Supplementation effects of pomegranate by-products on oxidative status, metabolic profile, and performance in transition dairy cows

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ABSTRACT

Recent studies demonstrated a high antioxidant capacity for pomegranate components due to their rich bioactive compounds, such as conjugated fatty acids and phenolics. The objective of the present study was, therefore, to assess whether pomegranate seed or pomegranate seed pulp (peel + seed) supplementation could be effective to improve antioxidant status, and hence metabolic profile and performance in periparturient dairy cows. After a 1-wk pretreatment period, Holstein cows (primiparous $n = 12$, multiparous $n = 18$) were assigned to 3 dietary treatments from 25 d before expected calving through 25 d postcalving. The dietary treatments included (1) control (CON); (2) diet supplemented with pomegranate seeds (PS; 400 g/cow per day); and (3) diet supplemented with pomegranate seed pulp (PSP; 400 g of seeds/cow per day + 1200 g of peels/cow per day). Compared with CON, supplementation with either PS or PSP had no effects on dry matter intake, rumen fermentation, and plasma concentrations of cholesterol, total protein, globulin, and aspartate amino transferase, but enhanced plasma total antioxidant activity, and lowered triacylglycerol, free fatty acids (FFA), and β -hydroxybutyrate at both pre- and postpartum periods. Plasma concentration of glucose, albumin, malondialdehyde (MDA) and blood superoxide dismutase (SOD) activity were not affected by dietary treatments at prepartum, whereas SOD activity increased and glucose, albumin, MDA, and FFA-to-albumin ratio decreased by feeding both by-products at postpartum period. In contrast to PS, supplementing PSP resulted in a greater decrease in plasma glucose and triacylglycerol concentration and higher increase in SOD activity. Energy- and fat-corrected milk yields were higher in cows fed PSP diet compared with those fed CON or PS diets, but content of milk fat, protein, and lactose were similar across the dietary treatments.

These findings indicated that dietary pomegranate by-products supplementation, in particular PSP, could improve antioxidant status, which was associated with a decline in lipid oxidation (FFA and β -hydroxybutyrate) and peroxidation (MDA) and an enhancement in glucose utilization as well as fat-corrected milk yield.

Key words: pomegranate peel, pomegranate seed, oxidative stress, transition cows

INTRODUCTION

During transition period, various changes in endocrine and metabolic status are required to accommodate parturition and lactogenesis in dairy cows. The increase in metabolic demand during the periparturient period is accompanied by increased rates of reactive oxygen species (ROS) production (Gaál et al., 2006). When an overload of ROS (e.g., O_2^- , $OH^\bullet$, H_2O_2) cannot be neutralized by antioxidant defenses, a condition referred to as oxidative stress ensues (Pham-Huy et al., 2008). Growing evidence has implicated oxidative stress as an inducer of tissue injury in several pathological conditions in transition dairy cows (Brzezinska-Slebodzinska et al., 1994; Gaál et al., 2006; Abuelo et al., 2016). Increased lipid peroxidation, decreased plasma antioxidant concentrations, and low antioxidative enzyme activity have been established as typical findings in the blood samples of transition dairy cows (Bernabucci et al., 2005). Hence, supplementation of external source of antioxidants, such as α -tocopherol, β -carotene, or micronutrients (e.g., Mn, Se, Zn) that increase the ability of antioxidant enzymes, are applied to prevent damages caused by ROS, in an attempt to decrease the incidence of postpartum diseases (Brzezinska-Slebodzinska et al., 1994; Yasui et al., 2014).

Pomegranate as a nutraceutical source has received considerable attentions, as it is a rich source of natural antioxidant compounds (Tzulker et al., 2007). About 50% of the fruit weight corresponds to the peel, and the edible part of pomegranate fruit consists of 40% arils and 10% seeds (Aviram et al., 2000). Previous studies (Singh et al., 2002; Tzulker et al., 2007) indicated greater antioxidant capacity of the pomegranate by-

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products compared with the juice. Pomegranate peel is a rich source of water-soluble polyphenols, including mainly hydrolyzable tannins (e.g., punicalagins and punicalins) as well as phenolic acids, flavonoids, and proanthocyanidins (Tzulker et al., 2007; Jurenka, 2008). These compounds are known to scavenge free radicals as well as having antimicrobial, antiatherosclerotic, anti-inflammatory, anticancer, immunomodulatory, and numerous health-promoting properties (Singh et al., 2002; Jurenka, 2008; Shabtay et al., 2008). Several investigations have demonstrated the beneficial effects of pomegranate-derived products on abundance of ruminal methanogenic activity (Jami et al., 2012), feed intake and weight gain (Shabtay et al., 2008), meat total phenolics and oxidative stability (Kotsampasi et al., 2014), and milk yield and milk antioxidant activity and essential fatty acids (FA) profile (Modarasi et al., 2011; Shabtay et al., 2012). The pomegranate seed, the other by-product of pomegranate juice processing, mainly contains conjugated FA, punicic acid (*cis*-9,*trans*-11,*cis*-13 octadecatrienoic acid) as well as phytoestrogens, sterols, γ -tocopherol, and hydroxyl benzoic acids (Fadavi et al., 2006; Liu et al., 2009). The presence of these components in pomegranate seed or conjugated FA have also been shown to possess antioxidant and anti-inflammatory activity in vitro (Singh et al., 2002) and in lactating dairy cows (Hanschke et al., 2016).

The rich functional compounds, such as phenolics and conjugated FA, in pomegranate peel and seed make these by-products an attractive component to improve antioxidant status, thereby improving health. Whereas sufficient information exists on biological effects of pomegranate components as a nutraceutical source in vitro, in humans, and in some cases in the ruminant diet, limited data are available to assess the potential antioxidant and metabolic effects of different pomegranate components on dairy cattle, particularly during the transition period. Therefore, we aimed to evaluate the effects of feeding pomegranate seed pulp and pomegranate seed on oxidative status, metabolic profile, and performance of transition dairy cows.

MATERIALS AND METHODS

Pomegranate By-Products

Pomegranate by-products (seeds and peels) were provided after juice production by a commercial juice-extracting factory (Doneh Co., Shahreza, Isfahan, Iran). Fresh pomegranate peels and seeds were separately air-dried in the sun for 7 d. Before feeding, the final DM was 88.0 and 81.3% for pomegranate seed and pomegranate peel, respectively. A stationary for-

age chopper was used to chop pomegranate peels, and pomegranate seeds were ground by a hammer mill using a 3-mm screen before inclusion in the diets.

Animals and Diets

The experiment was conducted at the farm facilities of the Lavark Research Station (Isfahan University of Technology, Isfahan, Iran) from April to August 2016. Thirty dry Holstein cows (18 multiparous with BW = 727 ± 22.3 kg, and 12 primiparous with BW = 608 ± 15.9 kg) were selected and housed in individual barn stalls (4×4 m) from 32 d prepartum and fed the same prepartum basal diet for 7 d. Holstein cows were blocked by parity, and randomly allocated to 1 of 3 diets containing (1) no pomegranate by-products (CON); (2) pomegranate seeds (PS); or (3) pomegranate seed pulp (PSP) containing a blend of pomegranate peels and seeds. Pomegranate seeds was supplemented at a rate of 350 g of DM per cow, which corresponded to 2.9 and 2.2% dietary DM for prepartum (predicted DMI = 12.0 kg/d) and postpartum (predicted DMI = 16.0 kg/d) diets, respectively. Pomegranate seed pulp was applied at rates of 1,350 g of DM per cow (350 g of pomegranate seeds + 1,000 g of peels DM), which corresponded to 11.2 and 8.4% dietary DM for prepartum and postpartum diets, respectively. The blend ratio for pomegranate peels and seeds was determined to be 4:1 based on previous reports in whole pomegranate pulp (Aviram et al., 2000). Pomegranate by-products were substituted for beet pulp in the diets as described in Table 1. Diets were formulated to be isocaloric and isonitrogenous and to meet NRC (2001) requirements of close-up or recommended amounts for fresh cows. Cows were fed the experimental dietary treatments from 25 d before the expected calving date to 25 d postpartum. Water was available at all times and wood shavings were used for bedding. The 3 diets were fed as a TMR once daily (0930 h) prepartum and twice daily (0930 and 1500 h) postpartum, and allowing for 5 to 10% refusal. After parturition, cows were milked 3 times daily at 0100, 0900, and 1700 h.

Sample Collection and Analysis

Milk yield of individual cows was recorded during postpartum period. Samples of milk were collected from 3 milkings every other day from 1 to 25 DIM. Samples were stored at 4°C with a preservative (sodium azide; 6 mg/mL) to determine milk composition (fat, protein, lactose, and TS) using an automated near-infrared spectroscopy analyzer (MilkoScan, 134 BN Foss Electric, Hillerød, Denmark). The yields of 3.5% FCM ($0.432 \times \text{milk yield} + 16.23 \times \text{fat yield}$)

and ECM ($12.82 \times \text{fat yield} + 7.13 \times \text{protein yield} + 0.323 \times \text{milk yield}$) were calculated according to NRC (2001) equations. Cows were weighed weekly and BCS was determined at d -25, 0, and 25 postpartum using a 5-point scale, where 1 = emaciated and 5 = obese.

Manual weighing of feeds and refusals were performed daily for individual cows throughout the experiment. The TMR and refusal samples were taken weekly before the morning feeding and frozen at -20°C for later analysis. After thawing, samples were composited, dried at 60°C for 48 h in a forced-air oven, and then ground to pass a 1-mm screen using a Wiley mill (Arthur Thomas Co., Philadelphia, PA). Samples were analyzed for DM (method 930.15; AOAC International, 2000), ash (method 942.05; AOAC International, 2000), N using the Kjeldahl method (990.03; AOAC Interna-

tional, 2000), ether extract (method 2003.05; AOAC International, 2000), and NDF using an ANKOM200 fiber analyzer (ANKOM Technology Corp., Fairport, NY) with heat-stable α -amylase.

To measure total phenolic and antioxidant activity in pomegranate by-products and TMR, 4 g of ground sample was extracted with 80 mL of aqueous methanol (80:20 vol/vol; dark bottle) in an ultrasonic bath for 30 min and then centrifuged ($10,000 \times g$, 10 min, room temperature). Extracts (1 mL) were diluted with aqueous methanol 80% (99 mL). Sample extract (1 mL) was mixed with 4 mL of 7.5% Na_2CO_3 , and 5 mL of the Folin-Ciocalteu reagent (1:10 diluted) and then centrifuged ($10,000 \times g$, 10 min, room temperature). After 30 min, the optical density was measured at 760 nm using a UV-Photometer (UV-Vis array Spectrophotometer,

Table 1. Ingredients and nutrient composition (DM basis) of prepartum and postpartum diets containing no pomegranate by-products (CON), pomegranate seed (PS), or pomegranate seed pulp (PSP)

Item	Prepartum			Postpartum		
	CON	PS	PSP	CON	PS	PSP
Ingredient, % of DM						
Corn silage	35.0	35.0	35.0	22.5	22.5	22.5
Alfalfa hay	11.0	11.0	11.0	18.8	18.8	18.8
Wheat straw	4.0	4.0	4.0	—	—	—
Beet pulp	10.8	8.42	—	8.13	6.31	—
Corn grain	10.6	10.6	10.6	16.9	16.9	16.9
Barley grain	12.2	12.3	11.4	9.4	9.4	8.8
Soybean meal	5.8	6.1	7.9	13.4	13.6	15.0
Canola meal	5.0	5.0	4.2	2.0	2.1	1.5
Fish meal	—	—	—	0.94	0.94	0.94
Soybean whole, extruded	4.2	3.3	3.3	3.8	3.1	3.1
Fat powder	—	—	—	0.94	0.94	0.94
Carbonate calcium	—	—	—	0.69	0.69	0.56
Magnesium oxide	—	—	—	0.31	0.31	0.31
White salt	—	—	—	0.31	0.31	0.31
Mineral premix ¹	0.67	0.67	0.67	0.63	0.63	0.63
Vitamin premix ²	0.67	0.67	0.67	0.63	0.63	0.63
Sodium bicarbonate	—	—	—	0.69	0.69	0.69
Pomegranate seed	—	2.92	2.92	—	2.19	2.19
Pomegranate peel	—	—	8.33	—	—	6.25
Chemical composition, % of DM (unless otherwise noted)						
DM	57.5	55.9	57.3	57.2	57.3	58.1
CP	13.3	13.5	13.0	17.5	17.2	17.6
NDF	42.3	41.4	42.1	34.9	35.3	33.4
Physically effective NDF	28.0	29.0	29.0	23.0	24.0	23.0
NFC ³	34.4	34.4	35.4	37.1	36.3	37.9
Ether extract	3.1	3.5	3.5	3.9	4.2	4.2
NE _L , Mcal/kg of DM	1.53	1.52	1.54	1.61	1.61	1.63
Calcium	0.48	0.46	0.53	0.81	0.81	0.80
Phosphorous	0.35	0.36	0.36	0.37	0.38	0.39
Total phenolic compounds, g/kg	0.280	0.563	1.18	0.519	0.855	1.36
Antioxidant activity						
Inhibition of radical activity, %	13.51	11.98	20.85	13.30	13.94	27.92
AAEAC, ⁴ mg/100 g	23.0	18.9	43.4	22.5	24.3	63.1

¹Contained per kilogram (DM basis) 0.8 g of iron; 16 g of zinc; 10 g of manganese; 4 g of copper; 0.15 g of iodine; 0.12 g of cobalt; and 0.08 g of selenium.

²Contained per kilogram (DM basis) 1,300,000 IU of vitamin A; 360,000 IU of vitamin D₃; 12,000 IU of vitamin E.

³NFC = $100 - (\% \text{ NDF} + \% \text{ CP} + \% \text{ fat} + \% \text{ ash})$ based on NRC (2001).

⁴Ascorbic acid equivalent antioxidant capacity.

Photonix Ar, 2017) with tannic acid as standard (1–100 mg/L; Bamdad et al., 2006). The antioxidant capacity of the extracts was measured using 1,1-diphenyl-2-picryl hydroxyl method. Five milliliters of a 0.1 mM methanolic solution of 1,1-diphenyl-2-picryl hydroxyl was mixed with 0.1 mL of sample extracts or ascorbic acid (2–100 mg/L). Control was prepared this way without any extracts. Changes in the absorbance of the samples were read at 517 nm. The percentage inhibition was calculated by the equation (control OD – sample OD)/control OD $\times$ 100. Chemical analysis of the feeds were performed in triplicates and means are presented in Table 1.

On d 25 postpartum, ruminal fluid were obtained 4 h after the morning feeding using a stomach tube attached to an Erlenmeyer flask connected to vacuum pump. After discarding initial sample (~100 mL), sample was strained through a 4-layers of cheesecloth and pH was determined immediately using a portable pH meter (HI 8314 membrane pH meter, Hanna Instruments, Villafraanca, Italy). A sample of the filtered ruminal fluid (5 mL) was added to 1 mL of metaphosphoric acid (250 g/L) and retained for short-chain fatty acids (SCFA) determination. Individual SCFA concentrations were measured by GC (CP-9002; Chrompack, Middelburg, the Netherlands) using a silica-fused column (CP-Wax Chrompack Capillary Column; Varian, Palo Alto, CA) with helium as a carrier gas. The initial and final oven temperatures were set at 55 and 195°C, respectively. Detector and injector temperatures were set at 250°C. Crotonic acid (1:7, vol/vol) was used as the internal standard.

For all cows, blood samples were collected 4 h after the morning feeding on d –14, 1, 7, 14, and 21 relative to expected calving. Blood samples were taken from coccygeal vessel using evacuated tubes containing anticoagulant heparin. Plasma samples were separated following centrifugation (2,000 $\times$ g, 20 min, room temperature) and stored frozen in plastic micro-tubes at –20°C for later analysis. Samples were analyzed for concentrations of albumin (photometric test using bromocresol green, 546 nm; Catalog number: 101500), total protein (Biuret method, 560 nm; Catalog number: 128500), triacylglycerol (TAG; enzymatic method using glycerol-3-phosphate oxidase-phenol 4 aminoantipyrine peroxidase, 505 nm; Catalog number: 132500), cholesterol (enzymatic method using cholesterol oxidase-phenol 4-aminoantipyrine peroxidase, 546 nm; Catalog number: 110500), glucose (enzymatic method using glucose oxidase-phenol 4-aminoantipyrine peroxidase, 505 nm; Catalog number: 117500), and aspartate aminotransferase (AST; International Federation of Clinical Chemistry and Laboratory Medicine method without pyridoxal phosphate, 340 nm; Catalog num-

ber: 118400) using commercial kits (Pars Azmun Co. Ltd., Karaj, Iran). The assay procedures followed the manufacturer's instructions (Pars Azmun Co. Ltd.; <http://parsazmun.ir/Products/Index/2022>) by an autoanalyzer (BT 1500, Biotecnica SpA, Rome, Italy). Globulin concentration was calculated by subtracting total protein from albumin. Concentrations of BHB (β -hydroxybutyrate dehydrogenase method, 340 nm), free fatty acids (FFA; RX Monza test), and total antioxidant capacity (TAC; metmyoglobin chromogen method) were measured calorimetrically by commercial kits (Randox Laboratories Ltd., Ardmore, UK) using the same autoanalyzer. Malondialdehyde (MDA) levels were measured based on the reaction of MDA with thiobarbituric acid as described by Zhang et al. (2006), and the absorbance was measured at 532 nm. For determination of superoxide dismutase (SOD) activity, a blood sample was centrifuged for 10 min at 2,000 $\times$ g at room temperature and then aspirated off the plasma. Erythrocytes were washed 4 times with 3 mL of 0.9% NaCl solution and centrifuging for 10 min at 2,000 $\times$ g (room temperature). After the final wash, erythrocytes were lysed using 2.0 mL of cold redistilled water and left at 4°C for 15 min. The lysate was diluted with 0.01 mol/L of phosphate buffer (pH 7.0). The activity of SOD was measured using xanthine and xanthine oxidase to generate superoxide radicals which react with iodonitrotetrazolium chloride (INT, 505 nm) using Randox kit (Catalog number: SD 125; Randox Laboratories) according to the manufacturer's protocol (http://www.tokyofuturestyle.com/pdf/randox_RANSOD.pdf).

Statistical Analysis

Two cows from the CON treatment and 1 cow from the PS treatment were removed because of displaced abomasum, lameness, and milking problems through the experiment. However, 1 cow was added to treatment 1 to have at least 9 replications. The blood metabolites data were analyzed for d –14 as prepartum data and d 1, 7, and 21 as postpartum. Data were analyzed for a completely randomized block design using the mixed model procedure in SAS (SAS 9.0 for windows, SAS Institute Inc., Cary, NC). Feed intake, BW change, and blood parameter data were analyzed separately for 2 discrete periods as prepartum (d –25 to 0 relative to calving) and postpartum (d 1 to 25 relative to calving) periods. Time was used as repeated measures and the first-order autoregressive variance-covariance matrix structure that provided the best fit according to Schwarz's Bayesian information criterion. Variables with repeated measurements (DMI, BW, milk yield, feed efficiency, and blood parameters) within the same cow were analyzed using the model

Table 2. Chemical composition, phenolic compounds, and antioxidant activity of pomegranate seed and pomegranate peel

Item	Pomegranate seed	Pomegranate peel
Chemical composition		
DM, % of sun dried	88.0	81.3
CP, % of DM	12.6	3.02
NDF, % of DM	49.6	19.7
ADF, % of DM	39.0	13.1
Ether extract, % of DM	12.1	1.94
Ash, % of DM	2.03	3.88
NFC ¹	8.33	68.9
Phenolics, g/kg		
Total phenolic compounds	1.61	25.5
Total tannins	1.31	21.9
Condensed tannins	0.11	3.62
Antioxidant activity		
Inhibition of radical activity (1,1-diphenyl-2-picryl hydroxyl), %	12.8	91.6
AAEAC, ² mg/100 g	21.1	240.2

¹NFC = 100 – (% NDF + % CP + % Fat + % Ash).

²Ascorbic acid equivalent antioxidant capacity.

$$Y_{ij(k)m} = \mu + P_i + T_j + C(T)_{jk} + D_l + T \times D + e_{ij(k)m},$$

where $Y_{ij(k)m}$ was each observation, μ was the overall mean, P_i was the fixed effect of parity i , T_j was the fixed effect of treatment j , $C(T)_{jk}$ was the random effect of cow k within treatment j , D_l was the fixed effect of time (day or week) l , $T \times D$ was the fixed interaction between time and treatment, and $e_{ij(k)m}$ was the residual. Rumen fermentation data were analyzed with the same mixed-effects model described above but without the effect of time period. The initial values of BW and BCS were considered as covariates. Data were presented as least squares means $\pm$ standard error of the mean. Orthogonal contrasts were used to determine the treatment and parity effects. Contrast statements included (1) CON versus pomegranate by-products (CON vs. PS and PSP); (2) PS versus PSP supplementation (PS vs. PSP); and (3) primiparous versus multiparous. The effects were considered significant when $P \leq 0.05$. A trend was considered to exist if $0.05 < P \leq 0.10$.

RESULTS

Pomegranate By-Products

Contents of DM, CP, ether extract, NDF, and phenolic compounds were different between pomegranate seeds and peels (Table 2). Pomegranate seed was high in fiber content (NDF = 49.6%), intermediate in fat (12.1%) and CP (12.6%), and low in total phenolic and tannin compounds. The concentration of NFC (68.9 vs. 8.3%) as well as total phenolic compounds (25.5 vs. 1.6 g/kg of DM) and tannin (21.9 vs. 1.3 g/kg of DM) was

found to be higher in pomegranate peels than pomegranate seeds. Pomegranate peels contained relatively low levels of NDF (19.7%), CP (3.0%), and ether extract (1.9%). Chemical composition of the 3 diets fed to dairy cows indicated similar energy and protein levels (Table 1). It was found that the inclusion of pomegranate by-products increased the content of total phenolic of experimental diets. The antioxidant activity of PS diets was similar to that of CON diet. However, the antioxidant activity were increased in both pre- and postpartum diets supplemented with pomegranate peels due to the high antioxidant activity of pomegranate peels (91.5% inhibition of radical activity).

Blood Metabolic Profile and Oxidative Indices

The mean values and the changes of plasma metabolic status of pre- and postpartum dairy cows are presented in Table 3 and in Figures 1, respectively. We found no effect of dietary pomegranate by-products on pre- and postpartum plasma concentrations of cholesterol, globulin, total protein, and aspartate aminotransferase (Table 3). Prepartum plasma glucose concentration was the same among the 3 treatments, but postpartum level was lower ($P = 0.02$; Table 3) for cows fed pomegranate by-products than cows fed CON. The lowest plasma glucose concentrations were observed in cows fed PSP diet on d 1 ($P = 0.07$), 7 ($P = 0.004$), and 21 ($P = 0.002$) (Figure 1). Pre- and postpartum plasma concentration of TAG was also lower for cows fed pomegranate by-products than for cows fed CON. A lower plasma TAG concentration was observed in cows fed PSP versus cows fed PS at postpartum ($P = 0.04$). Plasma albumin level did not differ significantly be-

Table 3. Blood metabolites and oxidative status of periparturient dairy cows fed diets containing no pomegranate by-products (CON), pomegranate seed (PS), or pomegranate seed pulp (PSP)

Item	Diet			SEM	P-value		
	CON	PS	PSP		CON vs. PS and PSP	PS vs. PSP	Primiparous vs. multiparous
Glucose, mg/dL							
Prepartum	68.5	67.5	69.8	1.53	0.94	0.37	0.001
Postpartum	64.0 ^a	61.6 ^a	54.3 ^b	1.73	0.02	0.02	0.65
Cholesterol, mg/dL							
Prepartum	94.9	80.6	84.1	11.0	0.12	0.70	0.66
Postpartum	139.2	134.5	120.6	7.17	0.20	0.18	0.52
Triacylglycerol, mg/dL							
Prepartum	23.9 ^a	20.2 ^{ab}	19.0 ^b	1.28	0.02	0.57	0.24
Postpartum	16.4 ^a	14.2 ^b	12.5 ^c	0.54	<0.001	0.04	0.28
Albumin, g/dL							
Prepartum	3.55	3.54	3.50	0.081	0.79	0.77	0.66
Postpartum	4.05 ^a	3.94 ^{ab}	3.82 ^b	0.063	0.04	0.20	0.01
Globulin, g/dL							
Prepartum	3.15	2.85	3.01	0.153	0.97	0.69	0.12
Postpartum	3.48	3.54	3.61	0.148	0.60	0.76	0.11
Albumin:globulin, g/g							
Prepartum	1.14	1.25	1.19	0.065	0.66	0.90	0.04
Postpartum	1.18	1.67	1.08	0.057	0.43	0.29	0.85
Total protein, g/dL							
Prepartum	6.70	6.37	6.53	0.195	0.92	0.66	0.21
Postpartum	7.54	7.51	7.42	0.157	0.72	0.70	0.02
Aspartate amino transferase, U/L							
Prepartum	51.0	45.0	40.8	6.78	0.36	0.70	0.58
Postpartum	56.3	56.0	51.6	1.81	0.26	0.10	0.16
Free fatty acids (FFA), mEq/L							
Prepartum	0.141 ^a	0.123 ^{ab}	0.106 ^b	0.0085	0.02	0.21	0.009
Postpartum	0.446 ^a	0.313 ^b	0.263 ^b	0.0299	<0.001	0.25	0.97
BHB, mmol/L							
Prepartum	0.862 ^a	0.636 ^{ab}	0.588 ^b	0.084	0.04	0.72	0.89
Postpartum	0.953 ^a	0.802 ^{ab}	0.709 ^b	0.0669	0.02	0.33	0.76
FFA:cholesterol ($\times 10^3$)							
Prepartum	1.45	1.58	1.26	0.579	0.87	0.15	0.09
Postpartum	4.02 ^a	3.07 ^{ab}	2.72 ^b	0.439	0.04	0.58	0.26
FFA:albumin ($\times 10^3$)							
Prepartum	39.9 ^a	34.8 ^{ab}	30.3 ^b	2.35	0.03	0.24	0.006
Postpartum	111.0 ^a	80.9 ^b	69.6 ^b	8.00	0.002	0.33	0.76
Total antioxidant capacity, nmol/L							
Prepartum	0.342 ^b	0.373 ^{ab}	0.396 ^b	0.0152	0.05	0.34	0.44
Postpartum	0.389 ^b	0.467 ^a	0.491 ^a	0.0169	0.02	0.31	0.08
Superoxide dismutase activity, U/L							
Prepartum	164.2	172.6	174.8	6.15	0.22	0.80	0.50
Postpartum	147.6 ^c	153.3 ^b	163.2 ^a	2.84	0.002	0.01	0.33
Malondialdehyde, μ mol/L							
Prepartum	1.65	1.55	1.08	0.180	0.16	0.11	0.20
Postpartum	1.70 ^a	1.50 ^{ab}	1.25 ^b	0.111	0.02	0.12	0.02

^{a-c}Means with different superscripts differ ($P < 0.05$).

tween the 3 dietary treatments at prepartum; however, cows supplemented with pomegranate by-products had lower ($P = 0.04$) plasma albumin level than cows fed CON during postpartum.

Results for plasma FFA concentration on d -14, 1, 7, and 21 relative to calving are shown in Figure 1. Plasma FFA concentrations increased at calving and gradually decreased after calving in cows receiving the PS and PSP diets, but remained almost constant in cows offered the CON diet to d 21 postpartum (Figure 1). Cows fed CON had greater FFA concentration dur-

ing prepartum ($P = 0.02$) and postpartum ($P < 0.001$) than cows fed the pomegranate by-products, with the lowest value observed in cows fed PSP (Table 3). This led to greater pre- ($P = 0.03$) and postpartum ($P < 0.002$) FFA-to-albumin ratio in cows fed CON than cows supplemented the pomegranate by-products despite the greater plasma albumin level in CON group. Moreover, cows on CON diet had a higher ($P = 0.04$) FFA-to-cholesterol ratio than cows on pomegranate by-products diets at postpartum, with no difference between PS and PSP. The highest plasma concentra-

tion of BHB was observed on d 1 after calving, with the highest value observed in cows on CON diet (Figure 1). The BHB concentration was lower at pre- ($P = 0.04$) and postpartum ($P = 0.02$; no treatment effects on d +7 and +21, Figure 1) in cows fed pomegranate by-products compared with cows fed CON, without any difference between PSP and PS diets (Table 3).

Plasma total antioxidant capacity was lower at prepartum than postpartum and unchanged up to d 1 post-calving and then increased in all cows (Figure 1). The mean TAC concentration was 0.377, 0.442, and 0.466 nmol/L for cows received the CON, PS, and PSP diets,

respectively (Table 3). Pre- and postpartum plasma levels of TAC were lower in cows received CON than cows supplemented with pomegranate by-products, with no difference between PS and PSP diets. The concentration of plasma MDA showed a trend opposite to that of TAC (Figure 1). The mean plasma concentration of MDA was 1.69, 1.50, and 1.21 $\mu\text{mol/L}$ for cows on CON, PS, and PSP diets, respectively. Cows on the CON diet had greater plasma MDA concentration at postpartum ($P = 0.02$) compared with cows on pomegranate by-products diets (Table 3). Blood SOD was greater at prepartum than postpartum and unchanged up to

Figure 1. Blood glucose, free fatty acids (FFA), BHB, superoxide dismutase (SOD), total antioxidant activity (TAC), and malondialdehyde (MDA) concentration of periparturient dairy cows fed diets containing no pomegranate by-products (CON), pomegranate seed (PS), or pomegranate seed pulp (PSP). Data were expressed as LSM $\pm$ SEM. * $P < 0.05$.

d 1 postcalving, but thereafter decreased in all cows (Figure 1). The mean SOD activity was 151, 159, and 166 U/L for the CON, PS, and PSP diets, respectively. Prepartum blood SOD activity was similar among the treatments (Table 3). Nevertheless, cows receiving the CON diet showed lower ($P = 0.002$) blood SOD activity than cows receiving the pomegranate by-products diets, with the highest value observed in cows receiving the PSP diet ($P = 0.01$). Primiparous cows had higher plasma glucose (prepartum; 72.7 vs. 64.4 mg/dL, $P = 0.001$) and MDA (postpartum; 1.64 vs. 1.32 $\mu\text{mol/L}$, $P = 0.02$) levels, but lower albumin (postpartum; 3.86 vs. 4.01 g/dL, $P = 0.05$), FFA (prepartum; 0.108 vs. 0.139 mEq/L, $P = 0.009$), FFA-to-albumin ratio (prepartum; $P = 0.006$), and TAC (postpartum; 0.432 vs. 0.467 nmol/L, $P = 0.08$) than multiparous cows (Table 3).

DMI, Milk Production, and Ruminal Fermentation

Dry matter intake, BW, BCS, milk yield, and milk composition of transition cows fed diets supplemented with pomegranate by-products are reported in Table 4. Prepartum DMI was 15.7, 15.4, and 16.8 kg/d and postpartum DMI was 16.7, 16.4, and 18.0 kg/d for cows fed CON, PS, and PSP diets, respectively. No differences were observed in pre- and postpartum DMI between CON and pomegranate by-products supplemented groups and between PS and PSP groups ($P > 0.05$). The actual DMI values were greater than the predicted DMI (12 and 16 kg/d for pre- and postpartum, respectively), which lead to slightly greater intake of pomegranate seed (0.4 vs. 0.35 kg/d) and pomegranate seed pulp (1.7 vs. 1.35 kg/d) than those predicted before the experiment. Initial BW and BCS were similar across dietary treatment. However, BW change (kg/d, from d -25 to 25 relative to calving) was significantly higher ($P = 0.05$) and BCS change tended to be greater ($P = 0.08$) in CON versus pomegranate-supplemented groups. No effect of pomegranate by-products supplementation on milk yield and composition were found across the dietary treatments. However, a trend ($P = 0.09$) for increased 3.5% FCM was observed when pomegranate by-products were supplemented in the diet. Yields of 3.5% FCM ($P = 0.02$), ECM ($P = 0.02$), milk fat ($P = 0.01$), TS ($P = 0.02$), protein ($P = 0.07$), and lactose ($P = 0.09$) were higher for cows fed the PSP diet than cows fed the PS diet, with no difference between CON and PS. As cows fed the PSP diet consumed numerically more DM and produced more milk, milk efficiency (milk yield/DMI) was similar across dietary treatments. Furthermore, cows fed diets containing pomegranate by-products during 180 d postpartum (1 record per month) tended to have a greater milk yield ($P = 0.08$) compared with those fed the CON diet. Initial BCS was

the same between primiparous and multiparous cows, but multiparous cows had greater DMI (18.7 vs. 15.4 kg/d, $P = 0.02$), milk production (44.7 vs. 32.8 kg/d, $P < 0.001$), and milk efficiency (2.43 vs. 2.15, $P = 0.05$) and tended to have higher milk fat content (3.45 vs. 3.07%, $P = 0.06$), as well as BW change ($P = 0.15$), than primiparous cows.

Ruminal pH did not differ between CON and pomegranate by-product-supplement groups or between PS and PSP (Table 5). Moreover, supplementation of pomegranate by-products in the diets did not affect total SCFA concentrations, the molar proportions of propionate, butyrate, valerate, isovalerate, or the acetate-to-propionate ratio.

DISCUSSION

Pomegranate By-Products

Iran is one of the world's largest producers and exporters of pomegranate fruit, with approximately 1 million tonnes of production in 2014 (Ministry of Agriculture, 2015). As a result of pomegranate juice production, a great quantity of pomegranate by-products (over 50%), mainly seeds and peels that contain high levels of moisture (~20% DM), are generated. The storage of these by-products require additional processing methods, such as ensiling, drying, or extracting their bioactive compounds before feeding. In our study, sun-dried pomegranate peels had 25.5 g/kg of DM phenolic compounds, similar to the phenolic content of pomegranate silage (22.5 g/kg of DM; Kotsampasi et al., 2014). The pomegranate seed used in the current study contained 12.1% fat. The oil content in pomegranates seed of 25 varieties grown in Iran ranged from 6.6 to 19.3% with punicic acid as the predominant FA (31.8 to 86.6%; Fadavi et al., 2006). We hypothesized that the supplementation of pomegranate seed or combined pomegranate seed and peels with rich bioactive compounds, such as conjugated FA and phenolics, might improve antioxidant status, thereby exerting positive influences on health and performance of dairy cows during transition period. In the present study, inclusion of pomegranate by-products into pre- and postpartum TMR, in particular PSP, increased dietary total phenolic contents (>2 times) and antioxidant activity (81.9%).

Oxidative and Metabolic Status

Under normal cellular metabolism, a balance exists between the levels of ROS and levels of endogenous antioxidants (Pham-Huy et al., 2008). However, the overproduction of ROS (e.g., during transition period

Table 4. Dry matter intake, BW, BCS, milk yield, milk composition, and feed efficiency of periparturient dairy cows fed diets containing no pomegranate by-products (CON), pomegranate seed (PS), or pomegranate seed pulp (PSP)

Item	Diet				P-value		
	CON	PS	PSP	SEM	CON vs. PS and PSP	PS vs. PSP	Primiparous vs. multiparous
DMI, kg/d							
Prepartum	15.7	15.4	16.8	0.62	0.64	0.12	0.02
Postpartum	16.7	16.4	18.0	1.05	0.69	0.29	0.009
BW ¹							
Initial, kg	708	692	733	21.9	0.98	0.13	<0.001
Change, kg/d	−2.93	−2.21	−2.26	0.268	0.05	0.90	0.15
BCS ¹							
Initial	3.47	3.44	3.39	0.086	0.65	0.69	0.97
Change	−0.720	−0.581	−0.442	0.099	0.08	0.73	0.99
Yield, kg/d							
Milk	37.1	38.4	41.6	1.96	0.21	0.18	<0.001
3.5% FCM ²	34.6 ^b	35.4 ^b	42.8 ^a	2.05	0.09	0.02	<0.001
ECM ²	35.4 ^b	36.1 ^b	43.4 ^a	2.03	0.09	0.02	<0.001
Fat	1.16 ^b	1.18 ^b	1.52 ^a	0.086	0.09	0.01	<0.001
Protein	1.16	1.18	1.38	0.076	0.22	0.07	<0.001
Lactose	1.77	1.85	2.13	0.109	0.12	0.09	<0.001
TS	4.54 ^b	4.69 ^b	5.58 ^a	0.248	0.06	0.02	<0.001
Yield, kg/d (records up to 180 d postpartum)							
Milk	39.6	43.2	44.0	1.75	0.08	0.75	0.001
Milk composition, %							
Fat	3.16	3.11	3.52	0.163	0.44	0.09	0.06
Protein	3.15	3.13	3.31	0.105	0.58	0.25	0.70
Lactose	4.79	4.99	5.10	0.186	0.28	0.68	0.71
TS	12.36	12.57	13.27	0.296	0.13	0.11	0.91
Milk efficiency, kg/kg							
Milk/DMI	2.19	2.36	2.33	0.112	0.26	0.82	0.05
FCM/DMI	2.07	2.24	2.36	0.141	0.18	0.53	0.02
ECM/DMI	2.11	2.28	2.40	0.133	0.16	0.50	0.02

^{a,b}Means within a row with different superscripts differ ($P < 0.05$).

¹Body weight and BCS changes during the transition period (d −25 to +25 relative to parturition).

²Yields of 3.5% FCM = $0.432 \times \text{milk yield} + 16.23 \times \text{fat yield}$ and ECM = $12.82 \times \text{fat yield} + 7.13 \times \text{protein yield} + 0.323 \times \text{milk yield}$ according to NRC (2001) equations.

due to augmented metabolic demands) overwhelms the neutralizing capacity of antioxidant mechanisms, and this might results in high lipid peroxidation and decreased level of antioxidant enzymes (Bernabucci et al., 2005; Bertoni et al., 2006; Pham-Huy et al., 2008). A recent study has provided increasing evidence that

oxidative stress has been associated with several health problems, especially during transition period (Abuelo et al., 2016). Our results demonstrated that supplementation with either PS or PSP had positive effects on antioxidant status, as indicated by enhanced blood TAC and SOD activity and depressed MDA level. Similar to

Table 5. Rumen fermentation of periparturient dairy cows fed diets containing no pomegranate by-products (CON), pomegranate seed (PS), or pomegranate seed pulp (PSP)

Item	Diet				P-value		
	CON	PS	PSP	SEM	CON vs. PS and PSP	PS vs. PSP	Primiparous vs. multiparous
pH	6.19	6.17	6.27	0.152	0.86	0.65	0.79
SCFA, ¹ mM	72.5	77.3	82.9	5.78	0.32	0.57	0.25
Acetate, %	65.8	66.3	64.0	2.76	0.83	0.55	0.63
Propionate, %	23.2	23.3	24.4	1.84	0.78	0.67	0.83
Butyrate, %	9.02	8.74	10.94	1.43	0.65	0.31	0.21
Valerate, %	1.72	1.52	1.52	0.107	0.12	0.99	0.02
Isovalerate, %	0.125	0.098	0.108	0.011	0.11	0.50	0.25
Acetate:propionate	3.08	2.98	2.79	0.385	0.68	0.72	0.77

¹Short-chain fatty acids.

these findings, herbal supplementation with rich polyphenol contents increased blood TAC and decreased MDA levels during the transition period (Hashemzadeh-Cigari et al., 2015). Previous studies also indicated that total phenolic contents and antioxidant activity in meat (Kotsampasi et al., 2014) and milk (Shabtay et al., 2012) were improved significantly when animals were fed diets containing pomegranate-derived supplements. Phenolic substances can prevent lipid peroxidation by scavenging free radicals or by activation of antioxidant enzymes such as glutathione reductase, glutathione peroxidase, and SOD (Tzulker et al., 2007; Wang et al., 2010). Moreover, conjugated FA, such as CLA or those found in PS (i.e., punicic acid) have been shown to be effective supplements in terms of lipid peroxidation by inhibition of prostaglandins biosynthesis (Singh et al., 2002; Hanschke et al., 2016). The results of our study indicated a larger improvement in antioxidant status and lipid peroxidation inhibitory in cows supplemented with both pomegranate seed and peel (PSP) compared with those supplemented with pomegranate seed alone. In accord, previous studies (Singh et al., 2002; Tzulker et al., 2007) demonstrated higher antioxidant activity (54%) of pomegranate peel than seed, which can be related to the high radical-scavenging activities of phenolic contents such as punicalagin or punicalin in pomegranate peel.

High plasma concentrations of BHB at prepartum (0.86 mM) and postpartum (d 1 = 1.03 mM and d 7 = 0.99 mM) were observed when cows were fed the CON diet. It has been suggested that prevalence rates of postpartum disorders (e.g., displaced abomasum) is higher when plasma BHB concentrations are above 0.6 to 0.8 mM at prepartum and 1.0 to 1.4 mM at postpartum (Ospina et al., 2013). Plasma BHB concentrations decreased when cows were fed diets supplemented with either PS (26% prepartum and 16% postpartum) or PSP (31% prepartum and 26% postpartum). Around calving, mobilization of FA from TAG stores in adipose tissue are favored due to increasing energy requirement and decreasing DMI (Contreras and Sordillo, 2011). In the liver, FFA can be completely oxidized to CO₂ to provide energy, partially oxidized to generate ketone bodies, or esterified to TAG and exported from the liver. An alternative pathway for liver FFA oxidation is an increase in the peroxisomal oxidation capacity, which contributes to the production of ROS and affects inflammatory responses (Sordillo and Raphael, 2013). In the current study, the reduction of BHB level was parallel with the decrease of FFA (33%) and MDA (20%) concentration by feeding diets containing either PS or PSP. Our results agree with other studies that reported a lower FFA concentration in dairy sheep (Chiofalo et al., 2012) and cattle (Hashemzadeh-Cigari et

al., 2015) supplemented with medicinal plant extracts. Bernabucci et al. (2005) demonstrated increased oxidative stress in cows with greater BCS loss concurrent with higher FFA and BHB concentrations postpartum. In our study, initial cow BW and BCS were similar among treatment groups, but cows fed the CON diet had greater BW (25%) and BCS (28%) loss than cows supplemented with pomegranate by-products. Whereas FFA are a major fuel source during periods of energy shortage, high concentrations of FFA are known to disrupt several inflammatory and immune functions (Sordillo and Raphael, 2013). Dietary supplementation with antioxidants can protect against the damaging influence of oxidants and downregulate mRNA expression of proinflammatory cytokines, which adversely affect lipolysis rate in transition cows (Bertoni et al., 2008; Sordillo and Raphael, 2013). Polyphenol compounds, such as hydrolysable tannins, anthocyanins, and ellagic acid, have been shown to correlate positively with antioxidant and anti-inflammatory activities in pomegranate peel and juice (Tzulker et al., 2007; Jurenka, 2008). Furthermore, it was previously shown that pomegranate seed oil possess antioxidant and anti-inflammatory activity by inhibiting cyclooxygenases and lipoxygenases (Singh et al., 2002).

Dietary supplementation with pomegranate by-products resulted in a significant decrease of postpartum blood glucose levels and the lowest values (d 1, 7, and 25) were observed when cows were fed the diet containing PSP. As dietary pomegranate by-product supplementation had no effect on DMI and ruminal fermentation pattern (i.e., propionate), lower blood glucose may be related to greater FCM yield, antioxidant status, or degree of insulin sensitivity. This would likely suggest an enhancement in insulin sensitivity in cows fed the PSP diet, which consequently increased glucose utilization and decreased fat mobilization and plasma non-esterified FA and BHB levels. It has been reported that quercetin (Gohlke et al., 2013) or herbal plant mixture (Hashemzadeh-Cigari et al., 2015) supplementation in cows resulted in changes in glucose metabolism, indicating increased insulin release or sensitivity; those authors suggested that the response might be responsible for improved performance response. Experimental evidence supports the idea ROS are an important trigger for insulin resistance, and reduced glucose consumption capacity and antioxidant supplementation can be used to decrease the consequences of insulin resistance (Houstis et al., 2006; Abuelo et al., 2016).

We found no effects of pomegranate by-products supplementation on plasma cholesterol, FFA-to-cholesterol ratio, total protein, globulin, and AST concentrations, and the values were within the ranges of normally fed cows. Supplementation with pomegranate by-products

lowered plasma TAG concentration in both the pre- and postpartum periods. In transition cows, adipose tissues are mobilized and FFA are transported to the liver where they are either oxidized or re-esterified to TAG and then are packed in very low density lipoproteins to export from the liver (Bertoni et al., 2006). Because plasma cholesterol concentration were well above the critical value (~ 2 mM or 77 mg/dL; Bertoni et al., 2008), the lower plasma TAG in supplemented cows may be related to lowered body fat mobilization (lower BW loss) or enhanced clearance from plasma lipoproteins. Sahin et al. (2006) observed that supplementation of antioxidants decreased elevated levels of plasma cholesterol and TAG under stress exposure. Albumin is a negative acute phase protein in plasma and contains domains to bind hydrophobic compounds, including FFA. Although albumin concentration was the lowest in cows fed diets containing pomegranate by-products, all the cows had albumin concentrations well above the critical value (3.0 g/dL) based on liver activity index (Bertoni et al., 2008). The ratio of FFA to albumin, which is as an index for predicting postpartum disease incidence (Contreras and Sordillo, 2011), was greater in cows fed CON than those fed pomegranate by-products, indicating cows supplemented with PSP and PS possibly had a slight improvement in liver function than unsupplemented cows. In humans, elevated FFA-to-albumin ratios are part of pathological changes of metabolic diseases such as type 2 diabetes (Cnop et al., 2001).

Intake and Lactation Performance

Dietary supplementation with PS or PSP had no effect on pre- and postpartum DMI, ruminal pH, SCFA concentrations, and molar proportions of individual SCFA. Inclusion of pomegranate extracts in the diet of dairy cows has been shown to improve DMI and rumen pH in some (Jami et al., 2012), but not all (Abarghuei et al., 2013; Shaani et al., 2016), studies. These differences may be due to different type and level of pomegranate supplements (pomegranate residues or extract), as well as different animal conditions. Pomegranate peel is rich in tannin compounds, which have been shown to have both adverse and beneficial effects, such as reducing ruminal protein degradation, DMI, and CH_4 production and increasing concentration of propionic acid. Moderate levels of tannin-containing feed supplementation improved nitrogen balance, milk production of ruminants without negatively affecting DMI (Hymes-Fecht et al., 2013). Jami et al. (2012) found that supplementing with 4% pomegranate peel extract to lactating cows improved ruminal pH, DMI, and nutrient digestibility. Our findings show that feeding PSP at level of 8 to 10%

in transition dairy cows (providing 50 g/d of tannin in the diet DM) was not excessive to reduce palatability or rumen microorganism activities, thereby decreasing SCFA production and DMI.

The supplementation of pomegranate by-products had no effect on milk yield and milk efficiency; however, supplementing diet containing PSP significantly increased FCM, ECM, and milk composition yields. Different effects of adding pomegranate-derived products on performance responses have been reported for growing cattle and lactating dairy cows (Shabtay et al., 2008, 2012; Jami et al., 2012; Abarghuei et al., 2013; Shaani et al., 2016). Although DMI was not affected by treatments in our experiment, the improvement in metabolic and antioxidative status in cows fed the PSP diet may be responsible in part for higher milk production performance. Shabtay et al. (2012) reported that mid-lactation cows with high SCC fed 4% concentrated pomegranate extract were more resistant to mastitis and produced 9.4% more milk than their control counterparts. Metabolic disorders are a key problem in the transition period of dairy cows and results in milk lost during the entire lactation. Wang et al. (2010) observed an improved antioxidative status and a tendency for increased FCM yield when a synthetic antioxidant was included during transition period.

We showed that blood level of MDA during postpartum was significantly greater and that of TAC and albumin was lower in primiparous cows compared with multiparous cows. However, primiparous cows produced less milk and showed a lower level of FFA than multiparous cows. These findings suggest that primiparous cows had more oxidative stress but less metabolic load around parturition. Ospina et al. (2010) indicated that milk yield was increased in primiparous cows and decreased in multiparous cows when FFA and BHB concentrations were greater than the critical values. A recent study by Omid et al. (2017) demonstrated that primiparous cows were more sensitive to oxidative stress as a result of lower TAC than multiparous cows, and supplemental dietary antioxidants (vitamin E and zinc) reduced oxidative stress and severity of udder edema in primiparous cows (NRC, 2001). New physiological experience (giving birth for the first time) and feeding management strategies (e.g., unexperienced high-grain diets) could be possible reasons for more susceptibility of primiparous cows (NRC, 2001; Humer et al., 2018).

CONCLUSIONS

Feeding pomegranate by-products during transition periods (8–10% diet DM) had no effect on DMI, but enhanced overall antioxidant capacity and declined lipid peroxidation (MDA). These beneficial effects were

associated with decreases in BW loss, FA mobilization, and oxidation (lowered FFA and BHB concentrations). However, larger improvements in antioxidant status and metabolic profile, as well as ECM production following PSP supplementation, were observed, possibly due to the feeding combined bioactive compounds (phenolics and conjugated FA) and a high antioxidant level of pomegranate peel.

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