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Effects of graded levels of dietary pomegranate peel on methane and nitrogen losses, and metabolic and health indicators in dairy cows

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ABSTRACT

This study aimed to quantify the effects of dietary inclusion of tannin-rich pomegranate peel (PP) on intake, methane and nitrogen (N) losses, and metabolic and health indicators in dairy cows. Four multiparous, late-lactating Brown Swiss dairy cows (796 kg body weight; 29 kg/d of energy corrected milk yield) were randomly allocated to 3 treatments in a randomized cyclic change-over design with 3 periods, each comprising 14 d of adaptation, 7 d of milk, urine, and feces collection, and 2 d of methane measurements. Treatments were formulated using PP that replaced on a dry matter (DM) basis 0% (control), 5%, and 10% of the basal mixed ration (BMR) consisting of corn and grass silage, alfalfa, and concentrate. Gaseous exchange of the cows was determined in open-circuit respiration chambers. Blood samples were collected on d 15 of each period. Individual feed intake as well as feces and urine excretion were quantified, and representative samples were collected for analyses of nutrients and phenol composition. Milk was analyzed for concentrations of fat, protein, lactose, milk urea N, and fatty acids. Total phenols and antioxidant capacity in milk and plasma were determined. In serum, the concentrations of urea and bilirubin as well as the activities of alanine aminotransferase (ALT), aspartate aminotransferase, glutamate dehydrogenase, alkaline phosphatase, and γ-glutamyl transferase were measured. The data was subjected to ANOVA with the Mixed procedure of SAS, with treatment and period as fixed and animal as random effects. The PP and BMR contained 218 and 3.5 g total extractable tannins per kg DM, respectively, and thereof 203 and 3.3 g hydrolyzable tannins. Total DM intake, energy corrected milk, and methane emission (total, yield, and intensity) were not affected by PP supplementation. The proportions of C18:2 *n*-6 and C18:3 *n*-3 in milk increased linearly as the amount of PP increased in the diet. Milk urea N, blood urea N, and urinary N excretion decreased linearly with the increase in dietary PP content. Total phenols and antioxidant capacity in milk and plasma were not affected by the inclusion of PP. The activity of ALT increased in a linear manner with the inclusion of PP. In conclusion, replacing up to 10% of BMR with PP improved milk fatty acid composition and alleviated metabolic and environmental N load. However, the elevated serum ALT activity indicates an onset of liver stress even at 5% PP, requiring the development of adaptation protocols for safe inclusion of PP in ruminant diets.

Key words: *Punica granatum*, hydrolyzable tannin, milk fatty acid, ruminant

INTRODUCTION

Millions of tons of fruit pomaces are produced annually worldwide and disposed of, which is problematic due to environmental pollution of landfills and high costs (Shalini and Gupta, 2010). Pomegranate shrub and tree (*Punica granatum* L.) is an important cash crop and has been cultivated since ancient times throughout the entire Mediterranean region of Asia, Africa, and Europe. Today, important pomegranate producers are India, China, Iran, Turkey, and the USA (Valero et al., 2015). During the industrial processing of pomegranate to juice, the peel, pulp, and seeds of the pomegranate fruit arise as by-products, and either are kept separately or are combined to form pomegranate pomace. Consequently, substantial amounts of these pomegranate by-products could be available each year as an alternative feed for livestock that does not compete with human food production (Shabtay et al., 2008).

Pomegranate peel (**PP**) is a rich source of plant secondary compounds, such as polyphenols, especially hydrolyzable tannins (**HT**), and thus a potential functional feed ingredient in ruminant diets for improving redox and health status (Gessner et al., 2017; Safari et

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al., 2018) as well as protein utilization (Shabtay et al., 2008). One of the main HT components is gallic acid, which reduced ruminal CP degradation in vitro supposedly by binding to feed protein (Wei et al., 2019). The PP contains also small amounts of condensed tannins (**CT**), that are known for their even more pronounced ability to bind protein by hydrophobic interactions in the rumen, thereby slowing down its rumen microbial degradation and increasing the dietary, ruminal undegradable protein fraction (Woodward et al., 2001). This inhibition of ruminal CP degradation was shown when PP fed to sheep reduced N excretion and mitigated **N** emissions from the manure in the form of nitrous oxide (Yurtseven et al., 2018) presumably due to reduced ruminal ammonia formation and urinary N losses. Nitrous oxide is a potent greenhouse gas emitted by livestock farming, as is methane (**CH_4**). Accordingly, PP extract has been shown to reduce in vivo CH_4 production from Murrah male buffalos without adversely affecting the efficiency of ruminal fermentation (Hundal et al., 2019). Bhatta et al. (2009) reported that HT suppressed the total population of methanogens in vitro by on average 12%. In our in vitro study, the addition of pomegranate pomace at 15% of DM as a substitute for hay resulted in a 14% reduction in CH_4 yield (per unit DM) compared with the control (Giller et al., 2021). Pomegranate-derived products can also be used to increase milk production and, alter the FA profile in milk fat by direct or indirect effect on ruminal biohydrogenation of fatty acids (**FA**) (Min and Solaiman, 2018; Buccioni et al., 2017). This includes a modulation of the proportion of linoleic and α-linolenic acid, which are considered beneficial for human health (Shabtay et al., 2012; Razzaghi et al., 2015). Importantly, HT as opposed to CT are degradable by ruminal microbes (Bhat et al., 1998). This degradation and the subsequent removal of the resulting HT metabolites from the rumen milieu by absorption questions the relevance of HT not only in terms of the above-mentioned effects but also with regard to the well-known antioxidant effects of polyphenols. Polyphenols may exert antioxidant properties directly by acting as free radical scavengers and metal chelators and indirectly by transcriptional activation of antioxidant enzyme expression (Zhou et al., 2019). In case of incomplete ruminal HT degradation, it is unclear if the absorption of the resulting metabolites may improve the systemic antioxidant status of ruminants. Despite the potential beneficial effects of HT intake, cattle poisoning has been reported after consuming large amounts of pomegranate (5 tonnes of whole and processed fruits offered to 35 steers), potentially due to the hepatotoxicity of the HT punicalagin (Hawes & Gill, 2018).

To our knowledge, the effect of PP or other pomegranate by-products on CH_4 emissions and N metabolism in dairy cows has not been studied to date and studies on the effects of HT-rich feed material in dairy cows are overall scarce (Herremans et al., 2020). We hypothesized that dietary inclusion of PP would: (1) mitigate CH_4 emissions; (2) shift N in the excreta from urine to feces without compromising the animals' performance; (3) enhance the antioxidant capacity of plasma and milk; and (4) improve the milk FA profile for human consumption. Moreover, we hypothesized that (5) including up to 10% of PP in the diet of dairy cows does not cause liver stress. Therefore, the main objective of our research was to test the effects of partially replacing a basal mixed ration (**BMR**) with 5 or 10% of PP on CH_4 and N losses, antioxidant capacity, and milk FA composition of dairy cows. Considering the large amount of HT contained in the PP, we also aimed at investigating the liver functionality of dairy cows.

MATERIALS AND METHODS

The experiment was performed at AgroVet-Strickhof (Eschikon, Lindau, Switzerland) from April to July 2021. The experimental protocol complied with the Swiss legislation for Animal Welfare and was approved by the Committee on Animal Experimentation of the Cantonal Veterinary Office of Zurich (ZH229/2020).

Experimental Design, Animals, and Diets

Four multiparous (4th lactation), late-lactating, pregnant Brown Swiss dairy cows were used in a randomized cyclic change-over design, including 3 dietary treatments and 3 periods. A late stage of lactation was chosen considering the unknown palatability and tolerance of this widely unknown feed. Per period, the 4 cows were randomly allocated to 1 of the treatments with 2 animals receiving the same treatment in each period and with all cows receiving each treatment once, resulting overall in 4 observations per treatment (Table S1). Each period lasted for 33 d and included 14 d of adaptation to the experimental diet, 7 d of sample collection, 2 d of CH_4 measurement, and 10 d of wash-out to minimize carry-over effects (Table S2). Cows were kept in a free stall barn during the first 7 d of the adaptation period and were then housed in a tie-stall with rubber mats for 14 d before they were moved to respiration chambers for 2 d CH_4 measurements. After that, the cows were transferred back to the free stall barn for a 10-d washout period to minimize carry-over effects between the experimental periods. Mastitis occurred in 1 cow in 1 quarter, was treated (Mamycin for 3 d and

Monocillin in the udder for 4 d) and cured on d 1 to 4 during the adaptation in period 2. Data from this cow were in the range of the other data. At the start of the experiment, cows were on average 86 ± 36 d pregnant, 261 ± 46 DIM (mean ± SD), producing 29 ± 2 kg of ECM/d, and weighing 796 ± 30 kg. Cows were fed a BMR composed (% of DM) of corn silage, 45; grass silage, 35; alfalfa, 9.4; concentrate (UFA-250, UFA, Sursee, Switzerland, Table 1), 9; shredded wheat straw, 1.3; mycotoxin binder, 0.2; NaCl, 0.1. Following recommendations by Agroscope (2021), energy (UFA-243, Table 1) and protein (UFA-249, Table 1) concentrates were provided according to the milk yield at a ratio of on average 43:56 by an automatic feeder (free stall) or given manually at each feeding event (tie-stall). Treatments were formulated using PP (purchased from Alfred Galke, Bad Grund, Germany; article 58302, batch 42031, harvested in Turkey in October 2020, sun dried, and disinfected with carbon dioxide) having a particle size of 5−10 mm and replacing 0% (control), 5%, and 10% of BMR on a DM basis (realized: 4.2% and 8.5%). All ingredients contained in the BMR were premixed before feeding, and the replacement was performed based on the DM of the BMR and PP. The 10% of PP were chosen as maximum dosage based on the findings by McSweeney et al. (1988) where the ingestion of 0.9 g HT/kg of BW (from yellow wood, *Terminalia oblongata*) led to signs of toxicity in 2 out of 4 sheep within 15 d. Pomegranate contains HT, of which one is punicalagin. Punicalagin was identified as one of the hepatotoxic components in *T. oblongata*. The realized maximum HT intake of the 4 animals in the present study was 0.67 g/kg of BW. The diet with only half of the maximum dosage (5% PP treatment) was tested to evaluate if a lower PP dose might already provide favorable effects on antioxidant capacity, CH_4 and N emissions at a concomitantly lower risk of liver stress.

Feeding, Weighing, Milking, and Blood Sampling

The cows had free access to water throughout the experiment. The BMR was provided on a weighing plate (custom-made model, Mettler-Toledo, Greifensee, Switzerland) per tie-stall. At each feeding event, the PP was mixed manually with the BMR on the weighing plates to achieve a homogenous distribution and intake of PP relative to BMR. The energy and protein concentrates were not mixed but added on top of the PP-BMR mixture. The total daily amounts of both the energy and protein concentrates and PP were split in half for morning and evening feeding. Cows were fed *ad libitum* in an amount generating at least 10% of daily feed refusal (as-fed basis). Feed was provided in equal amounts twice daily, at 0800 h and 1730 h. Refusals from the previous day were recorded using the weighing plates before the morning feeding to estimate DMI as the difference between DM offered and refused. Separate samples of each feed item (BMR, energy and protein concentrates, and PP) were taken 3 times during the sampling period. The 3 samples were pooled to provide 1 sample per feed item per experimental period, and stored at –20°C. These samples were dried at 60°C for 48 h, ground with a centrifugal mill (Model ZM1, Retsch GmbH, Haan, Germany) to pass a 1-mm screen, and stored at room temperature for subsequent analyses.

Cows were weighed at the start and end of each sampling period with a truckload scale (Waagen Döhrn, model Terra ET, Wesel, Germany) and an average body weight from each period was used for calculation. During the sampling period, cows were milked twice daily at 0630 h and 1630 h, and the milk of the individual cows was collected in buckets and weighed on a scale (ID2 Multirange, Mettler-Toledo) to record the daily milk yield. Milk samples were collected for 7 consecutive days, with daily milk samples pooled from evening milking and subsequent morning milking according to milk yield. Part of the mixed milk samples were preserved with Bronopol® for milk gross composition analysis at the end of each sampling period. For milk FA analysis, mixed milk samples (without Bronopol) were pooled to 2 samples per animal (sample 1: d 1–3, and sample 2: d 4–7) and period. On d 15 of each experimental period (d 1 of the collection period), blood samples were drawn from the jugular vein 2 h after the morning milking and collected into tubes containing EDTA (BD, Polymouth, UK) and clot-activator for plasma and serum collection, respectively. Blood samples were centrifuged (1,200 × *g*, 10 min, 4°C) and stored at −80°C until further analysis as described below.

Feces and Urine Collection

Total collection of feces and urine was performed from d 1 to 7 of the collection period. Feces were collected in steel trays located below a grid at the end of the tie-stall. Urine was collected separately from feces with urinals attached around the vulva of the cows. The urine was drained into a container, and a small subsample was diverted into a canister containing 30 g of 5 M sulfuric acid to prevent gaseous N losses. Feces and urine were weighed and sampled once per day. A proportion of 0.05 of the total feces as well as 25 mL and 50 mL of acidified and non-acidified urine, respectively, were collected and frozen at −20°C. After defrosting, acidified urine samples were analyzed individually, and samples of feces and non-acidified urine were pooled for each cow per period for analysis. Dried fecal samples

Table 1. Chemical composition of basal mixed ration, concentrates, pomegranate peel (PP), and experimental diets (as consumed) (% of DM, unless stated otherwise)

Item	Basal mixed ration[1]	Energy concentrate[2]	Protein concentrate[3]	Pomegranate peel	Diet consumed (% PP in diets)		
					0	5	10
DM (% of fresh matter)	39.0	87.8	88.2	87.2	44.2	45.9	47.9
OM	90.4	93.7	93.6	96.6	90.7	91.0	91.3
CP	13.6	24.8	44.1	3.60	16.0	15.5	15.2
Ether extract	2.89	7.77	5.31	0.42	3.26	3.16	3.07
NDF	44.9	20.3	23.7	22.9	42.5	41.7	40.9
ADF	34.3	14.8	18.4	18.2	32.4	31.9	31.3
ADL	4.15	4.42	4.34	5.58	4.17	4.23	4.28
NFC[4]	29.0	40.8	20.5	69.7	28.9	30.6	32.1
Gross energy (MJ)	17.9	19.5	20.6	17.1	18.1	18.1	18.1
Total phenols	1.29	0.499	0.689	23.7	1.22	2.06	2.93
Non-tannin phenols	0.935	0.460	0.571	1.82	0.89	0.93	0.96
Total tannins	0.349	0.039	0.118	21.8	0.32	1.13	1.96
Condensed tannins	0.017	0	0	1.54	0.02	0.07	0.13
Hydrolyzable tannins	0.333	0.039	0.118	20.3	0.31	1.06	1.83

[1]Composed of (% of DM) corn silage, 45; grass silage, 35; alfalfa, 9.4; concentrate, 9 (UFA-250, UFA, St. Margrethen, Switzerland); straw, 1.3; mycotoxin binder, 0.2; NaCl, 0.1. UFA 250 comprised soybean meal, 40%; wheat, 15%; rapeseed cake, 10%; corn fine flour, 8%; minerals (UFA 1117 TMR, Ca:P 2:1), 8%; corn gluten, 5%; dextrose, 4%; $NaHCO_3$, 3.5%; NaCl, 3%; limestone, 2.5%; Premix 74900, 0.2%; and Sel-Plex 2000, 0.1%. Mineral and vitamin content per kg UFA 250: Ca, 26.9 g; P,12.3 g; Mg, 10.7 g; vitamin A, 55,360 IU; vitamin D_3, 10,920 IU; vitamin E, 255,000 IU).

[2]The energy concentrate (UFA 243 PRIMA IPS, UFA, St. Margrethen, Switzerland) comprised wheat, rapeseed cake, dark distillers grains, soybean meal, corn, soft wheat bran, vegetable refined fatty acids (rapeseed/sunflower), sugar beet molasses, corn gluten, wheat bran, minerals, wheat starch. Mineral and vitamin content per kg UFA 243: Ca, 7.5 g; P, 5.5 g; Mg, 3.0 g; Na, 2.0 g; Zn, 60 mg; Mn, 60 mg; Fe, 30 mg; Cu, 10 mg; I, 0.85 mg; Co, 0.2 mg; Se, 0.2 mg; vitamin A, 7,500 IU; vitamin D_3, 1,500 IU; vitamin E, 25 IU

[3]The protein concentrate (UFA 249 PRIMA IPS, UFA, St. Margrethen, Switzerland) comprised corn gluten, soybean meal, dark distillers grains, rapeseed cake, soft wheat bran, wheat, sugar beet molasses, vegetable refined fatty acids (rapeseed/sunflower), soybeans, minerals, wheat starch. Mineral and vitamin content per kg UFA 249: Ca, 8.0 g; P, 6.5 g; Mg, 4.0 g; Na, 1.5 g; Zn, 60 mg; Mn, 60 mg; Fe, 30 mg; Cu, 10 mg; I, 0.85 mg; Co, 0.2 mg; Se, 0.2 mg; vitamin A, 7,500 IU; vitamin D_3, 1,500 IU; vitamin E, 25 IU

[4]NFC = 100 – (NDF + CP + EE + Ash); Ash = 100 – OM.

(60°C for 48 h) were ground through a 1-mm screen with the same centrifugal mill as that used for the feed samples.

Measurement of Methane Production

Four open-circuit respiration chambers (No Pollution, Industrial Systems Ltd., Edinburgh, UK) were used to measure CH_4 emissions from the individual cows during 2 d per period. During the first part of the 14-d adaptation in period 1, the cows were familiarized with the chamber by spending at least 4 h in the chamber. The chambers were 4.75 m wide, 3.25 m deep, and 2.5 m tall (38.6 m^3). Each chamber was fitted with 1 rear door for animal entrance, milking, and cleaning, and 1 front door used primarily for feeding. Feed and water intake were recorded electronically. The doors were opened twice daily for milking and feeding, and once on d 2 for cleaning. These openings were accounted for by interpolating about 2 × 10 min/d with values from adjacent times in which gas concentration had returned to equilibrium. Calibration was performed before the experiment and between experimental runs, using a pure N_2 gas (99.999%) at the start followed by a standard gas mixture containing 0.1% H_2 and 99.9% N_2. Then, a second standard gas mixture (0.08% CH_4, 20.9% O_2, 0.4% CO_2, and 78.62% N_2) was injected to let the instrument return to the expected concentrations. A CH_4 recovery test was performed 3 times for each chamber during the experiment. The CH_4 (99.9%) was injected at 0.35 L/min via a tube through the outside wall for 4 h. The measured concentration peaked after 1 to 2 h. The average recoveries in the 4 chambers were 100, 98, 98, and 99%, respectively. These values were used for adjustment of the CH_4 production data.

Laboratory Analyses

The chemical composition of feeds and feces was analyzed using standard procedures (AOAC International, 1997; van Soest et al., 1991). Contents of DM and ash were determined with an automatic thermogravimetric determinator (TGA-701, Leco, St. Joseph, MI, USA). The N content of feeds, fresh feces, milk, and acidified urine were analyzed using a C/N analyzer (Typ TruMac CN, Leco Coperation, St. Joseph, MI, USA: AOAC index no. 968.06). Crude protein was calculated either as 6.25 × N for feeds and dried feces or as 6.38

× N for milk. Ether extract (**EE**) was assessed in feeds by the Soxhlet method with an Extractor (Type E-500, Büchi, Flawil, Switzerland: AOAC index No. 963.15). A Fibertherm FT 12 (Gerhardt, Königswinter, Germany) was used to assess ash-corrected detergent fiber fractions. Heat-stable α-amylase (Sigma-Aldrich) was used with NDF analysis; ADL was determined sequentially after ADF analysis by incubation in 72% sulfuric acid for 3 h. Nonfiber carbohydrates were calculated as 100 - (NDF + CP + EE + ash). Gross energy (GE) concentration was determined in feed items and feces with a bomb calorimeter (C7000, IKA-Werke GmbH & Co. KG, Staufen, Germany). Phenol contents of urine and feces as well as feed were analyzed using the method of Makkar (2003a) modified by Jayanegara et al. (2012). Briefly, to measure total extractable phenols (**TEP**) and non-tannin phenols, a modified Folin-Ciocalteu method was used. The butanol-hydrochloride-iron method was applied to determine condensed tannins (**CT**). To calculate total tannins (**TT**) and HT, non-tannin phenols were deducted from TEP, and CT was deducted from TT, respectively. Total phenols (**TP**) in milk and plasma were analyzed based on Serafini et al. (1998). Values of TEP and non-tannin phenols as well as CT were expressed as gallic acid and leucocyanidin equivalents, respectively. Bronopol® stabilized milk was analyzed for concentrations of fat, protein, lactose and MUN using mid infrared spectroscopy (Milkoscan 4000, Foss Electric, Hillerød, Denmark). A Cobas® MIRA Plus (Roche Diagnostics, Mannheim, Germany) was used to quantify BUN. The total antioxidant capacity (**TAC**) in milk and plasma was measured by a commercial kit according to the manufacturer's instructions (OxiSelectTM, Cell Biolabs, San Diego, CA). Results are expressed in nmol/L of copper reducing equivalents (1 mmol/L of uric acid = 2189 μmol/L copper reducing equivalents). Activities of alanine aminotransferase (**ALT**), aspartate aminotransferase, glutamate dehydrogenase, alkaline phosphatase, and γ-glutamyl transferase as well as the concentration of bilirubin were determined in serum using commercially available kits from DiaSys Diagnostic Systems GmbH (Holzheim, Germany; ALT: ALAT (GPT) FS; aspartate aminotransferase: ASAT (GOT) FS; glutamate dehydrogenase: GLDH FS DGKC; alkaline phosphatase: Alkaline phosphatase FS IFCC 37°C; γ-glutamyl transferase: Gamma-GT FS (Szasz mod. /IFCC stand.); bilirubin: Bilirubin Auto Total FS) with an automated analyzer (Cobas® MIRA Plus instrument, Roche Diagnostics).

Milk FA were analyzed by GC (HP 6890 with flame-ionization detector, Hewlett Packard; equipped with a CP7421 column, 200 m × 0.25 mm, 0.25 μm; Varian). Milk (0.5 mL) was mixed with 5 mL of internal standards (n-heptane containing triundecanoin, tetradecenoic methylate and trivaleranoin). Sodium methylate was used for cold transesterification to FAME (Suter et al., 1997). Response factors made from 6:0, 13:0 and 19:0 triglyceride standards were used to adjust individual FA accordingly. The FAME were injected at a volume of 1.0 μL at a split 1:1 with a hydrogen flow of 1.7 L/min. The temperature regimen was as follows: The initial temperature of 60°C was held for 12 min, followed by an increase of 5°C/min to 170°C, kept for 60 min, increased by 5°C/min to 250°C and kept for 20 min. Identification of FAME was performed using a Supelco 37-component standard (Supelco Inc., Bellefonte PA, USA). Peak identification was further confirmed using chromatograms from Collomb and Bühler (2000).

Calculations and Statistical Analyses

The DMI of BMR, energy and protein concentrate, and PP was calculated based on the assumption that the proportion of BMR and PP was the same in the offered BMR-PP and in the refusals, and that energy and protein concentrates (provided on top of the BMR-PP mixture) were consumed completely (as confirmed by observations).

Data obtained more than once per period were averaged for each cow per period. The ECM yield was calculated according to Agroscope (2021).

$$\text{ECM (kg)} = \text{milk yield (kg)} \times [0.39 \times \text{fat (\%)} + 0.24 \times \text{protein (\%)} + 0.17 \times \text{lactose (\%)}]/3.14.$$

Apparent total-tract digestibility of each nutrient was calculated as the nutrient intake minus fecal nutrient excretion divided by nutrient intake. Nitrogen in acidified urine was corrected for the dilution by the acid. Energy turnover variables were calculated as follows:

$$CH_4 \text{ energy (MJ/d)} = CH_4 \text{ (L/d)} \times 0.03957 \text{ (Brouwer, 1965).}$$

$$\text{Urinary energy (MJ/d)} = 0.0348 \times \text{urinary C (g/d)} + 0.009 \times \text{urinary N (g/d) (Hoffmann und Klein, 1980).}$$

$$\text{ME (MJ/d)} = \text{gross energy (\textbf{GE}) intake (MJ/d)} - \text{fecal energy loss (MJ/d)} - CH_4 \text{ energy loss (MJ/d)} - \text{urinary energy loss (MJ/d).}$$

$$\text{Metabolizability} = \text{ME intake (MJ/d) / GE intake (MJ/d).}$$

Data were subjected to ANOVA with the Mixed procedure of SAS (version 9.4, SAS Institute, Cary, NC), considering PP treatment and period as the fixed and

animal as random effect. Linear and quadratic effects of increasing levels of PP were evaluated by orthogonal polynomial contrasts. Least squares means were reported and considered significantly different at $P < 0.05$ and trends at $0.05 \leq P < 0.10$.

RESULTS

Chemical Composition

The PP contained 70% NFC and was rich in TEP and particularly TT, whereof 86% were HT (Table 1). For the PP-supplemented diets as consumed, the contents of CP, EE, NDF, and ADF were lower, while those of NFC, TEP, TT, and HT were greater as compared with the zero PP control diet.

Feed Intake and Digestibility

The intake of BMR decreased linearly ($P = 0.014$) with an increasing proportion of dietary PP at constant total DMI (Table 2). Increasing the amount of PP in the diet resulted in a linear increase ($P = 0.030$) in ADL intake while intakes of CP, NDF, and ADF were not significantly affected. The effect of PP on N excretion observed in the present study is remarkable considering the unaffected TP excretion in urine and feces (data not shown). Total-tract apparent digestibility of DM, OM, CP, and EE decreased linearly ($P \leq 0.029$) with increasing dietary proportion of PP. Inclusion of PP did not affect body weight (Table 2).

Milk Yield, Composition and Antioxidant Capacity

Total milk and ECM yield, as well as proportions of milk fat, protein, and lactose, which averaged 22 kg/d, 25 kg/d, 4.88%, 3.96%, and 4.63%, respectively, were not significantly affected by dietary PP inclusion (Table 3). Likewise, the inclusion of dietary PP did not significantly affect milk TP concentration and TAC. Increasing the inclusion level of dietary PP, the proportions of C14:0 *iso* and C15:1 decreased linearly ($P \leq 0.029$) while the proportions of C18:2 n–6, C18:3 n–3 and C20:1 n–9 as well as the total n–6 FA increased linearly ($P \leq 0.029$). The total SFA, MUFA, PUFA and n–3 FA were not significantly affected by dietary PP.

Plasma Biomarkers and Antioxidant Capacity

The ALT activity in plasma increased linearly ($P = 0.004$) with increasing dietary PP proportion (Table 4). At unchanged plasma aspartate aminotransferase activity, the ratio of aspartate aminotransferase to ALT in plasma decreased linearly ($P = 0.004$) with increasing dietary PP proportion. With increasing dietary inclusion of PP, the activities of glutamate dehydrogenase and alkaline phosphatase in plasma increased and decreased linearly ($P \leq 0.037$), respectively, whereas no effect on plasma bilirubin concentration and γ-glutamyl transferase activity was observed. Feeding PP did not affect TP concentration and TAC in plasma.

Methane Production and Energy Turnover

There was no effect of the dietary treatment on total CH_4 emissions, as well as CH_4 yield and CH_4 emission intensity, expressed as CH_4 per unit intake and milk measured during the 7-d collection period and during the 2-d stay in the respiration chambers, respectively (Table 5). Intakes of GE, digestible energy, and ME were not affected by the PP treatment (Table 6). Fecal energy loss expressed as MJ/d and as percentage of GE intake increased linearly ($P = 0.036$) with increasing inclusion level of dietary PP. Increasing the proportion of dietary PP linearly reduced ($P = 0.036$) the apparent energy digestibility, whereas metabolizability was not significantly affected by dietary PP.

Nitrogen Turnover

The excretion of urinary N and the sum of fecal and urinary N decreased linearly ($P \leq 0.024$) with an increasing proportion of PP in the diet (Table 7). When expressed as a percentage of N intake, increasing the inclusion level of dietary PP linearly increased ($P = 0.042$) the excretion of fecal N and reduced ($P = 0.013$) that of urinary N, respectively, while the sum of fecal and urinary N excretion was not affected. Increasing dietary proportions of PP linearly decreased ($P = 0.042$) apparent N digestibility. Urinary N excretion as a percentage of N excreted in manure as well as MUN and BUN were linearly reduced ($P \leq 0.019$) with increasing dietary PP. There was no effect of the dietary treatment on body N retention or N use efficiency.

DISCUSSION

The phenolic and nutrient composition of the pomegranate product used in the present study show similarities with some and differences to other studies. These aspects are highlighted at the beginning of the discussion to facilitate understanding of the animal responses discussed in the following sections.

Phenol Composition of Pomegranate Peel

The batches of PP used in the present study and by Safari et al. (2018) substantially differed in their

Table 2. Effect of dietary pomegranate peel on intake, nutrient digestibility, and BW of the cows

	Pomegranate peel in diets[1] (%)				*P*-values[2]	
Item	0	5	10	SEM	L	Q
Intake (kg DM/d)						
Total[3]	20.7	21.3	21.0	0.56	0.319	0.110
Basal mixed ration	18.5	18.3	17.2	0.29	0.014	0.184
Energy concentrate	0.96	0.96	0.96	0.339	0.230	0.288
Protein concentrate	1.25	1.25	1.25	0.042	0.230	0.288
Pomegranate peel	0	0.80	1.60	0.036	<0.001	0.233
OM	18.7	19.4	19.1	0.52	0.229	0.147
CP	3.30	3.30	3.19	0.121	0.055	0.193
Ether extract	0.671	0.670	0.647	0.0320	0.032	0.173
NDF	8.88	8.73	8.65	0.179	0.213	0.164
ADF	6.69	6.77	6.55	0.134	0.232	0.163
ADL	0.861	0.901	0.897	0.0249	0.030	0.081
Apparent digestibility (%)						
DM	68.5	66.8	65.7	0.70	0.022	0.618
OM	70.4	68.5	67.5	0.76	0.020	0.537
CP	58.8	54.2	53.3	1.59	0.029	0.267
Ether extract	62.1	60.4	58.5	11.11	0.021	0.870
NDF	64.3	60.7	60.9	1.04	0.085	0.214
ADF	64.9	60.7	60.0	1.64	0.105	0.427
Gross energy	67.6	65.9	65.0	0.77	0.039	0.623
BW (kg)	789	798	801	15.5	0.227	0.662

[1]Proportion of basal mixed ration replaced.

[2]L, Q, linear and quadratic effect of pomegranate peel proportion.

[3]Total DMI (kg/d) during the 2-d stay in the respiration chamber was 19.5, 20.3, 20.6 for the diets with 0%, 5%, and 10% PP, respectively; SEM: 1.16; *P*-values L:0.389, Q: 0.772.

Table 3. Effect of dietary pomegranate peel on yield, composition and antioxidant properties of the milk of the cows

	Pomegranate peel in diets[1] (%)				*P*-values[2]	
Item	0	5	10	SEM	L	Q
Milk yield (kg/d)	22.4	22.7	22.1	2.31	0.701	0.514
ECM (kg/d)	26.0	25.7	24.7	1.36	0.339	0.768
Gross constituents (%)						
Fat	5.06	4.78	4.80	0.413	0.161	0.317
Protein	3.92	3.99	3.97	0.282	0.488	0.398
Lactose	4.56	4.67	4.67	0.116	0.230	0.510
Total phenols (mg/L)	29.2	29.4	29.2	1.56	0.870	0.777
Total antioxidant capacity[3] (nmol/L)	240	198	176	38.0	0.150	0.751
Fatty acids[4] (FA; g/100 g of total FA)						
C14:0 *iso*	0.315	0.295	0.280	0.010	<0.001	0.257
C15:1	0.328	0.298	0.286	0.0182	0.029	0.438
C18:2n–6 (LA)	1.33	1.50	1.56	0.113	0.002	0.147
C18:3n–3 (ALA)	0.559	0.644	0.677	0.0316	0.012	0.327
C20:1n–9	0.122	0.127	0.130	0.0024	0.029	0.748
Σ SFA	68.6	67.6	67.0	1.29	0.143	0.754
Σ MUFA	26.6	27.4	27.9	1.05	0.165	0.856
Σ PUFA	3.62	3.87	3.90	0.261	0.085	0.335
Σ n–6 FA	1.78	1.95	1.96	0.144	0.038	0.220
Σ n–3 FA	0.94	1.00	1.00	0.074	0.288	0.528
Σ n–6:n–3 FA	1.92	1.94	1.96	0.059	0.551	0.936

[1]Proportion of basal mixed ration replaced.

[2]L, Q, linear and quadratic effect of pomegranate peel proportion.

[3]Results are expressed in nmol/L of copper reducing equivalents (1 mmol/L of uric acid = 2189 μmol/L copper reducing equivalents).

[4]For individual fatty acids, only those mentioned in the results section are displayed. The extended fatty acid profile is presented in Supplementary Table S3.

Table 4. Effect of dietary pomegranate peel on serum metabolic markers as well as plasma total phenols and antioxidant capacity of the cows

	Pomegranate peel in diets[1] (%)				*P*-values[2]	
Item	0	5	10	SEM	L	Q
AST (U/L)	59.7	65.0	63.1	3.06	0.356	0.280
ALT (U/L)	23.5	50.2	59.3	4.95	0.004	0.169
AST/ALT	2.57	1.45	1.10	0.205	0.004	0.151
Bilirubin (μmol/L)	1.00	1.05	1.08	0.166	0.647	0.951
GLDH (U/L)	7.69	11.7	13.2	2.05	0.037	0.448
GGT (U/L)	18.6	20.9	21.2	2.23	0.134	0.429
AP (U/L)	147	123	117	67.5	0.032	0.321
Total phenols (mg/L)	266	316	299	18.7	0.280	0.222
Total antioxidant capacity (nmol/L)[3]	255	261	263	8.8	0.552	0.818

ALT, alanine aminotransferase; AP, alkaline phosphatase; AST, aspartate aminotransferase; GGT, γ-glutamyl transferase; GLDH, glutamate dehydrogenase.

[1]Proportion of basal mixed ration replaced.

[2]L, Q, linear and quadratic effect of pomegranate peel proportion.

[3]Results are expressed in nmol/L of copper reducing equivalents (1 mmol/L of uric acid = 2189 μmol/L copper reducing equivalents).

contents of TEP (240 vs. 26 g/kg DM) and TT (220 vs. 22 g/kg DM). As similar methods have been used for the analysis, this difference cannot be explained solely by the analytical methods. Growth conditions may affect tannin concentrations as reviewed by Aboagye and Beauchemin (2019) and thus may have contributed to the 10-fold higher polyphenol concentrations in the PP of the present study. Furthermore, the PP used in our study contained 20% of HT in DM, which is twice the amount of that found by Giller et al. (2021) in pomegranate pomace, a mixture of peel, seeds, and pulp. Indeed, according to Walid et al. (2012) the HT content of PP is 2 to 4 times higher than that of seeds (DM basis).

Nutritional Value of Pomegranate Peel

In the present study, the proportion of most nutrients in the PP batch used was comparable to that reported by Safari et al. (2018) (3.6 vs. 3.0% CP, 23 vs. 20% NDF, and 70 vs. 69% NFC in DM). The EE content was, however, comparatively low (0.42 vs. 1.94% of DM) in our batch. The similar total DMI and ME intakes among all 3 diets of the present study suggest that the energetic value of the PP was not much different from that of the BMR.

As BMR was exchanged by PP, the observed reduction in BMR intake was expected. Safari et al. (2018) and Shabtay et al. (2008) showed that the dietary inclusion of 11% of peel seed pulp DM (8% of PP and 3%

Table 5. Effect of dietary pomegranate peel on methane (CH_4) production of the cows

	Pomegranate peel in diets[1] (%)				*P*-values[2]	
Methane (CH_4)[3]	0	5	10	SEM	L	Q
Total (g/d)	462	458	455	19.8	0.723	0.994
Yield (g/kg of)						
DMI	22.3	21.5	21.7	0.47	0.370	0.444
OM intake	24.6	23.7	23.8	0.52	0.323	0.450
NDF intake	52.5	51.6	52.9	1.31	0.805	0.478
Digestible DM	32.6	32.3	33.1	0.69	0.675	0.529
Digestible OM	36.5	36.2	37.1	0.78	0.615	0.550
Digestible NDF	81.8	85.4	87.4	2.63	0.209	0.816
Emission intensity (g/kg of)						
Milk yield	20.7	21.3	22.3	2.55	0.579	0.926
ECM	18.0	18.3	19.1	1.32	0.584	0.863
Metabolic BW ($BW^{0.75}$)	3.09	3.05	3.03	0.132	0.662	0.932

[1]Proportion of basal mixed ration replaced.

[2]L, Q, linear and quadratic effect of pomegranate peel proportion.

[3]Methane was measured over 2 d; feed intake, digestibility, and milk variables were measured over 7 d. When expressing CH4 per unit DMI from the 2 d in the respiration chamber, the CH4 yield (g/kg) is 24.0, 22.5, 22.2 for the diets with 0%, 5%, and 10% PP, respectively (SEM: 0.91, L: $P = 0.145$, Q: $P = 0.573$).

Table 6. Effect of dietary pomegranate peel on energy metabolism of the cows

	Pomegranate peel in diets[1] (%)				*P*-values[2]	
Item	0	5	10	SEM	L	Q
Energy turnover (MJ/d)						
Intake						
Gross energy	375	386	379	10.7	0.438	0.140
Digestible energy	254	254	248	9.1	0.321	0.536
Metabolizable energy	216	218	213	7.7	0.562	0.471
Energy loss (MJ/d)						
Feces	121	131	132	2.5	0.010	0.066
Urine	11.9	10.1	9.7	1.19	0.150	0.588
Methane (CH_4)	25.6	25.3	25.1	1.10	0.723	0.994
Energy turnover (% of GE intake)						
Feces	32.4	34.1	35.0	0.77	0.036	0.641
Urine	3.18	2.64	2.52	0.305	0.149	0.537
Methane (CH_4)	6.80	6.57	6.63	0.142	0.441	0.454
Apparent digestibility (% of GE)	67.6	65.9	65.0	0.77	0.036	0.641
Metabolizability (% of GE)	57.6	56.7	55.8	0.66	0.132	0.996

[1]Proportion of basal mixed ration replaced.

[2]L, Q, linear and quadratic effect of pomegranate peel proportion.

of pomegranate seed, providing 50 g TT/d) and 20% of PP DM (94 g TT/d) did not affect the intake of transition dairy cows and feedlot calves, respectively. A slight intake promoting effect of supplementing 8.7% of PP in DM (54 g TT/d) in a high-concentrate (68% of DM) diet for primiparous, early lactating (46 ± 10 DIM) Holstein dairy cows has been reported by Akhlaghi et al. (2022). In addition to a potential interaction effect with the basal diet composition and individually different sensory perception of animals, this inconsistence may at least in part be due to variable tannin contents of different plant parts such as peel and seed as well as to their modification by the growth conditions (Walid et al., 2012; Aboagye and Beauchemin, 2019). A potential negative effect on DMI would have been likely due to the astringency of the tannins (Makkar, 2003b). Therefore, the lack of response in total DMI in the present study was unexpected considering the comparatively high TT content (2% of DM) and TT intake (412 g TT/d), indicating that other ingredients of PP have widely compensated for any adverse effects on palatability. However, astringency, and thus

Table 7. Effect of dietary pomegranate peel on nitrogen (N) metabolism of the cows

	Pomegranate peel in diets[1] (%)				*P*-values[2]	
Item	0	5	10	SEM	L	Q
g/d						
N intake	528	528	510	19.5	0.050	0.176
Fecal N	220	239	238	6.3	0.076	0.196
Urinary N	142	113	83.5	11.5	0.006	0.987
Fecal and urinary N	362	352	322	14.3	0.024	0.380
Milk N	129	135	125	5.8	0.455	0.102
Body N retention	36	41	63	14.4	0.265	0.647
% of N intake						
Fecal N	41.6	45.7	46.8	1.67	0.042	0.370
Urinary N	26.6	21.6	15.8	1.83	0.013	0.860
Fecal and urinary N	68.2	67.3	62.6	1.89	0.106	0.449
Milk N	24.7	25.5	24.8	0.86	0.935	0.474
Body N retention	7.20	7.19	12.6	2.682	0.229	0.457
Apparent N digestibility (%)	58.4	54.3	53.2	1.67	0.042	0.370
Urinary N (% of manure N)	39.5	32.4	24.7	2.84	0.019	0.949
N use efficiency[3] (%)	24.7	25.5	24.8	0.86	0.935	0.474
MUN (mg/dL)	9.09	8.32	6.77	0.332	0.004	0.310
BUN (mg/dL)	10.9	8.47	7.58	0.697	0.001	0.078

[1]Proportion of basal mixed ration replaced.

[2]L, Q, linear and quadratic effect of pomegranate peel proportion.

[3]Milk N/N intake.

feed palatability, is not only determined by TT content but also by the tannins' molecular structure (Soares et al., 2020). Astringency is caused by complex formation of tannins with salivary proteins, thereby reducing the lubricating effect of the saliva. A less pronounced protein-complexation by HT than by CT may thus explain the lack of feed intake reduction with HT-rich PP intake. Future research on biological effects of dietary tannins should therefore not only consider TT content but provide a more in-depth characterization of the tannin profile by at least providing the dietary proportions of HT and CT. In addition, a lack of response in total DMI has to be confirmed in cows at their peak of lactation fed a forage-based diet.

Increasing dietary PP content to 5% and 10% DM adversely affected the apparent total-tract digestibility of nutrients and energy, similar to what was reported for lambs and lactating cows that were fed PP and ensiled pomegranate pulp mixture, respectively, at 20% diet DM (Shaani et al., 2016; Karamnejad et al., 2019). For the apparent CP digestibility, this was expected due to the protein-binding and microbial activity-reducing effects of tannins in the rumen (reviewed by Makkar, 2003b). Soilman et al. (2022) showed in situ that dietary TT from PP at a concentration of only 0.05% DM vs. 2% of DM in our study already significantly reduced the ruminal degradability of DM and CP. Still, HT and CT proportions were not provided by Soilman et al. (2022), so we cannot exclude that a higher CT proportion in their PP might have contributed to the observed effects. Different from other nutrients, a decline in CP digestibility is not always unfavorable for the animal, as it often only reduces ruminal ammonia formation which, upon absorption, remains unused in metabolism. It has been even suggested that the tannin-protein complexes that are stable at ruminal pH may dissociate when the pH decreases below 3.5 (i.e., in the abomasum) or is greater than 8 (i.e., in the duodenum; Frutos et al., 2004) and thus improves supply with MP. However, the significant reduction in apparent digestibility of CP and the lack of response in N use efficiency observed in the present study indicate that tannin-protein complexes did not dissociate to a degree relevant for N use efficiency. Different from the expectations, Jami et al. (2012) found an improved nutrient digestibility in dairy cows fed 40 g/kg DM of a concentrated extract of pomegranate waste, providing approximately 9% of phenols, thereof 2.7% punicalagins and 2.5% ellagic acid. However, the basal diet offered in the study by Jami et al. (2012) was concentrate-based with only 38% DM of roughage. Consequently, the type of the basal ration (as already suggested by Wei et al., 2019), but also the experimental animals (e.g., breed, days in milk) and the nutritive value and tannin profile of the pomegranate by-product may contribute to differential effects of pomegranate by-products on nutrient digestibility. Tannins may also bind to polysaccharides and reduce their ruminal degradation, though to a lesser degree than with CP (Patra et al., 2012). Accordingly, the fiber (NDF, ADF) digestibility tended to decline with increasing dietary PP in the present study. This, together with the reduction in CP digestibility, would explain the decline in OM digestibility in the present study.

Despite the reduced nutrient digestibility and a slight increase in fecal energy loss with PP addition, the milk and ECM yield of the dairy cows in the present study were maintained. Similarly, in dairy cows fed pomegranate pulp silage DM at levels of 7.5 and 15% (Kotsampasi et al., 2017) or even 20% (Shaani et al., 2016), milk yield remained unaffected. Positive responses on ECM yield and milk components compared with the control group were reported by Safari et al. (2018) and Khorsandi et al. (2019), feeding a mixture of dried pomegranate peel (75%) and seed (25%) or ensiled pomegranate peel and seed (4:1) to dairy cows at levels of 11% or 30% of dietary DM. These ECM yield promoting effects could have been a result of a higher quality of the basal diet including much higher concentrate proportions used in these 2 studies compared with the present study (40 and 57 vs. 19% DM). Together, the results of the present study suggest that PP had a similar nutritional value as the BMR used. Because the present study used late-lactating cows, the nutritional value of pomegranate peel would have to be confirmed in cows at their peak of lactation.

Effects of Pomegranate Peel on Blood and Milk Phenols and Antioxidant Capacity

Polyphenols are known for their antioxidant effects and a positive correlation between both total polyphenols and HT and the antioxidant capacity of PP is reported (Shabtay et al., 2008). The lack of differences in plasma and milk TP may therefore explain the similar TAC among treatments in the present study. The unchanged TP in plasma and milk may in turn be explained by the ruminal degradation of HT (Bhat et al., 1998). With HT representing the majority of polyphenol compounds in PP, their complete or partial degradation would thus minimize polyphenol absorption from PP and prevent the increase in systemic TP. However, others described an improved antioxidant capacity in blood plasma (Safari et al., 2018) and milk (Akhlaghi et al., 2022) of dairy cows fed pomegranate-derived by-products. The contrasting results could be attributed to the degree of galloylation and conformation of the HT as well as their potential interactions

with the feed matrix, in addition to the different analytical methods used (Barbehenn and Constabel, 2011). A different adaptation to a prolonged exposure to the tannins (Mlambo et al., 2007), possibly associated with increasing ruminal degradation of HT, may be another reason.

Effects of Pomegranate Peel on Milk Fatty Acid Profile. The main PUFA of nutritional interest are linoleic (C18:2 n–6) and α-linolenic (C18:3 n–3) acid due to their importance for human nutrition and health (Simopoulos, 1999). When increasing the inclusion level of dietary PP, we observed a linear increase of these 2 milk FA. However, only total n–6 FA but not total n–3 FA increased in milk with PP feeding. A high intake of n–6 compared with n–3 FA is considered detrimental because of the pro- and anti-inflammatory effects, respectively, of these groups of FA. Still, the n–6/n–3 FA ratio of the milk remained unaffected with PP intake, indicating unchanged presence of precursors with inflammatory potential in the milk. Kotsampasi et al. (2017) reported the same changes in the FA profile in milk of dairy cows fed pomegranate pulp silage at levels of 7.5% and 15% of DM. Razzaghi et al. (2015) also observed an increase of C18:3 n–3 in milk fat of dairy goats fed diets supplemented with 12% DM of pomegranate seed pulp. These findings can be explained by the inhibitory effect of tannins from pomegranate by-products on the activity of *Butyrivibrio fibrisolvens*, which is involved in ruminal biohydrogenation of PUFA (Min and Solaiman, 2018). Moreover, Buccioni et al. (2017) found that HT could inhibit the last step of biohydrogenation. As a result, a higher proportion of MUFA, especially biohydrogenation intermediates, and PUFA is prone to escape the rumen following PP feeding and may be transferred to the milk. However, in the present study, biohydrogenation intermediates, total MUFA and PUFA did not significantly differ between treatments. This suggests that not exclusively an inhibition of FA biohydrogenation but other reasons such as the FA profile of the PP may have contributed to the observed increase in milk linoleic and α-linolenic acid proportion. Unfortunately, the FA profile of the PP used in the present study was not analyzed but proportions of linoleic and α-linolenic acid in dried pomegranate peel were reported to amount to 13.9% and 6.0% of total FA, respectively (Omer et al., 2019). The linear decrease of C14:0 *iso* observed in the present study indicates a certain effect of dietary PP on the de novo synthesis of FA in the mammary gland. This process is highly dependent on the 3 key enzymes acetyl-CoA carboxylase, FA synthase and acyl-CoA synthetase (Lee et al., 2017). Indeed, the HT tannic acid was shown to reduce expression of these enzymes in mammalian cells (Nagesh et al., 2020). Further research is required to confirm a potential inhibition of the enzymes' expression by dietary HT metabolites in the mammary gland in vivo.

Effects of Pomegranate Peel on Urinary Nitrogen Excretion, Nitrogen Balance and Methane Emission. After excretion, fecal N is relatively stable. In contrast, urinary urea N, which accounts for 50–90% of the N in cattle urine, can transform rapidly to ammonium (NH^{4+}) and subsequently to NH_3 (Spek et al., 2013). Shifting N excretion from urine to feces may, therefore, lower the adverse environmental impact of manure storage and distribution. The suppression of urinary N, associated with a trend for a higher excretion of fecal N, illustrates a shift of N partitioning from urine to feces with increasing levels of dietary PP in the present study. This compensation at least did not impair N use efficiency. As literature on the influence of pomegranate byproducts on N excretion of dairy cows is sparse, we compared our results with information on other tannin sources. Chestnut tannin (also high in HT) included at 1.5% of DM reduced urinary N losses in beef cattle (Aboagye et al., 2018). When combined with quebracho tannins (CT), chestnut tannins (0.75% each) were even more efficient in reducing urinary N (Aboagye et al., 2018). This indicates a more potent effect of CT than HT on ruminal N metabolism, further underlining the lower ruminal stability of HT compared with CT.

The effect of PP on N excretion observed in the present study is remarkable considering the unaffected urinary and fecal phenol excretion. The latter, in addition to the similar plasma phenol concentrations, supports the postulated intense degradation of HT by ruminal microbes while the small fraction of CT present in PP formed complexes with dietary proteins. This would allow part of the protein to by-pass microbial degradation in the rumen, thereby reducing ruminal ammonia formation. Still, also gallic acid, one of the main HT components, was shown to reduce ruminal CP degradation in vitro (Wei et al., 2019). The assumed reduced ruminal ammonia absorption and, thus, urea formation in the liver with increasing levels of PP is also confirmed by the concomitantly lower concentrations of BUN and MUN. Soilman et al. (2022) also showed a reduction of MUN in dairy cows fed 30 and 37.5 g PP/kg diet DM. Body N retention was quite high with 36 to 63 g/d, this even for cows that averaged 306 DIM, and having 796 kg of BW. Accordingly, a BWG of 2.3 kg/d would be expected from 63 g/d retained N in cows fed 10% PP, assuming body tissues contain 17% protein on average (NRC, 2001). In addition to the late stage of lactation, the cows were on average 131 d pregnant, thus the fetus and pregnant uterus likely accounted for a portion of the N retention observed. Still, the amount

of N apparently retained in the body tissues appears to be overestimated although we followed the recommendations for best practice (Hristov et al., 2019). These recommendations include 14 d of adaptation period and 7 d of total feces collection to minimize and account for daily variation in fecal output, sufficient acidification of the urine, and N analyses in fresh feces to avoid N volatilization during storage and the drying process (Brito et al., 2008). A similarly high body N retention of 58 g/d was reported by Reynolds et al. (2001) for multiparous cows averaging 233 DIM, 143 d of pregnancy, and 652 kg of BW. Brito et al. (2008) also reported a body N retention as high as 40 g/d in cows (198 DIM at start, unknown pregnancy stage, 643 kg of BW). These authors suggested N balance was overestimated because of unknown sources of N losses not accounted for. In their review, Hristov et al. (2019) stated that "there is no doubt that after more than 150 yr of collective experience, accurate measurements of N balance (...) continue to be exceedingly difficult to achieve (. . .) and would benefit from further development and innovation."

The lack of a CH_4 mitigating effect of dietary PP in the present study is consistent with the findings of Yurtseven et al. (2019), who showed that 5% PP in lambs' diet did not affect CH_4 emissions. However, this contrasts with the CH_4 mitigating effect of PP extract found in Murrah buffalos (Hundal et al., 2019), and with that of 15% pomegranate pomace DM demonstrated in vitro (Giller et al., 2021). Based on their results from a long-term and a short-term in vitro experiment with HT-derived gallic acid, Wei et al. (2019) suggested that methanogens may adapt to the presence of gallic acid over time. This would explain why pomegranate pomace showed a CH_4 mitigating effect during 24 h (Giller et al., 2021) and PP did not mitigate CH_4 after 21 d of feeding in the present study. However, the CH_4 mitigating effect of PP extract reported by Hundal et al. (2019) was observed after 45 d of feeding. Therefore, other factors than exposure time to HT must be involved in mediating CH_4 mitigating effects. Other HT-rich sources, such as the extracts of oak and chestnut tannin, have not been very effective in reducing CH_4 emissions (Focant et al., 2019; Aboagye et al., 2018), even though HT were found to exhibit direct effects against methanogens (Aboagye and Beauchemin, 2019). Up to 20 mg/g of the HT component gallic acid did also not mitigate CH_4 in vitro (Wei et al., 2019). Unlike PP rich in HT, *Acacia mearnsii* extracts rich in CT have been shown to reduce CH_4 emissions from bulls (Staerfl et al., 2012) and dairy cows (Denninger et al., 2020). Supplementing dairy cows with hazel leaves, containing 2% CT and 0.5% HT of dietary DM, reduced CH_4 yield by a magnitude of 25% (Terranova et al., 2021). Consequently, the absence of a tannin effect on CH_4 mitigation in the present study is likely attributed to the limited supply of dietary CT, the molecular structure of HT, and the assumed extensive degradation of HT by microbes in the rumen (Bhat et al., 1998; Makkar, 2003b). Therefore, no direct inhibitory effect on methanogenic archaea and protozoa population (Focant et al., 2019), or a toxic effect on methanogens (Jayanegara et al., 2012), occurred.

Effects of Pomegranate Peel on Liver Functionality

The doubled activity of ALT ($\geq$50 U/L) in serum of dairy cows consuming diets with 5 and 10% of PP for 15 d points toward a certain degree of liver cell stress, since this enzyme usually occurs in the cytoplasm of hepatocytes and is not present in serum at levels higher than 35 U/L (Rej, 1989; Gaina et al., 2020). As the activities of other liver enzymes (e.g., glutamate dehydrogenase) did not exceed the reference range, it seems that the liver stress in response to PP intake was still at an early stage. Removal of HT from the dairy cow diet at this early stage of HT intoxication can be assumed to allow the liver to recover, resulting in the normalization of systemic liver transaminase activity. Unfortunately, we did not monitor the serum ALT activity after the end of the study. However, even in the 2 animals (cows 2 and 3, Supplementary Table S1) receiving the unsupplemented control diet after the 10% PP diet according to the cyclic change-over design, serum ALT activity reduced from 68 and 71 U/L to 24 and 15 U/L, respectively, within 24 d (washout period and period before blood sampling). The finding of a potential hepatotoxicity of dietary PP in dairy cows is consistent with literature reporting toxicity of HT-rich feed material in ruminants (Hawes and Gill, 2018). Particularly after consumption of pomegranate fruits, acute periacinar hepatocellular necrosis was diagnosed in young Charolais-cross steers that eventually had led to death (Hawes and Gill, 2018). A distinct hepatotoxic effect was also shown for the HT punicalagin from *Terminalia oblongata* (yellow-wood) (Doig et al., 1990), which is an abundant HT in pomegranate peel as well (Sabraoui et al., 2020). In contrast to the rather stable CT, partial microbial degradation of HT in the rumen results in metabolites that seem to be the cause of liver toxicity going as far as liver necrosis (Bhat et al., 1998). A gradual introduction of dietary HT as opposed to a sudden high exposure has been proposed to enable the ruminal microbes to better adjust to the HT and to detoxify dietary HT metabolites at least partially in the metabolism (Bhat et al., 1998). Applied to PP, this indicates that such cautionary measure should already

be implemented before permanently adding 5% PP to the diet.

CONCLUSION

The combination of a small number of observations and using cows in late stage of lactation may have limited the understanding of the impact of pomegranate peel on variable responses. With this limitation in mind, our study confirms that pomegranate peel can be used as functional feed ingredient in ruminant diets to improve N turnover and milk fatty acid composition. However, the ruminal degradability of dietary HT highlights that the underlying mechanisms for these beneficial effects are still not understood and require mechanistic studies. In line with the presumed degradation of hydrolyzable tannins, the pomegranate peel intake lacked a favorable effect on systemic antioxidant capacity and CH_4 emission. The unchanged voluntary intake, metabolizability of the energy and animal productivity despite a reduced digestibility with hydrolyzable tannin intake at 1.8% DM points toward quite a high nutritional value of this feed. Because the present study used late-lactating cows, the nutritional value of pomegranate peel would have to be confirmed in cows at their peak of lactation, especially when fed a high-forage diet. It is not meaningful to aim for provoking greater effects by higher dietary proportions of pomegranate peel as the elevated plasma alanine aminotransferase activity points toward liver stress already at 5% inclusion level. Further research is required to understand the hepatotoxic effects of hydrolyzable tannins and their microbial metabolites and develop adaptation protocols allowing for a safe inclusion of pomegranate peel and other byproducts from pomegranate juice production into ruminant diets.

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