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Original Article

Processed barley grain mitigates the adverse effects of starch-rich diets in lactating Holstein cows

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Abstract

Background: High-starch diets, commonly formulated with barley grain (BG), can impair ruminal function and trigger systemic inflammation in lactating dairy cows. **Aims:** The present study investigated the effects of different forms of BG on rumen fermentation, immune-related gene expression, and systemic inflammation in Holstein cows fed starch-rich diets. **Methods:** Twenty-seven lactating Holstein cows (three fistulated and six non-fistulated per treatment) were randomly assigned to three dietary groups: untreated BG (BG_{CTRL}), BG processed with 5% lactic acid (BG_{LA}), or BG processed with sugar beet pulp extract (BG_{SBPE}). Cows received a basal total mixed ration for two weeks, followed by one week of high-starch experimental diets. **Results:** Cows fed BG_{LA} (5.97) and BG_{SBPE} (5.85) had significantly higher ruminal pH compared to BG_{CTRL} (5.55; $P<0.05$), along with shorter durations of pH<5.8 (61.35, 182.93, and 362.98 min/day for BG_{LA}, BG_{SBPE}, and BG_{CTRL}, respectively; $P<0.05$). The acidosis index was reduced from 18.10 in BG_{CTRL} to 8.13 in BG_{SBPE} and 3.05 in BG_{LA}. Also, BG_{LA} reduced ruminal *E. coli* abundance ($P<0.001$), while *Megasphaera elsdenii* was lower in BG_{SBPE} than in BG_{CTRL} ($P<0.001$). Both processed BG diets significantly decreased serum haptoglobin and serum amyloid A, and reduced mRNA expression of CD14, TLR4, and MD2. **Conclusion:** Processing BG with lactic acid or sugar beet pulp extract mitigates the adverse effects of high-starch diets on rumen health and systemic inflammation in lactating dairy cows.

Key words: Barley processing, Immune-related genes, Inflammation, Plant extract

Introduction

Energy-dense ingredients such as barley grain (BG), which are rich in rapidly fermentable carbohydrates like starch, are commonly included in the diets of high-producing lactating cows to meet their elevated energy demands (Nocek, 1997). However, such diets are associated with an increased risk of metabolic disorders, particularly subacute ruminal acidosis (SARA) (Nocek, 1997). This metabolic disorder is known to:

a) Reduce dry matter intake (DMI), milk fat, milk yield, and ruminal pH (Emmanuel *et al*., 2008; Plaizier *et al*., 2012)

b) Alter ruminal microbial diversity (Fernando *et al*., 2010; Petri *et al*., 2013)

c) Increase the concentration of short-chain fatty acids (SCFA) and the duration of ruminal pH below the SARA threshold (pH<5.8 for more than 5.24 h/day) (Zebeli *et al*., 2008)

d) Impair the ruminal fermentation profile (Plaizier *et al*., 2012; Zebeli and Metzler-Zebeli, 2012)

Excessive inclusion of BG in the diet can lead to a pronounced decline in ruminal pH, thereby disrupting the rumen microbial balance. This disruption favors the overgrowth of gram-negative bacteria such as *E. coli* and leads to the accumulation of lipopolysaccharides (LPS), which contribute to ruminal epithelial damage and systemic inflammation (Gozho *et al*., 2007; Khafipour *et al*., 2009a).

LPS is a potent inducer of the acute phase response (APR), characterized by elevated levels of acute phase proteins (APP), including serum amyloid A (SAA) and haptoglobin (Hp) in the bloodstream (Emmanuel *et al*., 2008; Iqbal *et al*., 2012b). Additionally, ruminal pH and

LPS concentration under SARA conditions may modulate mRNA expression of immune-related genes such as TLR4, CD14, and MD2 (Stefanska *et al*., 2018). To prevent such outcomes, effective dietary strategies, particularly the inclusion of chemically processed grains, are necessary. Processing BG with organic acids, such as lactic acid (LA), has shown potential to reduce starch degradation and moderate SARA, consequently decreasing APP levels (Iqbal *et al*., 2009; Iqbal *et al*., 2012b). In recent years, there has been growing interest in using plant-derived additives, such as extracts and essential oils, as natural antioxidants or microbial modulators in livestock diets (Aarabi *et al*., 2016). One such additive, sugar beet pulp extract (SBPE), has demonstrated promise in modifying starch fermentation dynamics in the rumen (Naseroleslami *et al*., 2018, 2022). The ethanolic extract of sugar beet pulp contains phenolic compounds with strong antioxidant properties (AbouSekken *et al*., 2013), and its high cation-exchange capacity may support buffering capacity in the gastrointestinal tract (McBurney *et al*., 1983). Based on these characteristics, it was hypothesized that treating BG with LA or SBPE would enhance ruminal fermentation (higher pH), improve blood metabolites and immune-related gene expression, and ultimately boost animal performance. Therefore, the present study aimed to investigate the effects of high-starch diets containing either untreated BG (BG_{CTRL}), BG processed with 5% LA (BG_{LA}), or BG treated with SBPE (BG_{SBPE}) on performance, ruminal and blood metabolites, and the expression of immune-related genes (CD14, TLR4, and MD2) in lactating Holstein cows.

Materials and Methods

Grain processing

Whole BG was treated with either 5% LA (85% excellent grade, Qingdao Derun Chemical Co., LTD) or SBPE, with untreated BG as the control (BG_{CTRL}). SBPE was prepared following the method of Naseroleslami *et al*. (2022). Twenty kg of molasses-free sugar beet pulp was mixed with 100 L of water in a plastic barrel, stirred at 30-min intervals for 48 h, and then filtered through cheesecloth. Approximately 1500 kg of BG was processed with either SBPE or LA. Each 250 kg batch was mixed with 12.5 L of SBPE or LA in a concrete mixer for 10 min, then stored in sealed plastic barrels for 48 h. The processed grain was spread on cotton sheets placed over mesh boxes to allow adequate air circulation and dried for 24 h in a ventilated laboratory room (25-28°C; relative humidity 35-45%) before storage in plastic bags.

Animals and experimental procedure

Twenty-seven multiparous Holstein cows were used in a 21-day experiment with a completely randomized design (CRD), including a 14-day adaptation period followed by a 7-day starch-rich feeding period. Cows were assigned to three treatments (BG_{CTRL}, BG_{LA}, and BG_{SBPE}; n=9 per group). Parity (ranging from 2 to 4), calving date, milk yield, and body weight were only considered to achieve a balanced distribution across groups. Eighteen lactating cows (102.4 ± 31.2 DIM, 36.61 ± 11.95 kg milk/day, 621.24 ± 65.58 kg BW) were used to evaluate production response, and nine ruminally cannulated lactating Holstein cows (474 ± 50 kg BW, 136 ± 7 DIM, 20.5 ± 3.5 kg milk/day) were used to evaluate rumen fermentation. Cows were housed in tie-stall barns with rubber mats, passive individual feeding systems, and free access to fresh water. The adaptation diet, formulated according to NRC (2001) to meet the requirements for 34 kg/day of 3.5% FCM with 25 kg DM intake, contained coarsely ground BG (108.4 g/kg DM) and was offered as a Total Mixed Ration (TMR); the full formulation is presented in Table 1. In the final week, cows received starch-rich diets containing 230 g/kg DM of untreated or processed BG. Diets were offered twice daily at 08:00 am and 8:00 pm, with 5% refusals targeted. The study protocol was approved by the Iranian Agricultural Research Center Animal Care and Use Committee (Project No. 44638).

Rumen fermentation responses

Rumen pH, SCFA concentrations, NH3-N concentration, and the abundances of *E. coli* and *Megasphaera elsdenii* were evaluated using cannulated cows. Rumen content was sampled with a 60-cm plastic pipe and a netted metal cylinder. The first 50 ml of rumen fluid was discarded, and the subsequent 50 ml was used for pH determination using a portable pH meter (Metrohm 744, Switzerland) over a 24-h monitoring period. Measurements were taken every 10 min during the first 2 h after feeding and every 30 min thereafter up to 24 h. Acidosis index was calculated as min/day with pH<5.8 divided by DMI (kg) (Penner *et al*., 2009). A pH value below 5.8 was considered the threshold for SARA, as reported by Plaizier *et al*. (2008). In addition, urine and feces samples were collected 6 h after the morning feeding, and their pH was immediately determined using the same portable pH meter (Metrohm 744, Switzerland). Samples collected at 0-, 4-, and 8-h post-feeding were used for SCFAs and NH3-N analysis. The SCFAs were preserved in metaphosphoric acid (250 g/L) and stored at 4°C, where they were analyzed via gas chromatography (CP-9002, Chrompack), using helium as carrier gas and crotonic acid as internal standard. NH3-N was determined by the phenol-hypochlorite method (Weatherburn, 1967).

DNA extraction

Relative abundances of *E. coli* and *M. elsdenii* were measured at 4- and 8-h post-feeding on the last two days of induction; the samples were transferred to sterile cryovials, frozen in liquid nitrogen, and stored at -80°C for the analysis. DNA extraction was performed following the protocol described by Minas *et al*. (2011) with minor modifications. In brief, rumen fluid samples were thawed at 32°C for 15 min; 400 µL of the fluid was mixed with CTAB solution and glass beads, then homogenized in two rounds of 30 s at 3000 rpm using a

Bioprep-24 Bead Homogenizer. The DNA purity and integrity were assessed using an Epoch microplate spectrophotometer and agarose gel electrophoresis, respectively. Primer sequences for *E. coli* and *M. elsdenii* were obtained from studies by Khafipour *et al*. (2009b) and Ozutsumi *et al*. (2006), respectively (Table 2).

Quantitative PCR

All qPCR conditions followed MIQE guidelines (Bustin *et al*., 2009). Quantitative PCR was performed in duplicate using RealQ Plus 2X master mix (Ampliqon, Denmark) on a BioRad CFX96 instrument (BioRad, USA). Amplification was performed in 96-well plates containing 3 µL DNA, 5 pmol primers, and 10 µL 2X master mix (total 20 µL). The PCR program included 95°C for 10 min, followed by 45 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 20 s. Melting curve analysis followed by linear heating from 60 to 90°C. An equal proportion of DNA from all samples was pooled to generate a DNA template for standard curve analysis. The standard curve consisted of two-fold serial dilutions of a pooled DNA template, spanning five data points, with three replicates for each dilution (Ghanipoor-Samami *et al*., 2018). Cq (Quantitative cycle) values of the standards were used to derive a standard curve, which shows the Cq values as a linear function of the

Table 1: Ingredients (% of DM) and chemical composition (% of DM) of the adaptation and experimental diets

Item	Experimental diets			
	Adaptation	BG_{CTRL}	BG_{LA}	BG_{SBPE}
Ingredients (% of DM)				
Corn silage	25.22	22.00	22.00	22.00
Alfalfa silage	5.69	5.00	5.00	5.00
Alfalfa hay	13.89	12.30	12.30	12.30
Wheat straw	1.79	0.90	0.90	0.90
Corn grain	8.22	7.31	7.31	7.31
Barley grain (native)	10.84	23.00	0.00	0.00
Barley grain (LA processed)	0.00	0.00	23.00	0.00
Barley grain (SBPE processed)	0.00	0.00	0.00	23.00
Soybean meal	7.39	8.50	8.50	8.50
Extruded soybean	2.14	1.90	1.90	1.90
Wheat bran	7.86	6.90	6.90	6.90
Yasminomax®	5.54	2.63	2.63	2.63
Wheat gluten	1.45	1.33	1.33	1.33
Cottonseed grain	2.99	2.66	2.66	2.66
Rapeseed meal	1.45	1.30	1.30	1.30
Fish meal	0.73	0.65	0.65	0.65
Sugar beet pulp	2.19	1.95	1.95	1.95
Molasses	0.61	0.54	0.54	0.54
DCP	0.16	0.10	0.10	0.10
Vitamin mineral mix	0.93	0.83	0.83	0.83
Bentonite	0.00	0.36	0.36	0.36
Salt	0.00	0.04	0.04	0.04
Sodium bicarbonate	0.73	0.00	0.00	0.00
Manganese oxide	0.16	0.00	0.00	0.00
Chemical composition (% of DM)				
DM (%)	58.00	59.10	59.10	59.10
OM (%)	94.00	94.00	94.00	94.00
Starch (%)	26.00	28.00	28.00	28.00
ME (MJ/kg DM)	11.40	11.50	11.50	11.50
CP	17.20	17.00	17.00	17.00
NDF	36.70	34.90	34.90	34.90
NFC	36.50	39.80	39.80	39.80

BG_{CTRL}: Unprocessed barley grain, BG_{LA}: Lactic acid 5% processed barley grain, BG_{SBPE}: Sugar beet pulp extract processed barley grain, DCP: Dicalcium phosphate and Limestone, CP: Crude protein, NDF: Neutral detergent fibre, NFC: Non-fibre carbohydrates, DM: Dry matter, OM: Organic matter, ME: Metabolizable energy, and Yasminomax®: Processed soybean meal treated with xylose; low ruminal degradability

Table 2: Primers used for relative quantification of microorganisms by real-time PCR

Target organism(s)	Primer	Sequence	Source	Product size (bp)
Escherichia coli	EcoliFimH2F[1]	GCCGGTGGCGCTTTATTTG	Khafipour *et al*. (2009)	114
	EcoliFimH2R[2]	TCATCGCTGTTATAGTTGTTGGTCT		
Megasphaera elsdenii	MegEls1F	GACCGAAACTGCGATGCTAGA	Ozutsumi *et al*. (2006)	129
	MegEls1R	CGCCTCAGCGTCAGTTGTC		

[1] F: Forward primer, and [2] R: Reverse primer

Table 3: Gene names and primer sequences for real-time quantitative PCR analysis

Target gene(s)	Primer name	Sequence	Source	Product size (bp)
TLR4	TLR4-F[1]	CCTTGCGTACAGGTTGTTCC	Danesh Mesgaran *et al*. (2021)	129
	TLR4-R[2]	GCCTAAATGTCTCAGGTAGTTAAAGC		
CD14	CD14-F	CACCACATTGCACACCTGTT	Danesh Mesgaran *et al*. (2021)	124
	CD14-R	TACTGGAGTGGGTTGCCATT		
MD2	MD2-F	GGAGAATCGTTGGGTCTGCT	Danesh Mesgaran *et al*. (2021)	92
	MD2-R	GCTCAGAACGTATTGAAACAGGA		
GAPDH	GAPDH-F	TCATTGAAGCCTTCACTACATGGTCT	Danesh Mesgaran *et al*. (2021)	147
	GAPDH-R	TGATGTTGGCAGGATCTCG		
RPS9	RPS9-F	TAGGCGCAGACGGGCAAACA	Danesh Mesgaran *et al*. (2021)	136
	RPS9-R	CCCATACTCGCCGATCAGCTTCA		

TLR4: Toll-like receptor 4, CD14: Cluster of differentiation 14, MD2: Myeloid differential protein 2, GAPDH: Glyceraldehyde-3-phosphate dehydrogenase, and RPS9: Ribosomal protein S9. [1] F: Forward primer, and [2] R: Reverse primer

natural logarithm of the specified amounts of DNA. The reaction efficiency was calculated using the slope of the standard curve (equation: efficiency = (10 (1/slope) - 1) 100. The reliability of the measurements was verified by evaluating the R^2 correlation coefficients (Riasi *et al*., 2024). Relative quantification of microorganisms was performed based on the ΔΔCt method.

Sampling and analysis

Feed intake and production responses

To obtain DMI, orts were collected daily during the high-starch diet period. Feed samples and orts were dried for 48 h at 95°C (AOAC, 1990). Daily DMI (kg/head) was determined by the difference between feed offered and orts. Daily milk production was recorded throughout the experiment, and milk samples were analyzed for nutrient content using Lactoscan S PFP, Bulgaria, during the adaptation and high-starch diet period in the milk laboratory of Pegah Dairy Company (Mashhad, Iran). Fat-corrected milk (3.5% FCM, kg/day) was calculated using the equation of (Tyrrell and Reid, 1965), FCM (3.5%) = (milk amount (kg) × 0.432) + (16.23 × fat (kg/d)); energy corrected milk (ECM) was calculated as presented by Iqbal *et al*. (2009), ECM (kg/d)= milk amount (kg) × (0.327 + 7.2 × % protein/100 + 12.96 × % fat/100), and Milk energy (Mcal/kg of milk) was obtained based on ME = 0.0929 × % fat + 0.0547 × % CP + 0.0359 × % lactose (Iqbal *et al*., 2009).

Blood chemistry

Blood samples were collected from the jugular vein 15 min before morning feeding and at 4, 6, and 8 h post-feeding on the last two induction days. Samples were used for blood chemistry, mRNA expression of CD14, TLR4, and MD2, and APP (Hp and SAA) analysis. At each sampling, 10 ml of blood was collected in vacuum tubes with a clot activator, clotted at room temperature for 30 min, and centrifuged at 1,900 ×g for 15 min. The serum was divided into two 1.5-ml aliquots and stored at -20°C. One aliquot was used for blood chemistry including glucose (GOD-PAP, https://parsazmun.de/GLUCOSE/), non-esterified fatty acids (NEFA, colorimetric method, Randox, County Antrim, UK), β-hydroxybutyric acid (BHBA, kinetic enzymatic method, Randox, County Antrim, UK), high-density lipoprotein (HDL, direct enzymatic colorimetric method, http://paadco.co), glutamic oxaloacetic transaminase (GOT, kinetic UV method based on IFCC recommendations, http://paadco.co), serum glutamic-pyruvic transaminase (SGPT, kinetic UV method based on IFCC recommendations, http://paadco.co). Samples at 6 h post-morning feeding were used to determine both Hp and SAA using an immunoturbidimetric assay by an ELISA reader (Epoch microplate reader, BioTek, USA) and commercial ELISA kits (enzyme-linked immunosorbent assay kit, Shanghai Korain Biotech Co.), respectively. On day 6 of the high-starch diet period, blood samples were collected in EDTA-containing vacuum tubes, snap-frozen, and stored at -80°C for mRNA expression analysis.

RNA isolation and real-time PCR

Snap-frozen whole blood samples were thawed and used for total RNA isolation with AccuZol™ (Bioneer, Korea) following the manufacturer's protocol. RNA purity and integrity were assessed using an Epoch microplate spectrophotometer and agarose gel electrophoresis, respectively. To remove any genomic DNA contamination, total RNA was treated with DNase I (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. Then, 1 µg of isolated RNA was used to synthesize cDNA with AccuPower® RT PreMix (Bioneer, Korea); all procedures were performed according to the manufacturer's instructions. Primer specifications for target genes MD2, TLR4, CD14, and reference genes GAPDH and RPS9 are in Table 3.

All qPCR reaction conditions followed MIQE guidelines. Reactions were conducted in duplicate using RealQ Plus 2X master mix (Ampliqon, Denmark) on a LightCycler® 96 instrument (Life Technologies, Roche Life Science, Basel, Switzerland). Amplification in 96-well plates (Eurogentec, Belgium) contained 2 µL of cDNA, 5 pmol of each primer, and 10 µL of the master mix (total 20 µL). PCR program: 95°C for 5 min, 40 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 20 s, followed by melting curve analysis from 60 to 90°C. An

equal proportion of cDNA from all samples was pooled to generate a cDNA template for standard curve analysis. The standard curve included two-fold serial dilutions of pooled cDNA template over five data points, with three replicates for each dilution (Ghanipoor-Samami *et al*., 2018). Cq (quantitative cycle) values of the standards were used to derive a standard curve, which shows the Cq values as a linear function of the natural logarithm of the specified amounts of cDNA. The reaction efficiency was calculated using the slope of the standard curve (equation: efficiency = (10 (1/slope) - 1) 100. The R2 correlation coefficients were considered to ensure reliability (Riasi *et al*., 2024). Relative quantification of TLR4, CD14, and MD2 expression was performed, with values normalized against the geometrical means of two reference genes, RPS9 and GAPDH (Danesh Mesgaran *et al*., 2021). Relative quantification of target genes was performed based on the ΔΔCt method, and the results are presented as arbitrary units.

Chemical analysis

Dietary ingredients (1 mm, Toos Shekan Khorasan, Mashhad, Iran) underwent chemical composition analysis and DM calculation (AOAC, 1990). Dry matter was determined after 24 h at 95°C (ISO 6496). Ash was determined after 3 h at 550°C (ISO 5984). Nitrogen was assessed using the Kjeldahl method (Kjeltec 2300 Autoanalyzer), with crude protein (CP) calculated as N × 6.25. The NDF and ADF concentrations were evaluated based on Van Soest *et al*. (1991). Starch content was analyzed using the anthrone/sulphuric acid method with glucose as a standard, estimated as 0.9 × glucose content (Cone, 1991).

Statistical analysis

Production response data were analyzed as a CRD using the Mixed procedure of SAS (ver. 9.4). Data related to cows' performance and DMI were pooled for both ruminal cannulated and non-cannulated cows. The same model was used for rumen fermentation responses (cannulated cows) and blood cells/metabolites (non-cannulated cows). Diet and cow effects were fixed and random, respectively. The cow was the experimental unit for all repeated-measures analyses. Bacterial data were analyzed in JMP® 4.0 (SAS Institute) using ANOVA by least-squares fit. Results are reported as least squares mean values, with significance at $P<0.05$ and trends at $0.05 \leq P < 0.10$.

Results

Rumen fermentation responses

As presented in Table 4, starch-rich diets significantly altered ruminal pH. Mean ruminal pH was higher in BG_{LA} (5.97), followed by BG_{SBPE} (5.85), and BG_{CTRL} (5.55, $P<0.001$). Nadir pH was lowest in BG_{CTRL} (5.14) and BG_{SBPE} (5.18) compared with BG_{LA} (5.41, $P<0.01$). The acidosis index was highest in BG_{CTRL} (18.10) and lowest in BG_{LA} (3.05). Duration below pH 5.8 was longest in BG_{CTRL} (362.98 min/d) compared with BG_{LA} (61.35 min/d, $P=0.001$) and BG_{SBPE} (182.93 min/d, $P=0.002$).

The relative abundance of *E. coli* and *M. elsdenii* in the rumen fluid was affected by time and diet. The relative abundance of these bacteria was lower in cows fed BGLA than in the other treatment groups ($P<0.001$). *M. elsdenii* was highest in BG_{CTRL}-fed animals (5.41, $P<0.001$). No significant difference in *E. coli* was observed between BG_{CTRL} and BG_{SBPE}. In BG_{CTRL}-fed cows, both *E. coli* and *M. elsdenii* increased from 4 h to 8 h post-feeding ($P<0.001$). In contrast, in BG_{SBPE}-fed cows, only *E. coli* increased at 8 h ($P<0.001$), while *M. elsdenii* showed a tendency to increase ($P=0.07$). In BG_{LA}-fed cows, *M. elsdenii* decreased at 8 h ($P<0.001$), and *E. coli* remained stable. Overall, *M. elsdenii* abundance was highest in BG_{CTRL} compared with the other groups (Table 5).

Figure 1 shows the effects of diet and sampling time on the molar proportions of total and individual SCFAs, as well as NH_3-N concentrations, which differed significantly among treatments. Total SCFA was influenced by time ($P=0.003$) and by the time×diet interaction ($P=0.05$). BG_{LA} and BG_{CTRL} increased total SCFA at 4 ($P=0.02$) and 8 h ($P<0.05$), while BG_{SBPE} showed a significant difference between 4 (129.75

Table 4: Ruminal pH parameters of fistulated lactating cows fed 3 different diets

Item	Experimental diets[1]			SEM[2]	P-value
	BG_{CTRL}	BG_{LA}	BG_{SBPE}		
Mean	5.55[c]	5.97[a]	5.85[b]	0.01	<.001
Minimum pH	5.14[b]	5.41[a]	5.18[b]	0.04	0.01
Maximum pH	6.52[a]	6.64[a]	6.38[b]	0.06	0.05
Time below pH 6 (min/d)	636.98[a]	197.35[c]	395.02[b]	30.85	0.002
Time below pH 5.8 (min/d)	362.98[a]	61.35[c]	182.93[b]	25.64	0.001
Acidosis index[3] (min/kg DMI)	18.10[a]	3.05[c]	8.13[b]	1.19	0.001
Urine pH	7.64	7.58	7.61	0.09	0.87
Fecal pH	6.44	6.36	6.49	0.14	0.81

[1] BG_{CTRL}: Control diet containing untreated barley grain, BG_{LA}: Treatment diet containing barley grain steeped for 48 h in an equal volume of 5% lactic acid (vol/vol), and BG_{SBPE}: Treatment diet containing barley grain steeped for 48 h in an equal volume of sugar beet pulp extract (vol/vol), [2] SEM: The largest standard error of the mean. Least-squares means with different superscripts ([a, b, c]) within a row indicate a significant difference between treatments ($P \leq 0.05$) based on Tukey analysis, and [3] Acidosis index: Duration of time with ruminal pH<5.8 per kg of DMI (min/kg DMI)

Table 5: Data showing the relative abundance of *Escherichia coli* and *Megasphaera elsdeni* in Holstein dairy cows subjected starch-rich diet by three different experimental diets

Organisms (a.u.)[2]	Experimental diets[1]						SEM[3]	P-value		
	BG_{CTRL}		BG_{LA}		BG_{SBPE}					
	4	8	4	8	4	8		Diet	Time	INT
Escherichia coli	3.32^{b}	4.25^{a}	2.01^{c}	2.09^{c}	3.24^{b}	4.40^{a}	0.37	<0.001	<0.001	0.01
Megasphaera elsdenii	3.19^{b}	7.63^{a}	1.53^{a}	0.71^{b}	2.11^{z}	2.58^{y}	0.17	<0.001	<0.001	<0.001

[1] BG_{CTRL}: Control diet containing untreated barley grain, BG_{LA}: Treatment diet containing barley grain steeped for 48 h in equal quantity of 5% lactic acid (vol/vol), and BG_{SBPE}: Treatment diet containing barley grain steeped for 48 h in equal quantity of sugar beet pulp extract (vol/vol), [2] Values are presented as relative abundance in arbitrary units (a.u.), and [3] The largest standard error of the mean. Least-squares means with different superscripts ($^{a, b}$) within a row indicate significant differences among sampling times within the same experimental diet ($P \leq 0.05$). Superscripts ($^{y, z}$) show a trend ($0.05 < P \leq 0.10$) based on Tukey's test

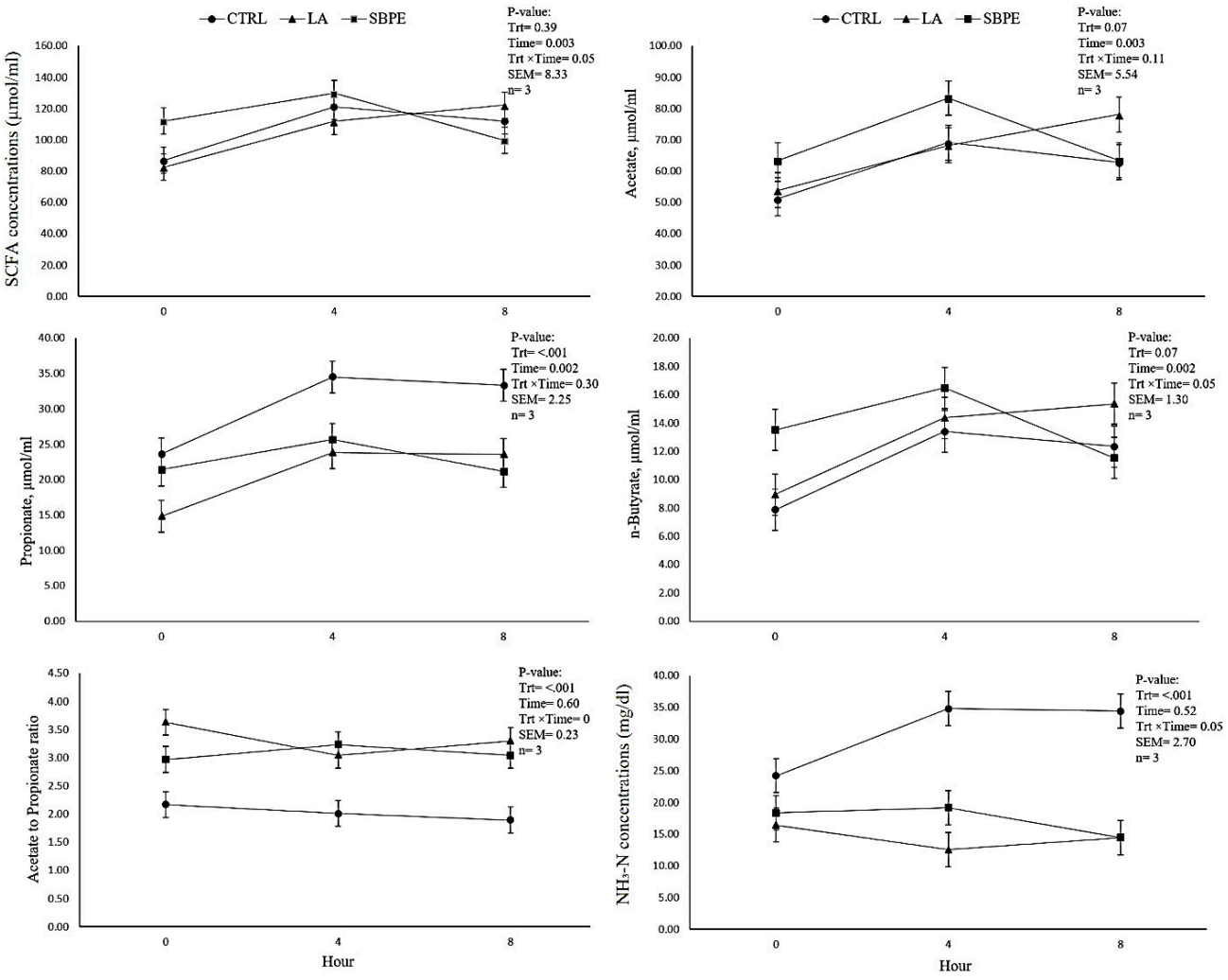

Fig. 1: Total and individual SCF concentrations (µmol/ml) and NH3-N concentrations (mg/dl) in the rumen fluid of lactating Holstein dairy cows fed diets based on native barley grain (BG_{CTRL}; ●), grain steeped in 5% lactic acid solution (LA; ▲) and grain steeped in sugar beet pulp extract (BG_{SBPE}; ■) prior to morning feeding (0), 4 and 8 h after morning feeding (LSM ± SEM; n=3; diet: Effect of the experimental diets; Time = Effect of sampling hour, Diet × Time= Effect of treatment by sampling hour interaction)

µmol/ml) and 8 h (99.70 µmol/ml, P=0.02). The acetate concentration was significantly affected by diet (P=0.001) and by the time×diet interaction (P=0.001). BG_{SBPE} had a higher acetate concentration (80.94 µmol/ml) compared with BG_{CTRL} (61.03 µmol/ml, P<0.001) and BG_{LA} (66.74 µmol/ml, P=0.002). Time and diet significantly affected ruminal propionate concentration (P<0.001). Cows fed BG_{LA} (20.76 µmol/ml) or BG_{SBPE} (22.73 µmol/ml) had lower propionate concentrations compared with those fed BG_{CTRL} (30.46 µmol/ml). Diet (P=0.07, a trend), time (P=0.002), and their interaction (P=0.05) significantly affected n-Butyrate concentration. BG_{SBPE} increased n-Butyrate (13.84 µmol/ml) compared with BG_{CTRL} (11.20

µmol/ml), with no significant changes in BG_{LA}-fed animals (12.89 µmol/ml). Diet (P=0.002) and time×diet interaction (P=0.001) significantly affected n-Valerate molar proportion. Cows fed BG_{LA} (1.48 µmol/ml) had lower ruminal n-Valerate than those fed BG_{CTRL} (1.70 µmol/ml, P=0.01) and BG_{SBPE} (1.79 µmol/ml, P=0.001). The acetate to propionate ratio was higher in BG_{LA} (3.32) and BG_{SBPE} (3.08) compared with BG_{CTRL} (2.02, P<0.01). NH3-N concentration (mg/dl) was influenced by diet (P<0.001) and by the time×diet interaction (P=0.05). BG_{LA} and BG_{SBPE} significantly decreased NH3-N compared with BG_{CTRL} (14.5, 17.33, and 31.13 mg/dl, respectively).

Production responses

Data of DMI, milk yield and composition, and feed efficiency are reported in Table 6. Cows fed BG_{SBPE} had a higher DMI (P=0.03) compared with those fed BG_{CTRL} and BG_{LA} (22.49, 20.05, and 20.41 kg/d, respectively). Experimental diets showed numerical tendencies (0.05<P<0.10) to alter milk production (P=0.09) and the daily yields of fat (P=0.07), protein (P=0.08), lactose (P=0.07), 3.5% FCM (P=0.09), ECM (P=0.09), and ME (P=0.09). These tendencies were not statistically significant but indicate noticeable numerical differences among treatments. Briefly, BG_{SBPE} caused a significant increase in milk yield relative to BG_{CTRL} (P=0.04). As compared with BG_{CTRL}, feeding BG_{SBPE} (P=0.04) or BG_{LA} (P=0.09) increased the 3.5% FCM. Animals fed BG_{SBPE} had higher ECM compared with BG_{CTRL} (P=0.04). Milk energy was also affected by diet, with BG_{SBPE} showing an increase (P=0.04) and BG_{LA} showing a tendency for an increase (P=0.09) relative to BG_{CTRL}. Feeding BG_{LA} led to an increase in milk fat yield (P=0.03), while BG_{SBPE} tended to increase it (P=0.08) as compared to BG_{CTRL}. Milk protein and lactose yield were significantly increased in animals fed BG_{SBPE} compared with those of BG_{CTRL} (P=0.03). Cows fed BG_{LA} had higher milk fat percentage (P=0.01) and fat to protein ratio (P=0.01) when compared with the other groups.

Blood chemistry

The results of the blood chemistry analysis are represented in Fig. 2. The effects of diet (P=0.04), time (P<0.001), and their interaction (P=0.02) on blood glucose concentration were significant. Providing a starch-rich diet either with BG_{LA} (66.17 mg/dl; P=0.05) or BG_{SBPE} (65.12 mg/dl; P=0.02), as compared with BG_{CTRL} (72.92 mg/dl), caused a decrease in glucose concentration. The concentration of SGPT was affected by the experimental diets (P<0.001), time (P=0.08), and their interaction (P<0.001). Feeding BG_{LA} (32.21 IU/L; P<0.001) and BG_{SBPE} (27.29 IU/L; P<0.001) led to a lower SGPT when compared with BG_{CTRL} (33.50 IU/L). The difference between BG_{LA} and BG_{SBPE} was also significant, with BG_{LA} showing higher values than BG_{SBPE} (P<0.001).

Blood mRNA expression and acute phase protein responses

The effects of starch-rich diets (BG_{CTRL}, BG_{LA}, and BG_{SBPE}) on mRNA expression of CD14, TLR4, and

Table 6: Effects of different barley grain treatments on DMI (kg/d), milk yield (kg/d) and composition (%), and feed efficiency in lactating cows

Item	Experimental diets[1]			SEM[2]	P-value		
	BG_{CTRL}	BG_{LA}	BG_{SBPE}		Diet	Day	INT
DMI (kg/d)	20.05^{b}	20.11^{b}	22.49^{a}	0.67	0.03	0.15	0.33
Milk yield (kg/d)							
Milk	31.33^{b}	32.13^{bz}	35.68^{ay}	1.37	0.09	<0.001	0.16
FCM	29.64^{bz}	33.36^{by}	34.20^{a}	1.48	0.09	<0.001	0.14
ECM	30.19^{b}	33.45^{ab}	34.75^{a}	1.40	0.09	<0.001	0.13
Milk energy	20.36^{bz}	22.79^{ay}	23.46^{a}	0.96	0.09	<0.001	0.13
Fat	0.99^{bz}	1.20^{a}	1.17^{ay}	0.06	0.07	<0.001	0.13
Protein	1.03^{b}	1.09^{ab}	1.17^{a}	0.04	0.08	<0.001	0.12
Lactose	1.53^{b}	1.60^{ab}	1.75^{a}	0.06	0.07	<0.001	0.14
Milk composition (%)							
Fat	3.18^{b}	3.74^{a}	3.24^{b}	0.13	0.01	0.21	0.40
Protein	3.30	3.36	3.30	0.08	0.86	0.95	0.51
Lactose	4.90	4.98	4.91	0.13	0.89	0.93	0.49
Fat: protein ratio	0.97^{b}	1.11^{a}	0.98^{b}	0.04	0.02	0.25	0.52
Feed efficiency							
Milk/DMI	1.56	1.59	1.60	0.05	0.86	<0.001	0.13
FCM/DMI	1.48^{z}	1.65^{y}	1.53	0.07	0.21	<0.001	0.11
ECM/DMI	1.51	1.66	1.56	0.07	0.30	<0.001	0.11

DMI: Dry matter intake, 3.5% FCM (Tyrrell and Reid, 1965) = (milk amount (kg) × 0.432) + (16.23 × fat (kg/d)), ECM (Iqbal *et al*., 2009) = milk amount (kg) × (0.327 + 7.2 × % protein/100 + 12.96 × % fat/100), Milk energy (Mcal/kg of milk) (Iqbal *et al*., 2009) = 0.0929 × % fat + 0.0547 × % CP + 0.0359 × % lactose, [1]BG_{CTRL}: Control diet containing untreated barley grain, BG_{LA}: Treatment diet containing barley grain steeped for 48 h in equal quantity of 5% lactic acid (vol/vol), and BG_{SBPE}: Treatment diet containing barley grain steeped for 48 h in equal quantity of sugar beet pulp extract (vol/vol), and [2] The largest standard error of the mean. Least-squares means with different superscripts ($^{a, b}$) within a row indicate a significant difference between treatments (P≤0.05), and y and z show a trend (P≤0.10) based on Tukey analysis

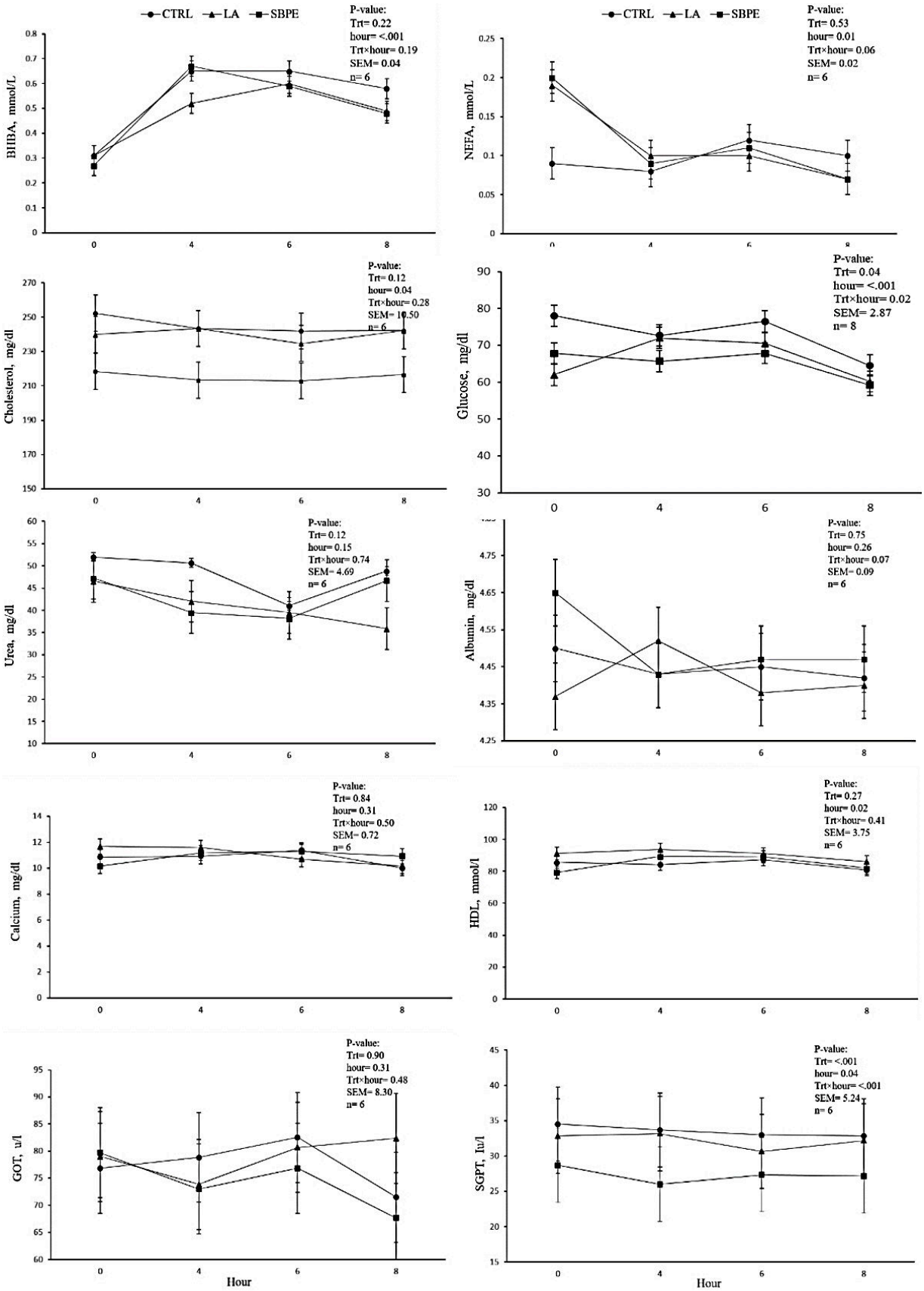

Fig. 2: Variation in blood metabolite concentrations including BHBA (mmol/L), NEFA (mmol/L), HDL (mmol/L), GOT (U/L), and SGPT (U/L) in serum of lactating Holstein dairy cows fed diets based on native barley grain (BG_{CTRL}; ●), grain steeped in 5% lactic acid solution (BG_{LA}; ▲) and grain steeped in sugar beet pulp extract (BG_{SBPE}; ■) before morning feeding (0), 4, 6 and 8 h after morning feeding (LSM ± SEM; n=6; Diet: Effect of the experimental diets; Time: Effect of sampling hours, Diet × Time: Effect of treatment by sampling hour interaction)

Table 7: Expression patterns of selected genes (CD14, TLR4, and MD2) in the whole blood, acute phase proteins, Calcium, and high-density lipoprotein of lactating Holstein dairy cows fed with three different diets at 6 h after morning feeding

Indexes[1]	Experimental diets[2]			SEM[3]	P-value
	BG_{CTRL}	BG_{LA}	BG_{SBPE}		
Gene expression (a.u.)[4]					
CD14	4.29[a]	2.04[b]	2.54[b]	0.32	0.001
TLR4	1.58[a]	0.78[b]	0.92[b]	0.17	0.01
MD2	1.66[a]	0.73[b]	0.57[b]	0.19	0.03
Acute phase proteins					
Haptoglobin (µg/ml)	41.43[a]	32.68[c]	38.41[b]	0.68	<0.001
Serum amyloid A (µg/ml)	10.32[a]	4.83[b]	4.30[b]	0.33	<0.001
Metabolites					
High-density lipoproteins (mg/dl)	90.00	92.60	92.40	2.98	0.79
Calcium (mg/dl)	12.00	11.50	11.95	0.60	0.80

[1] CD14: Cluster of differentiation 14, TLR4: Toll-like receptor 4, MD2: Myeloid differential protein 2, [2] BG_{CTRL}: Control diet containing untreated barley grain, BG_{LA}: Treatment diet containing barley grain steeped for 48 h in equal quantity of 5% acid lactic (vol/vol), and BG_{SBPE}: Treatment diet containing barley grain steeped for 48 h in equal quantity of sugar beet pulp extract (vol/vol), [3]The largest standard error of the mean. Least-square means with different superscripts ([a, b, c]) within a row indicate significant differences between treatments ($P \leq 0.05$) based on Tukey analysis, and [4]Values are presented in arbitrary units (a.u.)

MD2 and the concentration of APP (Hp and SAA) 6 h post-morning feeding are presented in Table 7. Compared with BG_{CTRL}, cows fed BG_{LA} and BG_{SBPE} showed markedly reduced expression of the selected genes ($P<0.01$). However, the mRNA expression of these genes was not significantly different between BG_{LA} and BG_{SBPE}. In response to BG_{LA} and BG_{SBPE} feeding, both Hp and SAA concentrations were lower in comparison with BG_{CTRL} ($P<0.001$).

Discussion

Rumen fermentation responses

Starch-rich diets challenge ruminal fermentation by lowering pH and increasing the risk of SARA. In our study, cows fed processed BG (BG_{LA} or BG_{SBPE}) maintained higher ruminal pH and exhibited a reduced acidosis burden compared with BG_{CTRL}, indicating improved fermentation stability. The low mean pH and elevated acidosis index in BG_{CTRL} are consistent with the well-documented risk of SARA under rapidly fermentable starch diets (Zebeli *et al*., 2008). Processing BG with LA has been reported to reduce microbial access to starch granules, increase resistant starch, and enhance starch bypass to the small intestine, thereby lowering the acid load in the rumen (Zebeli *et al*., 2008; Iqbal *et al*., 2009; Deckardt *et al*., 2014). Similarly, treating BG with SBPE has been shown to decrease ruminal starch degradability and increase post-ruminal starch flow (Naseroleslami *et al*., 2022), which could explain the higher ruminal pH and lower acidosis index observed in BG_{SBPE}-fed cows. Collectively, these mechanisms help to maintain a healthier ruminal environment and mitigate the severity of acidosis in cows receiving processed BG diets.

The main end products of ruminal fermentation, SCFAs, exhibit relative proportions that are strongly associated with ruminal health and nutrient utilization. In our study, processed BG diets shifted the SCFA profile toward a more favorable pattern compared with BG_{CTRL}. Feeding BG_{SBPE} increased acetate and butyrate concentrations and resulted in a higher acetate-to-propionate ratio. Although lactate was not measured, previous reports indicate that part of ruminal lactate can be converted to acetate (Giesecke and Stangassinger, 1980), which may partially explain the higher acetate in BG_{SBPE}-fed cows. The higher butyrate concentration in BG_{SBPE}-fed cows may have contributed to the improved pH, since butyrate fermentation generates fewer protons than acetate (Chamberlain *et al*., 1985; Oba, 2011). Similar patterns have been reported when sugar beet pulp was included in the diet, with shifts toward greater acetate and butyrate and lower propionate (Petri *et al*., 2019). Although sugar beet pulp and its extract differ in composition, it is plausible that soluble components of the extract contributed to the more favorable SCFA profile observed in our study. A greater A:P ratio is also indicative of more stable ruminal fermentation conditions (Zebeli *et al*., 2008). Although BG_{LA} did not significantly change total SCFA concentration, cows receiving this diet also showed numerically higher acetate and butyrate and a significantly greater A:P ratio than BG_{CTRL}, which agrees with the healthier ruminal environment observed in this group. Conversely, BG_{CTRL}-fed cows exhibited the highest propionate concentrations but also the lowest ruminal pH, suggesting that excessive starch fermentation favored propionate production at the expense of ruminal stability. Collectively, these findings indicate that processing BG with LA or SBPE not only alleviated ruminal acidosis but also promoted SCFA profiles consistent with improved fermentation balance.

In line with previous research (Khafipour *et al*., 2009b), our results showed a higher proportion of *E. coli* and a higher acidosis index in BG_{CTRL} compared with BG_{LA} and BG_{SBPE}. Interestingly, the relative abundance of *M. elsdenii* was also greater in B_{GCTRL}. *M. elsdenii* is a key lactate-utilizing bacterium (Counotte and Prins, 1981); this may represent a compensatory microbial response to the more acidic environment in BG_{CTRL}. However, lactate was not measured in our study, and

thus, no direct link between *M. elsdenii* and lactate concentrations can be confirmed. It is possible that under severe acidotic conditions, lactate production exceeded utilization capacity, leading to an imbalance, as suggested by previous reports (Nagaraja and Titgemeyer, 2007). The lower abundance of *E. coli* in BG_{SBPE} may be attributed to the more stable ruminal conditions and higher pH in this group. Sugar beet pulp has previously been shown to modify the rumen microbiota, reducing lactate-producing bacteria and promoting acetate producers (Petri *et al*., 2019). Although our study used an extract rather than the pulp itself, it is plausible that soluble bioactive components in SBPE contributed to the higher pH and more favorable microbial profile. Nevertheless, the exact mechanism of these effects remains unclear and requires further investigation.

Ruminal NH_3-N concentration was lower in cows fed BG_{LA} and BG_{SBPE} compared with BG_{CTRL}. NH_3-N reflects the balance between dietary protein degradation and microbial protein synthesis (O'Connor *et al*., 1993); this reduction suggests more efficient microbial capture of ammonia under processed BG diets. The interpretation is consistent with our earlier findings (Kheirandish *et al*., 2022), in which processing BG with LA or SBPE increased microbial protein synthesis *in vitro*. Although in Naseroleslami *et al*. (2022) processed BG with SBPE was associated with higher NH_3-N, likely due to differences in basal diet composition and energy synchronization, the starch-rich diet in the present study appeared to improve synchrony of carbohydrate and nitrogen release, thereby enhancing microbial protein yield (Malekjahani *et al*., 2017).

Productive and metabolic responses

One of the undesired consequences of ruminal acidosis is a reduction of DMI (Emmanuel *et al*., 2008; Khafipour *et al*., 2009b). In the present study, BG_{SBPE} significantly increased DMI, which was accompanied by a higher milk yield. This observation agrees with findings by Naseroleslami *et al*. (2022), who also reported greater feed intake in cows receiving SBPE-treated BG. The higher intake could be attributed to improved rumen environment and nutrient digestibility in BG_{SBPE}, which may have reduced the risk of subacute acidosis and promoted better rumen function. Propionate is the primary precursor for glucose in ruminants. The BG_{CTRL} group showed higher ruminal propionate concentrations, which coincided with elevated serum glucose compared with the processed groups. In contrast, cows fed BG_{LA} and BG_{SBPE} had lower propionate, consistent with their lower serum glucose concentrations, which may reflect reduced substrate supply for hepatic gluconeogenesis (Aschenbach *et al*., 2010). However, this reduction should not be interpreted as a disadvantage, since the processed BG groups maintained a healthier ruminal environment with higher pH and a reduced acidosis burden, suggesting more stable fermentation. These findings suggest that the slight decrease in glucose may be compensated for by enhanced rumen function and a reduced risk of SARA, thereby contributing to improved animal health and productivity. Although the hypophagic effect of increased propionate and blood glucose levels might contribute to decreased DMI in BG_{CTRL}, it should be noted that DMI is likely regulated by multiple interacting factors beyond glucose and propionate, including rumen pH, fermentation stability, diet digestibility, passage rate, and inflammatory status (Oba and Allen, 2003; Allen *et al*., 2005). Thus, the lower intake observed in BG_{CTRL} is more plausibly explained by prolonged ruminal acidosis and impaired rumen function than by glucose-mediated satiety alone. In contrast, the stable ruminal environment in BG_{SBPE}-fed cows likely encouraged greater intake.

Moreover, BG_{LA} feeding did not significantly change DMI, which agrees with previous studies (Iqbal *et al*., 2009; Zebeli *et al*., 2012). This may be because LA treatment stabilizes ruminal fermentation without markedly enhancing palatability or nutrient digestibility to a level sufficient to increase feed intake. Thus, BG_{LA} appears to maintain intake at a stable level rather than stimulating a significant increase.

Regarding milk production parameters, reductions in milk production and milk fat percentage have been considered consequences of SARA and starch-rich diets (Nocek, 1997). Feeding BG_{SBPE} significantly increased milk yield compared with BG_{CTRL}, consistent with previous observations (Naseroleslami *et al*., 2022). This improvement may be attributed primarily to enhanced fermentation stability and greater nutrient availability. Although BG_{LA} did not significantly affect milk yield, it improved milk fat yield and the fat-to-protein ratio relative to BG_{CTRL}. This response can be linked to the healthier ruminal environment observed in BG_{LA}-fed cows, which showed the highest mean ruminal pH (5.97) and the shortest duration below pH 5.8 (61.35 min/day). This is consistent with the report of Krause *et al*. (2002b), who observed that higher mean ruminal pH and shorter time below pH 5.8 were positively correlated with milk fat percentage. Since acetate and butyrate are major precursors of milk fat, and a higher A:P ratio favors fat synthesis, the numerical increases in acetate and butyrate, together with the significantly higher A:P ratio in the BG_{LA} group compared with BG_{CTRL}, are consistent with the improved milk fat yield and fat-to-protein ratio observed in this group .Conversely, BG_{CTRL}-fed cows had the lowest mean pH (5.55) and longest duration below 5.8 (362.98 min/day), which was associated with depressed milk fat percentage. These observations support the view of Zebeli *et al*. (2008) that milk fat content and percentage may serve as indicators of a healthy rumen milieu. Overall, these results suggest that improved rumen environment and fermentation stability in processed BG diets supported greater nutrient intake and, consequently, enhanced productive performance.

Cows fed BG_{LA} and BG_{SBPE} had lower blood glucose concentrations, likely due to lower propionate levels in ruminal fluid (Iqbal *et al*., 2012a). Both processed BG groups (BG_{LA} and BG_{SBPE}) also showed lower serum GPT activity, an indicator of hepatocellular damage

(Huang *et al*., 2006). This finding suggests reduced liver stress compared with BG_{CTRL}, in agreement with earlier reports linking high-grain feeding to elevated GOT and GPT activities as markers of liver injury under SARA conditions *(*Guo *et al*., 2017). Sugar beet pulp has been shown to exert hepatoprotective effects, partly through its antioxidant properties (AbouSekken *et al*., 2013), which may explain the lower GPT levels in BG_{SBPE}. Similarly, LA processing of BG has been reported to improve metabolic profiles and mitigate stress responses in dairy cows (Iqbal *et al*., 2012b), which may explain the lower GPT in BG_{LA}. Thus, the reduced GPT observed in both processed BG groups may indicate less tissue damage and inflammatory stress. However, further research is needed to clarify the mechanisms.

Lower blood urea concentrations in BG_{LA} were expected since processing BG with LA reduces protein degradation in the rumen (Kheirandish *et al*., 2022). Processing BG with organic acids (LA, citric acid, tannic acid) and heat has been shown to reduce protein degradation and ammonia concentration (Khol-Parisini *et al*., 2015). Our previous trial also showed that LA processing increased microbial protein concentration without significantly affecting $N\text{-}NH_3$ levels (Kheirandish *et al*., 2022). Applying LA in processed BG may affect the energy release rate, synchronizing energy and nitrogen liberation, thus boosting microbial yield (Kheirandish *et al*., 2022). This resulted in a reduction in ammonia-N concentration, shifting from protein degradation to microbial protein production (Rodríguez *et al*., 2007).

Blood mRNA expression and acute phase protein responses

Our study investigated whether processing BG with LA or SBPE could mitigate the adverse effects of starch-rich diets by altering ruminal starch degradation and modulating immune-related gene expression. We found that processing BG with these organic substances mitigated the negative impacts of starch-rich diets, as reflected by reduced mRNA expression of CD14, TLR4, and MD2, along with lower circulating APPs. These changes suggest a potential improvement in systemic immune status, likely associated with a healthier ruminal environment. It is well established that SARA promotes the production of harmful microbial by-products, such as LPS, which trigger inflammatory responses (Zebeli and Metzler-Zebeli, 2012; Stefanska *et al*., 2018). For example, grain-enriched diets supplying 30-45% BG of DM were shown to reduce ruminal pH and increase microbial toxins by 6- to 14-fold (Emmanuel *et al*., 2008). In our study, cows fed BG_{LA} or BG_{SBPE} diets exhibited downregulated expression of CD14, TLR4, and MD2 compared with BG_{CTRL}. By maintaining higher ruminal pH, these treatments may have limited LPS release and its binding to the LPS receptor complex. Recognition of LPS by the CD14/TLR4/MD2 complex activates pro-inflammatory cytokine pathways, which are upstream mediators of APR and APP production (Ceciliani *et al*., 2012; Tothova *et al*., 2014). Consistent with this finding, Stefanska *et al*. (2018) reported negative correlations between ruminal pH and the expression of CD14, MD2, and TLR4, as well as serum concentrations of SAA and Hp. Taken together, our findings suggest that feeding BG_{LA} or BG_{SBPE} can help mitigate immune activation by stabilizing rumen fermentation and lowering the systemic inflammatory response. Nevertheless, further research is warranted to fully elucidate the metabolic consequences of feeding LA- and SBPE-treated BG to dairy cows.

Including LA- or SBPE-processed BG in diets may help maintain a higher ruminal pH, possibly by altering the rate or extent of starch degradation, as suggested by previous studies. This could promote more stable fermentation and support improvements in milk fat yield and the fat-to-protein ratio. In our study, SBPE-processed BG was associated with a more favorable SCFA profile, characterized by lower propionate and higher acetate and butyrate concentrations. Furthermore, both BG_{LA} and BG_{SBPE} modulated immune responses by down-regulating the expression of CD14, TLR4, and MD2, and by lowering circulating SAA and Hp levels. In conclusion, these findings indicate that BG processing, especially with SBPE, may alleviate certain adverse effects of starch-rich diets and promote better ruminal and systemic health in dairy cows. Further research is warranted to confirm these mechanisms and their implications under practical feeding conditions.

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Conflict of interest

The authors declare no conflict of interest.

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Use of generative AI and AI-assisted technologies statements

During the preparation of this manuscript, the authors used Grammarly for language editing and improvement of grammar and readability.

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