the presence of condensed tannin increased the molar proportion of propionate, a glucogenic precursor formed in the rumen which may have been expected to increase blood glucose concentration (Makkar et al., 1995). Blood total protein concentration is an indicator of the long-term protein status of dairy cows (Topps and Thompson, 1984) and no effect on total protein content of blood in our study represents the normal protein. Similarly, Nasri and Ben Salem (2012) reported that oral administration of *Agave Americana* extracts (containing 120, 240 and 360 mg saponins/kg DM) or *Quillaja saponaria* (containing 120 mg saponins/kg DM) to Barbarine female lamb had no effect on blood total protein concentration. In contrast, Sinclair et al. (2009) showed that inclusion of whole-crop pea silages differing in their tannin content decreased blood total protein. The addition of PPE to dairy cow diets decreased blood cholesterol concentration. It is well established that saponins form insoluble complexes with cholesterol (Nasri and Ben Salem, 2012; Shi et al., 2004). It is generally accepted that the principal action of saponins on blood cholesterol is by a direct binding between saponins and dietary cholesterol in the gut, which prevents its absorption from the small intestine. Secondly, a binding between saponins and bile acids in the gut may lead to decrease the enterohepatic circulation of bile acids and increase cholesterol excretion with feces. Saponins form micelles with cholesterol. The hydrophobic portion of the saponin (the aglycone or sapogenin) attaches (lipophilic bonding) to the hydrophobic sterol nucleus, in a stacked micellar combination (Oakenfull and Sidhu, 1989). These results are consistent with the findings of Nasri and Ben Salem (2012) who illustrated administration of *Agave Americana* extracts to Barbarine female lamb decreased blood cholesterol concentration. Contrasting results were reported by Chiofalo et al. (2012) who noted dietary supplementation of 600 and 1200 mg extract of *Rosmarinus officinalis* L. per day had no effect on blood cholesterol concentration of dairy ewes. BUN concentrations were normal range of 6–27 mg/dl. In the present experiment, a significant effect of PPE addition on BUN was observed. Lowered BUN may be due to decrease proteolysis in rumen and decline in ammonia production and reduced absorption of ammonia from the rumen and enter to blood. Similarly, it was shown that blood urea nitrogen, ruminal ammonia, and urinary N loss were lower when sheep and goats were fed legumes that contained tannins (Woodward, 1988). Additionally, other studies showed that saponins could decrease ammonia production in the rumen and thus affecting blood urea concentration (Francis et al., 2002; Wina et al., 2005). In contrast, Nasri and Ben Salem (2012) reported that BUN concentration was not affected by oral administration of *Agave Americana* extracts or *Quillaja saponaria*. These researchers reported that absence of effect of this extract on blood urea could be due to the low concentrations of saponins administered to lambs.

For all treatments, MUN concentrations were within the normal range of 10–14 mg/dl. MUN concentrations are closely related to blood urea N concentrations and can be used as an indicator of protein utilization (Cannas et al., 1998).

4.3. Milk fatty acids composition

There is limited information on the use of secondary metabolites (such as phenolics, tannins and saponins) to alter the ruminal biohydrogenation process and manipulate milk fatty acid profile (Benchaar and Chauinard, 2009). Total SFA were lower in the milk of PPE-supplemented cows compared with animals not received PPE, that may be due to inhibit ruminal biohydrogenasion (Vasta et al., 2009; Khiaosa-Ard et al., 2009). Similarly, Vasta et al. (2009) had shown that tannin supplementation lowered the accumulation of SFA in milk of sheep. Dietary saturated fat intake has been shown to increase low density lipoprotein cholesterol, and therefore has been associated with increased risk of cardiovascular disease (Siri-Tarino et al., 2010; Steijns, 2008). Therefore, lowered SFA can be useful. PPE supplement increased EPA and DHA, indicating that secondary metabolites may protect lipids of ruminal biohydrogenation (Vasta et al., 2009) and inhibit growth and metabolism bacteria that are capable of biohydrogenation (Vasta et al., 2009; Mapiye et al., 2010). Therefore protection of fatty acids from ruminal biohydrogenation or inhibition of ruminal bacteria activity by PPE metabolites could have produced higher escape of fatty acids from rumen to tissue and milk. Additionally, once this fatty acids such as C18:3 *c* (n-3) is converted by desaturase and elongase enzymes into nutritionally important long-chain (20–22) polyunsaturated fatty acids such as EPA and DHA (Brenna et al., 2009; Harris et al., 2009).Thus, apart from

ruminal production, the higher amount of EPA and DHA obtained in milk fat of cows fed the PPE might have arisen from increased endogenous biosynthesis of this fatty acid from C18:3 *c* (n-3) in the breast tissue. Recent reviews have reported that the metabolic conversion efficiency of C18:3 *c* (n-3) to n-3 PUFA in humans is low and as result n-3 PUFA are now regarded as dietary essential (Molendi-Coste et al., 2011; Harris et al., 2009). Even with the limited capacity of metabolic conversion of C18:3 *c* (n-3) to n-3 PUFA (Burdge and Calder, 2005; Givens, 2010), it has many roles in human health that are independent from its conversion to n-3 PUFA (Zhao et al., 2007; Hassan et al., 2010). The long chain n-3 PUFA plays a significant role in prevention of certain diseases and disorders such as cardiovascular diseases, hypertension, type 2 diabetes, irritable bowel syndrome, muscular degeneration, rheumatoid arthritis, asthma, psychiatric disorders and several cancers (Givens, 2010; McAfee et al., 2010; Micha et al., 2010). To decrease the risk of these diseases in humans, nutritionists recommend dietary intake for C18:3 *c* (n-3) of between 1.1 and 2.5 g/d and between 200 and 600 mg/d for EPA plus DHA (Smit et al., 2009; USDA and HHS, 2010; Molendi-Coste et al., 2011). Similarly, Mapiye et al. (2010) reported that beef fed with *Acacia karroo* (contains 7.4 g condensed tannin/kg DM) have more EPA and DHA comparing to those fed control diet. In contrast, Cabbidu et al. (2009) showed that in sheep fed *Sulla* (contain 25–27 g condensed tannin/kg DM), DHA content was lower comparing to sheep fed Sulla and polyethylene glycol, but EPA was not significant.

Addition of PPE decreased ω6/ω3 ratio in cow's milk. It has been reported that decreased ω6/ω3 ratio has a valuable influence on the healthiness of dairy products (Cabbidu et al., 2009). A lower ratio of ω6/ω3 fatty acids is more desirable in reducing the risk of many of the chronic diseases of high prevalence for example; it had suggested a protective effect of omega-3 fatty acids on breast cancer risk (Simopoulos, 2002). Decrease in ω6/ω3 ratio may be due to inhibits ruminal biohydrogenation by secondary metabolites (Cabbidu et al., 2009). Similar to this study, Mapiye et al. (2010) showed that beef supplemented with *Acacia karroo* have lower ω6/ω3 ratio from steers that received control diet. Additionally, Turner et al. (2005) reported that using *birdsfoot trefoil* (*Lotus corniculatus*) in dairy cows diet declined ruminal biohydrogenation and increased ω3 fatty acids. However, Cabbidu et al. (2009) showed that in sheep fed *Sulla* (contain 25–27 g condensed tannin/kg DM), had higher ω6/ω3 ratio comparing to sheep fed *Sulla* plus polyethylene glycol. These researchers illustrated that this can be explained by the higher biohydrogenation activity of ruminal bacteria in the polyethylene glycol group, due to the partial inactivation of the tannins.

5. Conclusions

Adding PPE lowered BUN, MUN and blood cholesterol. Lower MUN suggested improved utilization of N when PPE was supplemented. Milk from cows fed PPE400 and PPE800 had significantly lower SFA, desirable ω6/ω3 ratio and higher content of EPA and DHA than milk from cows that received no PPE. It was suggested that milk from cows supplemented with PPE could be a healthier food from a human nutrition perspective. Feeding PPE to lactating cows could be a valuable mean for increasing the supply of PUFA to milk and consequently, improve the nutrition value of milk for human consumption.

Conflicts of interest

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

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