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Original Research Article

Dietary supplementation of sodium butyrate enhances lactation performance by promoting nutrient digestion and mammary gland development in dairy cows

Jing Zhang, Lijun Bu, Yapeng Liu, Wenjie Huo, Chengqiang Xia, Caixia Pei, Qiang Liu*

College of Animal Sciences, Shanxi Agricultural University, Taigu 030801, China

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ABSTRACT

This experiment was to evaluate the influence of sodium butyrate (SB) addition on milk production, ruminal fermentation, nutrient digestion, and the development and metabolism regulation of the mammary gland in dairy cows. Forty Holstein dairy cows averaging 710 ± 18.5 kg body weight, 72.8 ± 3.66 d in milk (DIM), and 41.4 ± 1.42 kg/d milk production were divided into four treatments blocked by DIM and milk production. Treatments were control group, low SB, medium SB, and high SB with 0, 100, 200 and 300 g/d of SB addition per cow, respectively. The study lasted for 105 d. Production of milk, milk protein and lactose quadratically increased ($P < 0.05$), while fat-corrected milk, energy-corrected milk and milk fat yields linearly increased ($P < 0.05$) with increasing SB addition. The digestibility of dietary dry matter, organic matter, and crude protein linearly increased ($P < 0.05$), whereas the digestibility of ether extract, neutral detergent fibre, and acid detergent fibre quadratically increased ($P < 0.05$). Ruminal pH quadratically decreased ($P = 0.04$), while total volatile fatty acids (VFA) quadratically increased ($P = 0.03$) with increasing SB addition. The acetic acid to propionic acid ratio increased ($P = 0.03$) linearly due to the unaltered acetic acid molar percentage and a linear decrease in propionic acid molar percentage. Ruminal enzymatic activity of carboxymethyl-cellulase and α-amylase, populations of total bacteria, total anaerobic fungi, total protozoa, *Ruminococcus albus, R. flavefaciens, Butyrivibrio fibrisolvens*, *Fibrobacter succinogenes,* and *Ruminobacter amylophilus* linearly increased ($P < 0.05$). Blood glucose, urea nitrogen, and non-esterified fatty acids linearly decreased ($P < 0.05$), while total protein concentration linearly increased ($P = 0.04$). Moreover, the addition of SB at 200 g/d promoted ($P < 0.05$) mRNA and protein expression of PPARγ, SREBF1, ACACA, FASN, SCD, CCNA2, CCND1, PCNA, Bcl-2, GPR41, and the ratios of p-Akt/Akt and p-mTOR/mTOR, but decreased ($P < 0.05$) mRNA and protein expressions of Bax, caspase-3, and caspase-9. The results suggest that milk production and milk fat synthesis increased with SB addition by stimulating rumen fermentation, nutrient digestion, gene and protein expressions concerned with milk fat synthesis and mammary gland development.

1. Introduction

The fat content along with the composition of fatty acids in milk are primary parameters for evaluating milk quality in the dairy industry (Bernard et al., 2008). The fatty acids in milk are derived from de novo synthesis of acetate and β-hydroxybutyrate (BHBA) in the bovine mammary gland, from fatty acids absorbed by the bovine mammary gland from the diet or the mobilization of body fat reserves (Sheng et al., 2015). Furthermore, butyrate can be absorbed by the rumen epithelium and rapidly metabolized to BHBA (Naeem et al., 2012), which in turn elevates the blood BHBA

* Corresponding author.
E-mail address: liuqiangabc@sxau.edu.cn (Q. Liu).
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concentration (Izumi et al., 2019). Additionally, BHBA is not only a precursor of milk fat synthesis, but also an important carrier of energy transfer from liver tissue to peripheral tissue under low glucose conditions (Ali et al., 2021; Sun et al., 2019).

Research has elucidated that the addition of butyrate to the dairy cows' diet increases milk fat content and production (Huhtanen et al., 1998; Izumi et al., 2019) and nutrient digestibility (Huhtanen et al., 1993; Fukumori et al., 2020), but reduces blood glucose content (Herrick et al., 2018; Urrutia et al., 2019; Halfen et al., 2021), urea nitrogen content, and non-esterified fatty acid (NEFA) content (Urrutia et al., 2019; Halfen et al., 2021). However, findings vary regarding the influence of butyrate addition on dry matter (DM) intake, milk yield, and ruminal fermentation response because of variations in the administration dose of butyrate and dietary composition. Additionally, sodium butyrate (SB) elevates the expression of genes concerned with lactation in bovine mammary epithelial cells (BMEC) in vitro (Chen et al., 2019; Cheng et al., 2020). However, less is known about microbial enzyme activity, microflora composition, and the expression of genes and proteins concerned with fatty acid synthesis and the proliferation and apoptosis of mammary glands, following SB addition in the dairy cow diet.

In this study, we hypothesized that the addition of SB could elevate the production of milk and milk fat by stimulating rumen fermentation, nutrient digestion, and the expression of genes and proteins concerned with fatty acid synthesis, cell proliferation, and apoptosis in bovine mammary glands. Therefore, we aimed to evaluate the underlying mechanism of SB addition on milk production and quality.

2. Materials and methods

2.1. Animal ethics statement

This experimental design and research proposal was approved by the Animal Care and Use Committee of Shanxi Agriculture University, Taigu, Shanxi, China. All animal experiments complied with the ARRIVE guidelines.

2.2. Holstein cows, experimental design, and total mixed ration

Forty primiparous Holstein dairy cows, averaging 710 ± 18.5 kg body weight (BW), 72.8 ± 3.66 d in milk (DIM), and 41.4 ± 1.42 kg/d milk production, were divided into four treatments blocked by DIM and milk yield. The treatment groups were as follows: control group, low SB (LSB), medium SB (MSB) and high SB (HSB), with 0, 100, 200 and 300 g/d SB per cow, respectively. The SB additive (feed grade, 990 g/kg; Yikang Chemical Co., Ltd, Wuhan, China) was hand-mixed into the top one-third of the daily ration. Total mixed ration (TMR, Table 1) was formulated for lactating dairy cows based on NRC (2001). The feeding experiment was conducted for 105 d, comprising a 15-d adaptation period and then a 90-d sampling period. Cows were housed in a naturally ventilated, two-row, head-to-head free-stall barn, milked three times per day (at 05:30, 13:30, and 20:30), fed the same standard diet ad libitum, and had free access to water.

2.3. Sampling and data collection

During the sampling period, the cows were weighed at 16:00 on d 1 and 90. The TMR provided and refusals for each dairy cow were determined daily to estimate DM intake. The samples were collected every 10 d and kept at −20 °C for later determination. Milk production was measured daily for each cow and averaged each week for a time course analysis. Milk samples were collected weekly and kept at 4 °C with antimicrobial agent 2-bromo-2-nitropropane-1, 3-diol for later determination. At 06:30 and 18:30 during d 70 to 87, all cows were dosed with 5 g of chromic oxide powder placed in a gelatin capsule as a digestion marker. During d 78 to 87, about 250 g of faecal samples were collected from each cow's rectum at 07:00, 13:00, 19:00, and 01:00, and kept at −20 °C for later analysis. During d 88 to 89, samples of TMR, refusals, and faeces, were collected for each cow, dried at 55 °C for 72 h, and ground to pass through a 1 mm screen with a cutter mill (Beishengwei Experimental Instrument Co., Ltd., Changzhou, China).

Table 1
Ingredients and chemical composition of the basal diet (%, dry-matter basis).

Item	Contents
Ingredients	
Corn fodder silage	25.00
Alfalfa hay	12.00
Oat hay	13.00
Corn grain, ground	25.60
Wheat bran	6.00
Soybean meal	9.10
Rapeseed meal	2.50
Cottonseed cake	5.00
Calcium carbonate	0.50
Salt	0.50
Dicalcium phosphate	0.30
Mineral and vitamin premix[1]	0.50
Chemical composition	
Organic matter	94.5
Crude protein	16.6
Ether extract	3.24
Neutral detergent fibre	31.2
Acid detergent fibre	19.3
Calcium	0.71
Phosphorus	0.44
NE_L[2], MJ/kg	6.58

[1] Per kilogram premix: 20,000 mg Fe, 1,600 mg Cu, 8,000 mg Mn, 7,122 mg Zn, 1,200 mg I, 60 mg Se, 20 mg Co, 820,000 IU vitamin A, 300,000 IU vitamin D, and 10,000 IU vitamin E.
[2] Net energy for lactation (NE_L) was estimated based on NRC (2001).

At 06:30, 9:30, 12:30 and 15:30 on d 44 and 89 of the sampling period, rumen fluid from each cow was collected using an oral stomach tube. The initial 150 mL of ruminal fluid was abandoned to avoid saliva contamination, and the next 200 mL was reserved. The ruminal pH of each cow was measured once using a portable pH meter (ST3100/F, Zhejiang Scientific Equipment Co. LTD, Zhejiang, China). The samples of rumen fluid were filtered through four layers of medical gauze. Exactly 5 mL of filtrate was mixed with 1 mL of 250 g/L meta-phosphoric acid and kept at −20 °C for the determination of volatile fatty acids (VFA). Exactly 5 mL of filtrate was mixed with 1 mL of 20 g/L sulfuric acid and kept at −20 °C for the analysis of ammoniacal nitrogen. For microbial DNA extraction and analyses of microbial enzymatic activity, 50 mL of filtrate was put into liquid nitrogen and then kept at −80 °C.

At 10:30 on d 90 of the sampling period, blood samples for each dairy cow were taken from the coccygeal vessel into 10 mL evacuated tubes (Hunan Liuyang Medical Instrument Co. Ltd, Liuyang, China). To separate the serum, blood samples were centrifuged at 2,000 × *g* and 4 °C for 12 min. Samples of serum were kept at −20 °C.

For each cow in groups supplemented with 0 and 200 g SB per cow per d, mammary tissue biopsies were carried out from 16:00 to 20:00 on d 90 of the sampling period. About 1 g of secretory tissues in the mammary gland of each cow was collected via surgical biopsy based on the method of Farr et al. (1996) from the midpoint section of the rear quarter. Tissue biopsy samples for total RNA extraction were rapidly frozen in liquid nitrogen and kept in a low temperature refrigerator at −80 °C.

2.4. Chemical analyses

The content of DM (method 934.01), nitrogen (method 976.05), ether extract (EE) (method 973.18) and crude ash (method 942.05) content in TMR, refusal, and faeces samples were measured based on AOAC (2000). The content of organic matter (OM) was estimated as the difference between DM and crude ash. The content of neutral detergent fibre (NDF) was determine as elaborated by Van Soest et al. (1991) using heat-stable alpha-amylase and sodium sulfite. The content of acid detergent fibre (ADF) was analysed based on AOAC (2000, method 973.18). The content of fat, true protein, and lactose in the milk were analysed using a Milko Scan FT-120 unit (Foss Electric, Hillerød, Denmark) based on AOAC (2000, method 972.16). The content of chromium in the faeces was measured using atomic absorption spectrophotometry (Williams et al., 1962). Ruminal VFA concentrations were analysed using gas chromatography (GC-7890; Shandong Jinpu Analytical Instrument Co. Ltd, Jinan, China). Ammoniacal nitrogen content was analysed according to AOAC (2000). The enzymatic activities of ruminal cellobiose, carboxymethylcellulose (CMC), α-amylase, xylanase, pectinase, and protease were measured as elaborated by Agarwal et al. (2002). Total protein, glucose, urea nitrogen, NEFA, and insulin content in serum were analysed using a Konelab auto-analyser (Thermo Fisher Scientific Oy, Vantaa, Finland) and an enzyme-linked immunosorbent assay test assay kit (Beijing Meilian Biotechnology Co. Ltd, Beijing, China).

2.5. Extraction of microbial DNA and real-time PCR

Exactly 1.5 mL of homogeneous ruminal fluid was used to extract microbial DNA using the repeated bead-beating plus column (RBB + C) as elaborated by Yu and Morrison (2004). The integrity and purity of the extracted DNA were checked via agarose gel electrophoresis and a NanoDrop 2000 Spectrophotometer (Thermo Scientific, NanoDrop Technologies), respectively. The target microbial population comprised total bacteria, protozoa, anaerobic fungi, and methanogens bacteria, *Ruminococcus* (*R.*) *flavefaciens*, *R. albus*, *Butyrivibrio* (*B.*) *fibrisolvens*, *Fibrobacter* (*F.*) *succinogenes*, *Ruminobacter* (*Rb.*) *amylophilus* and *Prevotella* (*P.*) *ruminicola*. The target microbial primer set sequences were designed based on the reference (Denman and McSweeney, 2006; Stevenson and Weimer, 2007) and listed in Table 2. For absolute quantification of gene copy number, ten sample-derived standards were prepared from the microbial DNA treatment pool by conventional PCR. The purification and quantification of the PCR product was conducted by using the PureLink Quick Gel Extraction and PCR Purification Combo Kit (Thermo Fisher Scientific Co. Ltd), and by using a spectrophotometer, respectively. The mass concentration and the length of the PCR product was used to calculate the copy number of each standard (Yu and Morrison, 2004). The quantification of the target DNA was carried out by using 10-fold serial dilutions from 10^1 to 10^8 DNA copies. The amplification and detection of real-time PCR were conducted in a Chromo 4 system (Bio-Rad, USA) in triplicate. The 20 μL reaction mixture included 10 μL of SYBR Premix Taq II (TaKaRa), 2 μL of DNA template, 0.8 μL of each primer (10 μmol/L), 0.4 μL of ROX Reference Dye II (TaKaRa), and 6.0 μL of dH_2O. The PCR cycling conditions were detailed as follows: 1 cycle at 50 °C for 2 min and 95 °C for 2 min for the original denaturation step, followed by 40 cycles at 95 °C for 15 s, anneal at annealing temperature (Table 2) of each target microbial DNA for 30 s, and extension at 60 °C for 1 min.

2.6. Extraction of mammary gland RNA and quantitative real-time PCR

The total RNA was isolated from 100 mg mammary gland tissues using the Trizol Reagent RNA extraction kit (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. The concentration and quality of the extracted RNA were measured using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Rockland, DE, USA). For all preparations, the ratio of absorbance at 260 and 280 nm was close to 2.0. The denaturing agarose gel electrophoresis and ethidium bromide staining was used to check the integrity of the RNA. The cDNA was synthesized from 500 ng total RNA of each sample per 10 μL sample reaction using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories GmbH, Munich, Germany) following the manufacturer's specification. The reaction was conducted at 37 °C for 15 min and 85 °C for 5 min. To determine possible contamination with genomic or environmental DNA, a negative control reaction without reverse transcriptase was carried out for each sample (Kuzinski et al., 2011).

The quantification of mRNA abundance for fatty acid synthase (*FASN*), acetyl-coenzyme A carboxylase-α (*ACACA*), fatty acid-binding protein 3 (*FABP3*), peroxisome proliferator-activated receptor γ (*PPARγ*), stearoyl-CoA desaturase (*SCD*), sterol regulatory element-binding factor 1 (*SREBF1*), proliferating cell nuclear antigen (*PCNA*), Cyclin A2 (*CCNA2*), Cyclin D1 (*CCND1*), Bcl-2 apoptosis regulator (*Bcl-2*), Bcl-2 associated X (*Bax*), caspase-3, caspase-9 and

Table 2
The PCR primers used for real-time PCR of microbial DNA.

Target species	Primer sequence (5′–3′)	GenBank accession no.	Annealing temperature, °C	Size, bp
Total bacteria	F: CGGCAACGAGCGCAACCC R: CCATTGTAGCACGTGTGTAGCC	CP058023.1	60.0	147
Total fungi	F: GAGGAAGTAAAAGTCGTAACAAGGTTTC R: CAAATTCACAAAGGGTAGGATGATT	GQ355327.1	57.5	120
Total protozoa	F: GCTTTCGWTGGTAGTGTATT R: CTTGCCCTCYAATCGTWCT	HM212038.1	59.0	234
Total methanogens	F: TTCGGTGGATCDCARAGRGC R: GBARGTCGWAWCCGTAGAATCC	GQ339873.1	60.0	160
R. albus	F: CCCTAAAAGCAGTCTTAGTTCG R: CCTCCTTGCGGTTAGAACA	CP002403.1	60.0	176
R. flavefaciens	F: ATTGTCCCAGTTCAGATTGC R: GGCGTCCTCATTGCTGTTAG	AB849343.1	60.0	173
B. fibrisolvens	F: ACCGCATAAGCGCACGGA R: CGGGTCCATCTTGTACCGATAAAT	HQ404372.1	61.0	65
F. succinogenes	F: GTTCGGAATTACTGGGCGTAAA R: CGCCTGCCCCTGAACTATC	AB275512.1	61.0	121
Rb. amylophilus	F: CTGGGGAGCTGCCTGAATG R: GCATCTGAATGCGACTGGTTG	MH708240.1	60.0	102
P. ruminicola	F: GAAAGTCGGATTAATGCTCTATGTTG R: CATCCTATAGCGGTAAACCTTTGG	LT975683.1	58.5	74

glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was carried out by qRT-PCR using the iCycler and the iQ-SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA). The PCR primers of target genes for the real-time PCR were designed using Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) as shown in Table 3. Subsequently, relative quantitative PCR was carried out on a MxPro-Mx3000P multiplex quantitative PCR system (Stratagene, La Jolla, CA) in triplicate. The 20 μL reaction mixture comprised 2 μL of cDNA, 10 μL of SYBR Premix Taq II (TaKaRa), 0.8 μL of Forward Primer (10 μmmol/L), 0.8 μL of Reverse Primer (10 μmmol/L), 0.4 μL of ROX Reference Dye II (TaKaRa), and 6.0 μL of nuclease-free water. The following PCR cycling conditions were used: 1 cycle at 95 °C for 20 s for the original denaturation step, 45 cycles at 95 °C for 20 s, annealing at annealing temperature (Table 3) for 30 s, and extension at 62 °C for 20 s (Denman and McSweeney, 2006). The expression of the target gene was measured relative to the *GAPDH* level by the $2^{-\Delta\Delta CT}$ approach (Livak and Schmittgen, 2001). The relative mRNA expression level of each target gene was standardized to that in the control group.

2.7. Western blot analysis

Six mammary tissue samples were selected randomly from the same block of two groups, from which total protein was extracted from 100 mg of ground bovine mammary tissue using radioimmunoprecipitation assay buffer (RIPA buffer) containing protease/phosphatase inhibitors (Roche Diagnostics, Shanghai, China). After homogenization and 20 min of incubation on ice, the sample was centrifuged at 10,000 to 14,000 × *g* at 4 °C for 3 to 5 min. The supernatant, i.e. the extracted protein, was carefully collected and transferred into a new 1.5-mL Eppendorf tube. The Pierce BCA Protein Assay Kit (NCI3225CH, Thermo Fisher Scientific, Waltham, MA, USA) was used to analyse protein content based on the manufacturer's instructions, in which the reference protein was bovine serum albumin (5 mg/mL). The β-actin was used as a loading control and equal quantities of protein (20 μg) were separated by a 12% SDS-PAGE in running buffer. The separated proteins were transferred to nitrocellulose membranes (1704271; Bio-Rad, Hercules, CA, USA) in a buffer containing 2.9 g/L of glycine, 5.8 g/L of trimethylol aminomethane, and 20% methanol at 110 V for 90 min for proteins bigger than 40 kDa, and 110 V for 60 min for proteins less than 40 kDa. The membranes were blocked with 6% (w/v) bovine serum albumin in Tris-buffered saline with Tween (TBST; 50 mmol/L Tris, pH 7.6, 150 mmol/L NaCl, and 0.1% Tween 20) for 2 h at room temperature. Next, the membranes were incubated with a primary antibody for mouse anti-Cyclin A1 (1:2000; cat. no. NB100-2660; Novusbio Biologicals LLC), mouse anti-PCNA (1:2000; cat. no. 2586; Cell Signaling Technology, Inc., Danvers, MA, United States), rabbit anti-Akt (1:2000; cat. no. 9272; Cell Signaling Technology, Inc., Danvers, MA, United States), rabbit anti-p-Akt_{Ser473} (1:2000; cat. no. bs-0876R; BIOSS, Beijing, China), rabbit anti-mTOR (1:2000; cat. no. bs-1992R; BIOSS, Beijing, China), rabbit anti-p-mTOR $_{Ser2448}$ (1:2000; cat. no. bs-3495R; BIOSS, Beijing, China), rabbit anti-Bcl-2 (1:2000; cat. no. bs-20351R; BIOSS, Beijing, China), mouse anti-Bax (1:2000; cat. no. bs-0127M; BIOSS, Beijing, China), rabbit anti-Caspase-3 (1:2000; cat. no. bs-0081R; BIOSS, Beijing, China), rabbit anti-Caspase-9 (1:2000; cat. no. bs-0049R; BIOSS, Beijing, China), rabbit anti-G-protein-coupled receptor 41 (GPR41, 1:2000; cat. no. bs-16076R; BIOSS, Beijing, China), rabbit anti-PPARγ (1:2000; cat. no. bs-4590R; BIOSS, Beijing, China), rabbit anti-p-ACACA (1:2000; cat. no. bs-12954R; BIOSS, Beijing, China), rabbit anti-ACACA (1:2000; cat. no. bs-11912R;

Table 3
The PCR primers of target genes for the real-time PCR assay.

Gene	Primer sequence (5′–3′)	GenBank accession no.	Annealing temperature, °C	Size, bp	PCR amplification efficiency, %
ACACA	F: CATCTTGTCCGAAACGTCGAT R: CCCTTCGAACATACACCTCCA	AJ132890	58.0	101	100.0
FASN	F: AGGACCTCGTGAAGGCTGTGA R: CCAAGGTCTGAAAGCGAGCTG	NM001012669	62.0	85	102.5
SCD	F: TCCTGTTGTTGTGCTTCATCC R: GGCATAACGGAATAAGGTGGC	AY241933	58.0	101	101.6
PPARγ	F: AACTCCCTCATGGCCATTGAATG R: AGGTCAGCAGACTCTGGGTTC	NM181024.2	60.0	323	103.4
SREBF1	F: CTGACGACCGTGAAAACAGA R: AGACGGCAGATTTATTCAACTT	NM001113302	60.0	334	102.8
FABP3	F: GAACTCGACTCCCAGCTTGAA R: AAGCCTACCACAATCATCGAAG	DN518905	60.0	102	99.9
CCNA2	F: ACCACAGCACGCACAACAGTC R: AGTGTCTCTGGTGGGTTGAGGAG	NM001075123.1	64.0	87	101.7
CCND1	F: GCCGAGGAGAACAAGCAGATCATC R: CATGGAGGGCGGGTTGGAAATG	NM001046273.2	63.0	96	100.9
PCNA	F: ACATCAGCTCAAGTGGCGTGAAC R: GCAGCGGTAAGTGTCGAAGCC	NM001034494.1	64.0	101	102.0
Bcl-2	F: TGTGGATGACCGAGTACCTGAA R: AGAGACAGCCAGGAGAAATCAAAC	NM001076533.1	60.0	127	102.6
Bax4	F: TTTTGCTTCAGGGTTTCATCCAGGA R: CAGCTGCGATCATCCTCTGCAG	NM173894.1	62.0	174	100.0
Caspase-3	F: AGAACTGGACTGTGGCATTGAG R: GCACAAAGCGACTGGATGAAC	NM001077840.1	60.0	165	102.2
Caspase-9	F: CCAGGACACTCTGGCTTCAT R: CGGCTTTGATGGGTCATCCT	NM001205504.2	60.0	70	101.4
GAPDH	F: CCTGGAGAAACCTGCCAAGT R: AGCCGTATTCATTGTCATACCA	NM001034034.2	59.0	215	100.7

ACACA = acetyl-coenzyme A carboxylase-α; *Bax4* = Bcl-2 associated X; *Bcl-2* = Bcl-2 apoptosis regulator; *CCNA2* = Cyclin A2; *CCND1* = Cyclin D1; *FASN* = fatty acid synthase; *FABP3* = fatty acid-binding protein 3; *GAPDH* = glyceraldehyde-3-phosphate dehydrogenase; *PCNA* = proliferating cell nuclear antigen; *PPARγ* = peroxisome proliferator-activated receptor γ; *SCD* = stearoyl-CoA desaturase; *SREBF1* = sterol regulatory element-binding factor 1.

BIOSS, Beijing, China), rabbit anti-FASN (1:2000; cat. no. bs-60347R; BIOSS, Beijing, China), rabbit anti-SCD1 (1:2000; cat. no. bs-3787R; BIOSS, Beijing, China), and rabbit anti-β-actin (1:10000; cat. no. 4970; Cell Signaling Technology, Inc., Danvers, MA, United States) diluted in TBST, and incubated overnight at 4 °C. The membranes were washed five times with 1× TBST for 5 min to remove unbound antibodies and incubated with goat anti-rabbit (1:5000; cat. no. E-AB-1003; Elabscience, Wuhan, Hubei, China) or goat anti-mouse (1:5000; cat. no. RS0001; Immunoway, Plano, TX, United States) secondary antibodies diluted in TBST at 25 °C for 2 h. The intensity of each band was quantified using the Super Signal West Pico Chemiluminescence substrate (Thermo Fisher Scientific, Inc.) and ImageJ software (version 1.4.3.67). The relative protein expression level of the target protein is the ratio of the intensity value of the target protein to the intensity value of the corresponding β-actin.

2.8. Statistical analyses

Dietary net energy (NE) was estimated by multiplying the $NE_{L\text{-}3X}$ density of the feed ingredient by its dietary content (NRC, 2001). Energy-corrected milk (ECM) was estimated as 0.3246 × milk yield + 12.86 × fat yield + 7.04 × protein yield (NRC, 2001). The 4% fat-corrected milk (FCM) was estimated as 0.4 × milk yield + 15 × fat yield. The feed efficiency was calculated as milk yield (actual milk and ECM yield) divided by dietary DM intake for each cow. Data for DMI, milk production, feed efficiency and milk fatty acid composition ($n = 10$ per group) were analyzed using the following statistical model:

$$Y_{iklm} = \mu + \beta V_k + B_i + W_j + C_{k(i)} + S_l + WS_{jl} + E_{ijkl},$$

where Y_{iklm} represents the dependent variable; μ represents the overall mean; β represents the regression coefficient; V_k represents the covariate measurement; B_i represents effect of block i; W_j represents effect of week j; $C_{k(i)}$ represents effect of cow k within block i; S_l represents effect of SB treatment l; WS_{jl} represents interaction between week and SB treatment; E_{ijkl} represents residual error.

Data for ruminal fermentation, microbial enzymatic activity and microbiota ($n = 10$ per group) were analyzed using the following statistical model for which there were repeated measurements over time (pH, VFA, enzymatic activity and microbiota):

$$Y_{iklm} = \mu + \beta V_k + B_i + D_j + C_{k(i)} + S_l + DS_{jl} + E_{ijkl} + T_m + ST_{ml} + W_{ijklm},$$

where Y_{iklm} represents the dependent variable; μ represents the overall mean; β represents the regression coefficient; V_k represents the covariate measurement; B_i represents effect of block i; D_j represents effect of day j; $C_{k(i)}$ represents effect of cow k within block i; S_l represents effect of SB treatment l; DS_{jl} represents interaction between day and SB treatment; E_{ijkl} represents whole plot error; T_m represents effect of time m; ST_{ml} represents interaction between SB treatment and time; W_{ijklm} represents subplot error.

Data for nutrient digestibility and blood parameters ($n = 10$ per group) were analyzed using the following statistical model:

$$Y_{iklm} = \mu + B_i + C_{k(i)} + S_l + E_{ikl},$$

where Y_{iklm} represents the dependent variable; μ represents the overall mean; B_i represents effect of block i; $C_{k(i)}$ represents effect of cow k within block i; S_l represents effect of SB treatment l; E_{ikl} represents residual error.

The spatial covariance structure SP was used for estimating covariance and the subject of the repeated measurements was defined as cow (block × day × treatment). Linear and quadratic orthogonal contrasts were performed using the CONTRAST statement of SAS with coefficients estimated based on the dose of SB addition. The mRNA expression data ($n = 10$ per group) and Western blotting data ($n = 6$ per group) were analysed using the SigmaPlot version 12.5 (Systat Software, Inc., San Jose, CA, USA) statistical analysis package. Statistical differences in means among treatments were analysed using Student's t-test. Effects of the factors were considered significant at $P < 0.05$ unless other trends were declared at $P < 0.10$.

3. Results

3.1. Feed intake, milk production, and feed efficiency

Although dietary DM intake was not impacted, production of actual milk increased quadratically ($P = 0.04$) with increasing SB addition (Table 4). Yields of 4.0% FCM ($P = 0.02$) and ECM ($P = 0.02$) linearly increased with increasing doses of SB. Milk fat content increased linearly ($P < 0.01$) and milk protein content tended to increase quadratically ($P = 0.07$), but milk lactose content did not change following SB addition. Hence, the production of milk fat linearly increased ($P < 0.01$), and a quadratic increase was detected for yield of milk protein ($P = 0.03$) and milk lactose ($P = 0.04$) with an increasing dose of SB additives. The feed efficiency described as either milk yield to dietary DM intake ratio or ECM yield to dietary DM intake ratio also increased quadratically ($P < 0.01$) with increasing SB addition.

3.2. Digestibility coefficient and ruminal fermentation

The total tract digestibility of dietary DM ($P = 0.04$), OM ($P = 0.02$), and CP ($P < 0.01$) quadratically increased with increasing SB addition (Table 5). Moreover, the digestibility of dietary EE, NDF, and ADF increased linearly ($P = 0.03$) with increasing SB addition. Ruminal pH quadratically decreased ($P = 0.04$), whereas ruminal total VFA content quadratically increased ($P = 0.03$) with increasing SB addition. The molar percentage of acetate was unaffected by SB addition, while the molar percentage of propionate decreased ($P = 0.03$) linearly with increasing SB addition. Finally, the acetic acid to propionic acid ratio increased linearly ($P = 0.03$) with increasing SB addition. Moreover, a linear elevation pattern ($P = 0.05$) appeared in the butyrate molar percentage, but not in the isovalerate molar percentage, which linearly decreased ($P = 0.03$). Nevertheless, no influence was found for the molar percentages of valerate and isobutyrate with SB addition. Ammoniacal nitrogen content in the rumen linearly decreased ($P = 0.04$) with increasing SB addition.

3.3. Microbial enzymatic activity and microbiota

The CMC activity increased ($P = 0.04$) linearly with increased SB addition (Table 6). The activity of α-amylase decreased ($P < 0.01$) linearly with increasing doses of SB (Table 6). Populations of total bacteria, total protozoa, total anaerobic fungi, *R. flavefaciens*, *R. albus*, *Butyrivibrio fibrisolvens*, and *Fibrobacter succinogenes* linearly increased ($P < 0.05$) with increasing doses of SB. Nevertheless, the *Rb. amylophilus* population linearly decreased ($P = 0.03$) with increasing doses of SB. Populations of total methanogens and *P. ruminicola* were not impacted by SB additives.

3.4. Blood metabolites

A linear decrease was observed for blood glucose ($P = 0.04$), urea nitrogen ($P = 0.04$), NEFA ($P = 0.02$), and insulin ($P = 0.02$)

Table 4
Effects of sodium butyrate (SB) addition on dry matter intake, lactation performance, and feed efficiency in dairy cows.

Item	Treatments[1]				SEM	Contrast, *P*-value	
	Control	LSB	MSB	HSB		Linear	Quadratic
DM intake, kg/d	22.5	22.1	21.8	21.9	0.20	0.09	0.50
Milk production, kg/d							
Actual	35.9	36.7	37.1	36.9	0.39	0.45	0.04
FCM[2]	33.0	34.6	36.9	37.4	0.46	0.02	0.47
ECM[3]	35.1	37.3	39.3	39.3	0.44	0.02	0.34
Fat	1.24	1.32	1.46	1.51	0.024	<0.01	0.14
True protein	1.07	1.19	1.21	1.18	0.017	0.32	0.03
Lactose	1.80	1.91	1.91	1.90	0.026	0.89	0.04
Milk composition, g/kg							
Fat	34.5	36.0	39.7	40.8	0.41	<0.01	0.84
True protein	29.9	32.5	32.7	32.0	0.35	0.24	0.07
Lactose	50.4	52.1	51.4	51.4	0.44	0.56	0.36
Feed efficiency[4], kg/kg							
Milk yield to DM intake ratio	1.59	1.66	1.70	1.68	0.006	0.09	<0.01
ECM yield to DM intake ratio	1.57	1.69	1.80	1.79	0.007	0.07	<0.01

[1] Control, low SB (LSB), medium SB (MSB), and high SB (HSB) with 0, 100, 200, and 300 g/d SB per cow, respectively.
[2] Fat-corrected milk (FCM) was estimated as 0.4 × milk yield + 15 × fat yield.
[3] Energy-corrected milk (ECM) was estimated as 0.3246 × milk yield + 12.86 × fat yield + 7.04 × protein yield.
[4] Feed efficiency was estimated as milk yield (milk and ECM yield) divided by DM intake for each dairy cow.

Table 5
Effects of sodium butyrate (SB) addition on nutrient digestibility and rumen fermentation in lactating dairy cows.

Item	Treatments[1]				SEM	Contrast, *P*-value	
	Control	LSB	MSB	HSB		Linear	Quadratic
Nutrient digestibility, %							
Dry matter	67.5	70.8	72.3	71.9	0.52	0.14	0.04
Organic matter	68.9	72.1	73.6	73.2	0.50	0.09	0.02
Crude protein	73.3	75.3	79.4	77.5	0.69	0.02	<0.01
Ether extract	68.4	70.4	74.3	73.9	1.11	0.03	0.12
Neutral detergent fibre	51.5	54.9	58.4	59.8	0.93	0.03	0.17
Acid detergent fibre	43.4	45.5	48.3	48.6	1.21	0.03	0.24
Rumen fermentation							
pH	6.80	6.69	6.46	6.58	0.034	0.45	0.04
Total VFA, mmol/L	99	103	117	113	8.0	0.38	0.03
Acetate	61.3	61.0	62.0	60.7	0.84	0.69	0.86
Propionate	21.4	20.6	18.1	18.1	0.78	0.03	0.76
Butyrate	12.8	13.9	15.5	16.6	0.28	0.05	0.38
Valerate	1.45	1.68	1.65	1.85	0.084	0.15	0.92
Isobutyrate	1.33	1.32	1.36	1.31	0.014	0.93	0.57
Isovalerate	1.72	1.58	1.43	1.48	0.086	0.03	0.16
Acetate-to-propionate ratio	2.87	2.96	3.43	3.34	0.130	0.03	0.79
Ammonia N, mg/dL	11.9	10.6	9.27	9.38	0.913	0.04	0.39

VFA = volatile fatty acids.
[1] Control, low SB (LSB), medium SB (MSB) and high SB (HSB) with 0, 100, 200 and 300 g/d SB per cow, respectively.

concentrations with increasing SB addition (Table 7). However, blood total protein content linearly increased ($P = 0.04$).

3.5. Expression of genes and proteins concerned with fatty acid synthesis

With regard to the synthesis of fatty acids, dietary addition of SB at 200 g/d increased the mRNA expressions of *PPARγ* ($P < 0.01$), *SREBF1* ($P = 0.03$), *ACACA* ($P < 0.01$), *FASN* ($P = 0.04$), *SCD* ($P = 0.03$), and *FABP3* ($P = 0.02$) (Fig. 1A). The protein expression of PPARγ ($P < 0.05$), sterol regulatory element-binding protein 1 (SREBP1; $P < 0.01$), FASN ($P = 0.03$), p-ACACA/ACACA ($P = 0.04$), and SCD ($P < 0.01$) were uniformly increased through the addition of 200 g/d of SB (Fig. 1B and C).

3.6. Expression of genes and proteins concerned with cell proliferation and apoptosis

The *CCNA2* and *CCND1* genes encode for Cyclin A2 and D1, respectively. Dietary addition of SB at 200 g/d significantly increased the mRNA expression of *CCNA2* ($P < 0.01$), *CCND1* ($P < 0.01$), and *PCNA* ($P = 0.04$) (Fig. 2A). The protein expression of Cyclin A1 ($P = 0.03$) and PCNA ($P < 0.01$) was uniformly increased by 200 g/d of SB (Fig. 2B and C). Meanwhile, the mRNA expression of genes concerned with the inhibition of apoptosis, such as *Bcl-2* ($P = 0.03$), and the *Bcl-2/Bax* ratio ($P = 0.04$) was significantly increased SB addition at 200 g/d. The mRNA expression of genes concerned with promoting apoptosis, such as *Bax* ($P = 0.03$), caspase-3 ($P = 0.02$), and caspase-9 ($P < 0.01$) was decreased by SB addition at 200 g/d (Fig. 2A). Meanwhile, the protein expression of Bcl-2 and the Bcl-2/Bax ratio were increased ($P < 0.01$) by SB addition at 200 g/d, whereas the protein expression of Bax, caspase-3, and caspase-9 was decreased ($P < 0.01$) by SB addition at 200 g/d (Fig. 2B and C).

As a receptor for butyrate, the GPR41 regulates cell proliferation and apoptosis via the Akt/mTOR signalling pathway (Meng et al., 2023; Zhou et al., 2021). Addition of SB at 200 g/d increased ($P < 0.01$) the protein expression of GPR41. The ratio of p-Akt to Akt and ratio of p-mTOR to mTOR were also increased ($P < 0.01$) by SB addition at 200 g/d (Fig. 3A and B).

Table 6
Effects of sodium butyrate (SB) addition on rumen microbial enzyme activity and microflora in dairy cows.

Item	Treatments[1]				SEM	Contrast, *P*-value	
	Control	LSB	MSB	HSB		Linear	Quadratic
Enzyme activity							
Carboxymethyl-cellulase, μmol glucose/min per mL	0.219	0.284	0.364	0.375	0.0172	0.04	0.88
Cellobiase, μmol glucose/min per mL	0.141	0.161	0.158	0.180	0.0083	0.17	0.95
Xylanase, μmol xylose/min per mL	0.613	0.797	0.679	0.708	0.0451	0.69	0.43
Pectinase, μmol D-galactouronic acid/min per mL	0.412	0.420	0.415	0.506	0.0223	0.17	0.36
α-Amylase, μmol maltose/min per mL	0.572	0.539	0.434	0.391	0.0274	<0.01	0.90
Protease, μg hydrolysed protein/min per mL	0.895	0.930	0.849	0.615	0.0713	0.17	0.37
Microflora, copies/mL							
Total bacteria, $\times 10^{11}$	5.82	6.96	7.15	7.84	0.646	0.04	0.44
Total anaerobic fungi, $\times 10^{7}$	1.93	2.27	2.81	2.87	0.399	0.04	0.51
Total protozoa, $\times 10^{5}$	5.10	6.34	6.98	6.96	0.697	0.03	0.42
Total methanogens, $\times 10^{9}$	5.05	5.07	5.12	5.30	0.483	0.43	0.77
R. albus, $\times 10^{8}$	4.44	5.05	5.61	5.70	0.286	0.02	0.40
R. flavefaciens, $\times 10^{9}$	3.68	4.09	4.52	4.56	0.551	0.05	0.35
F. succinogenes, $\times 10^{10}$	3.05	4.14	4.62	4.85	0.432	0.03	0.38
B. fibrisolvens, $\times 10^{9}$	3.76	4.36	5.10	5.25	0.445	0.03	0.41
P. ruminicola, $\times 10^{9}$	8.37	7.49	7.15	7.40	1.723	0.21	0.46
Rb. amylophilus, $\times 10^{8}$	3.54	3.42	3.05	2.99	0.208	0.03	0.51

[1] Control, low SB (LSB), medium SB (MSB), and high SB (HSB) with 0, 100, 200, and 300 g/d SB per cow, respectively.

Table 7
Effects of sodium butyrate (SB) addition on blood metabolites in lactating dairy cows.

Item	Treatments[1]				SEM	Contrast, *P*-value	
	Control	LSB	MSB	HSB		Linear	Quadratic
Glucose, mmol/L	10.30	9.95	8.91	8.26	0.278	0.04	0.52
Total protein, g/L	84.2	85.1	98.8	98.4	1.96	0.04	0.66
Urea nitrogen, mmol/L	10.6	9.97	9.40	8.73	0.302	0.04	0.54
NEFA, μmol/L	959	933	874	871	17.5	0.02	0.15
Insulin, mIU/L	37.8	35.3	33.6	34.2	0.85	0.02	0.26

NEFA = non-esterified fatty acids.
[1] Control, low SB (LSB), medium SB (MSB), and high SB (HSB) with 0, 100, 200, and 300 g/d SB per cow, respectively.

4. Discussion

4.1. Dry matter intake, milk yield, and feed efficiency

Although dietary DM intake was not impacted by SB addition, production of actual milk, FCM, ECM, fat, true protein, and lactose increased. Kowalski et al. (2015) reported that addition of SB at 300 g/d from 30 d preparturition to 60 d post-parturition did not impact dietary DM intake and the amount of milk produced after delivery. However, Urrutia et al. (2019) demonstrated that the supplementation of 2.5% calcium butyrate of DM in the basal diet decreased DM intake by 2.6 kg/d and actual milk production by 1.65 kg/d by 7 d of treatment compared to the control. Although milk yield responses to butyrate addition vary in different studies, an increased milk fat production or content with butyrate addition was often reported in dairy cows (Huhtanen et al., 1993, 1998; Miettinen and Huhtanen, 1996). Nevertheless, some studies reported no differences in yield, the percentage of any milk components (Herrick et al., 2018), or a decline in milk fat yield (Urrutia et al., 2019). Moreover, research has not demonstrated a consistent effect of increasing butyrate supply on milk protein content. Huhtanen et al. (1993) found a positive linear response of milk protein content with the ruminal infusion of butyrate from 0 to 600 g/d in dairy cows, whereas Urrutia et al. (2019) found no impact of 2.5% calcium butyrate of DM added in the basal diet on milk protein content in dairy cows. The disagreement in findings was ascribed to the different administration modes and doses of butyrate in these studies. For example, the supplemented dose of butyrate used in this study (from 0.45% to 1.35% of dietary DM) was lower than that used by Urrutia et al. (2019). Although DM intake had no differences among treatments in the current experiment, the tendency for a quadratic increase in milk protein content with SB addition was due to the increased DM and CP digestibility, and the likely increased microbial protein evidenced by the decreased rumen ammonia content (Cummins and Papas, 1985).

4.2. Nutrient digestion and rumen fermentation

The increased nutrient digestibility with SB addition was due to enhanced rumen fermentation and improved milk production. Huhtanen et al. (1993) demonstrated that the digestibility of dietary DM, OM, CP, and NDF in dairy cows increased linearly with an increase in the infusion rate of butyrate. Another study reported that cows fed butyrate at 1.1% of dietary DM tended to increase digestibility of dietary DM and OM compared to the control group, but starch, NDF, and CP digestibility were not impacted by butyrate addition (Fukumori et al., 2020). Supplementation of butyrate increased nutrient digestibility by promoting gastrointestinal function. Previous studies showed that ruminal infusion or dietary addition of butyrate stimulated the growth of ruminal epithelium, increased the length of the ruminal papilla (Mentschel et al., 2001), accelerated ruminal epithelial blood flow (Rémond et al., 1993), and increased VFA absorption (Storm et al., 2011). Furthermore, Guilloteau et al. (2010) reported that butyrate supplementation stimulated digestive enzyme secretion.

In the current experiment, it is possible that sodium butyrate promoted the growth and propagation of rumen bacteria and the secretion of microbial enzymes, and promoted the digestion of fibre, thus leading to the increase in VFA and the decrease of rumen pH. The quadratically decreased ruminal pH was related to the

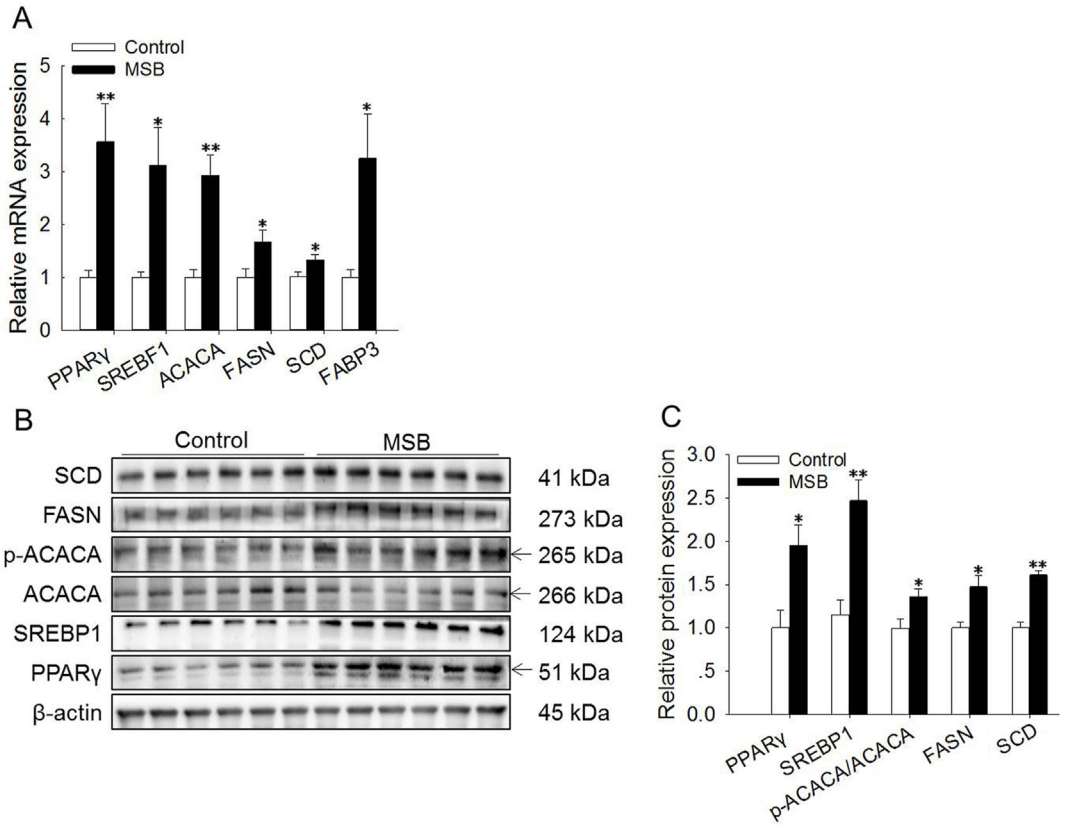

Fig. 1. Effects of dietary medium sodium butyrate (MSB) addition on fatty acid synthesis related mRNA and protein expression in the bovine mammary gland. (A) The mRNA expression levels of peroxisome proliferator-activated receptor (*PPARγ*), sterol regulatory element-binding factor 1 (*SREBF1*), acetyl-coenzyme A carboxylase-α (*ACACA*), fatty acid synthase (*FASN*), stearoyl-CoA desaturase (*SCD*), and *FABP3* in bovine mammary glands treated with SB (0 and 200 g/d SB per cow). Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as the reference gene. $n = 10$ per group, and values are the mean ± SEM. The relative mRNA expression level of each target gene was standardized to that in the control group. (B) Western blot analysis of PPARγ (the specific bands with an arrow), sterol regulatory element-binding protein 1 (SREBP1), ACACA (the specific bands with an arrow), FASN, and SCD in bovine mammary gland tissues. β-Actin was used as a loading control. $n = 6$ per group, selected randomly from the same block of two groups. (C) Mean ± SEM of immunopositive bands of PPARγ, SREBP1, ACACA, FASN, and SCD. The relative protein expression level of each target protein was standardized to that in the control group. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus the control group.

quadratically increased total VFA content with increasing SB addition. The increased total VFA and butyrate concentrations were ascribed to the increased dietary NDF and ADF digestibility and suggested that cellulolytic bacteria populations and enzymatic activity increased following SB addition. Moreover, the linearly increased ratio of acetic acid to propionic acid implied that the rumen fermentation pattern tended towards higher acetic formation with increasing SB addition. Ruminal butyrate concentration increased by 63.3% and 135.9% for dairy cows when 1 and 2 g of butyrate per kg BW were added, respectively, in comparsion with the control group (Herrick et al., 2018). Similarly, Fukumori et al. (2020) reported that dairy cows fed butyrate at 1.1% of dietary DM had increased ruminal butyrate concentrations compared with the control cows. The linearly decreased ammoniacal nitrogen content in the rumen should be principally ascribed to increased bacterial protein synthesis with SB addition (Cummins and Papas, 1985).

4.3. Microbial enzyme activity and microflora

Fibre materials in cow diets are degraded to acetate in the rumen by ruminal bacteria, protozoa, and fungi secreting cellulolytic enzymes (Orpin, 1984). Ruminal fungi can degrade feed lignocellulosic tissues, and about 30% of fibre digestion and 10% of the VFA production could be due to ruminal protozoa (Wang and McAllister, 2002). Therefore, the linear elevation in ruminal CMC activity with increasing SB doses was due to the linearly increased populations of total bacteria, total anaerobic fungi, total protozoa, *R. flavefaciens*, *R. albus*, *B. fibrisolvens*, and *F. succinogenes*, which supported improved ruminal fermentation and increased digestibility of dietary NDF and ADF. The linear reduction in α-amylase activity was in keeping with the observed decrease in the *Rb. amylophilus* population and indicated that SB addition can inhibit starch degradation in the rumen. The results further confirmed the decreased propionate molar percentage with increasing SB doses. The unaltered ruminal protease activity was mainly related to the unchanged *P. ruminicola* population, and it was further confirmed that microbial protein synthesis was promoted as the observed linear decline in ammoniacal nitrogen content with increasing SB addition (Cummins and Papas, 1985).

4.4. Blood metabolites

Earlier studies have illuminated the role of butyrate involved in the regulation of glucose metabolism (Storry and Rook, 1965), and have shown the inhibition of butyrate for hepatic uptake of propionate in vitro (Demigné et al., 1986). The linear decrease in blood glucose following SB addition is in keeping with other research (Herrick et al., 2018; Urrutia et al., 2019; Halfen et al., 2021). The linear decrease in insulin content with increasing SB addition may depend on the dose of SB, the physiological stage of the cow, and the duration of SB administration. Blood urea nitrogen (BUN) and

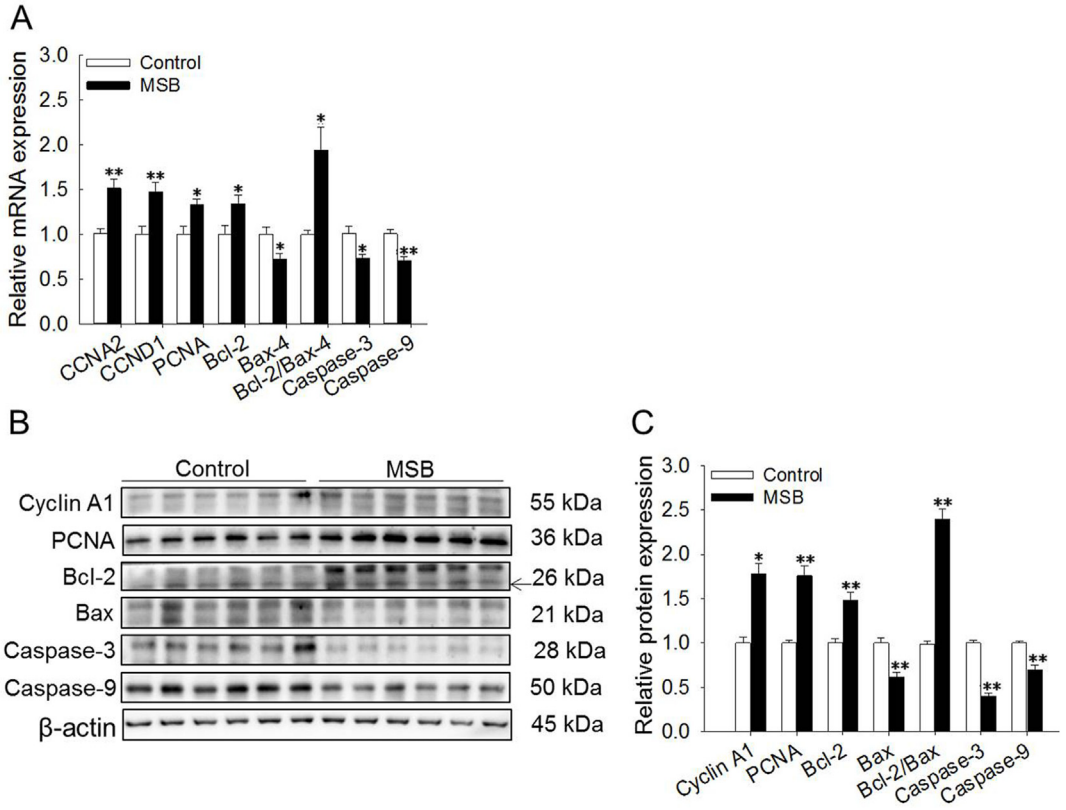

Fig. 2. Effects of dietary medium sodium butyrate (MSB) addition on proliferation and apoptosis-related mRNA and protein expression in the bovine mammary gland. (A) The mRNA expression levels of Cyclin A2 (*CCNA2*), Cyclin D1 (*CCND1*), proliferating cell nuclear antigen (*PCNA*), Bcl-2 apoptosis regulator (*Bcl-2*), Bcl-2 associated X (*Bax*), *Bcl-2/Bax*, caspase-3, and caspase-9 in bovine mammary glands treated with SB (0 and 200 g/d SB per cow). Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as the reference gene. $n = 10$ per group, and values are the mean ± SEM. The relative mRNA expression level of each target gene was standardized to that in the control group. (B) Western blot analysis of Cyclin A1, PCNA, Bcl-2 (the specific bands with an arrow), Bax, Bcl-2/Bax, caspase-3, and caspase-9 in bovine mammary gland tissues. β-Actin was used as a loading control. $n = 6$ per group, selected randomly from the same block of two groups. (C) Mean ± SEM of immunopositive bands of Cyclin A1, PCNA, Bcl-2, Bax, Bcl-2/Bax, caspase-3, and caspase-9. The relative protein expression level of each target protein was standardized to that in the control group. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus the control group.

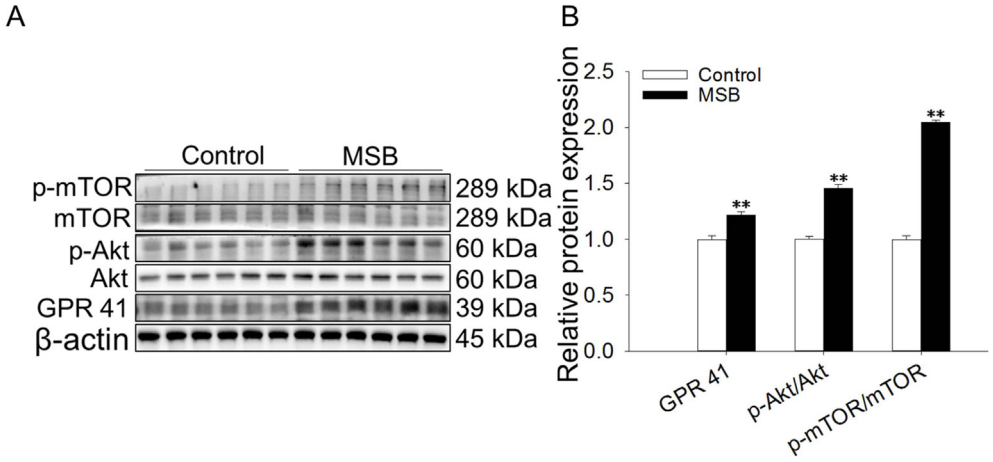

Fig. 3. Impacts of dietary medium sodium butyrate (MSB) addition on the Akt-mTOR signalling pathway of the bovine mammary gland. (A) Western blot analysis of p-Akt, Akt, p-mTOR, mTOR and G-protein-coupled receptor 41 (GPR41) in bovine mammary glands treated with SB (0 and 200 g/d SB per cow). β-Actin was used as a loading control. $n = 6$ per group, selected randomly from the same block of two groups. (B) Mean ± SEM of the immunopositive bands of GPR41, p-Akt/Akt and p-mTOR/mTOR. The relative protein expression level of each target protein was standardized to that in the control group. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus the control group.

total protein content are indicators of the efficiency of protein utilization, and BUN is also related to energy balance and plasma NEFA (Broderick and Clayton, 1997; Rastani et al., 2006). The increased total protein content and decreased BUN content with SB addition observed in current studies may suggest higher utilization of dietary protein or lower body protein mobilization. The linear reduction in NEFA concentrations with butyrate addition was in agreement with other research (Urrutia et al., 2019; Halfen et al., 2021), and indicated that butyrate might modulate the lipolysis rates of NEFA and trigger ketone body formation. The addition of SB

could increase peripheral blood BHBA concentration in dairy cows (Izumi et al., 2019), the precursor of milk fat synthesis (Sun et al., 2019; Ali et al., 2021), and lead to lipolysis inhibition mediated by β-hydroxybutyrate through activating adipose nutrient-sensing receptors (Mielenz, 2017). However, Herrick et al. (2018) found no reductions in plasma NEFA with ruminal butyrate infusion.

4.5. Expression of genes and proteins implicated in fatty acid synthesis

In the bovine mammary gland, both PPARγ and SREBF1 control the mRNA expression of *FASN*, *ACACA*, *SCD* and *FABP3* (Bionaz and Loor, 2008; Ma and Corl, 2012). Either FASN or ACACA takes part in the synthesis of fatty acids from acetic acid and BHBA (Bionaz and Loor, 2008), and is positively related to the secretion of $C_{4\text{-}16}$ fatty acids (Bernard et al., 2008). The SCD protein is answerable for catalysing the synthesis of monounsaturated fatty acids from saturated fatty acids (Sheng et al., 2015). The FABP3 protein is concerned with the absorption and intracellular transport of long chain fatty acids in BMEC (Sheng et al., 2015). The increased mRNA and protein expressions of FASN, ACACA, PPARγ, and SREBF1 with SB addition indicated that the genes and proteins concerned with the synthesis of short chain fatty acids and medium chain fatty acids were up-regulated and supported the elevation in milk fat yield and percentage. A recent study that found SB addition tended to increase 4% FCM yield and increased the milk fat content (Izumi et al., 2019). The increased *FABP3* mRNA expression suggested that the genes involved in the synthesis, absorption, and transport of long chain fatty acids in the bovine mammary gland were stimulated by SB addition. Moreover, other studies have found increased mRNA expression of *PPARγ*, *SREBF1*, *FASN*, *ACACA*, and *SCD* with acetate and BHBA addition in vitro (Sheng et al., 2015).

4.6. Expression of genes and proteins implicated in cell proliferation and apoptosis

Stimulating cell proliferation and suppressing cell apoptosis in the bovine mammary gland should be the critical control point that accelerates lactation persistence in lactating dairy cows. Cyclin D modulates the transformation from the G1 phase of the cell cycle to the S phase, while Cyclin A is considered essential for initiating and completing DNA replication in the S phase (Zhang et al., 2018; Lim and Kaldis, 2013). Additionally, PCNA is an indispensable cofactor for proteins involved in DNA replication and repair (Park et al., 2016). Moreover, other research has demonstrated that cyclin D3 and PCNA take part in adjusting the proliferation of mammary epithelial cells (Li et al., 2020; Zhang et al., 2018). In the current experiment, SB addition increased the mRNA expression of cyclins (*CCNA2* and *CCND1*) and *PCNA*, and the protein expression of Cyclin A1 and PCNA, indicating that SB addition excites the proliferation of BMEC. Traditionally, most of mammary development and cell growth and differentiation occurs before parturition and then net growth during lactation is expected to be minimal. In view of the results of the current experiment, it could provide a way to promote research of mammary gland development of perinatal cows in the future.

The Bcl-2 family proteins take part in the modulation of apoptosis cell death and comprise anti-apoptosis (e.g., Bcl-2) and pro-apoptosis (e.g., Bax) members (Shamas-Din et al., 2013). Since Bcl-2 prevents apoptosis by inhibiting the activity of Bax, a high Bcl-2/Bax ratio indicates suppressed apoptosis. Therefore, this ratio could indicate the status of cellular apoptosis of BMEC. Both caspase-3 and caspase-9 play an important role in the execution phase of cell apoptosis (Pisani et al., 2020). The reduction in their expression suppresses the initiation of apoptosis. In this study, the increased mRNA and protein expression of Bcl-2 and the Bcl-2/Bax ratio, and the decreased mRNA and protein expression of Bax, caspase-3, and caspase-9 with SB addition, indicated that supplementation of SB inhibited the expression of apoptotic markers in the bovine mammary gland. Therefore, above findings indicated that SB very likely modulates the proliferation of the bovine mammary gland by stimulating the expression of proliferative markers and inhibiting the expression of apoptotic markers. Since the number of mammary gland cells proliferating in cows has not been measured, whether there is stimulation of other types of cells deserves further verification.

The Akt signalling pathway has been implicated in modulating the proliferation and apoptosis of diverse cells, for example, breast cancer cells (Huang et al., 2021) and HC11 cells (Meng et al., 2017). Furthermore, the mTOR signalling pathway participates in adjusting the proliferation of diverse cells (Li et al., 2017). Recent research has reported that SB may be a ligand for the GPR41, which regulates apoptosis and inflammation (Li et al., 2019; Zhou et al., 2021). Moreover, the addition of SB decreased apoptosis through the PI3K/Akt pathway in LPS-induced MAC-T cells (Li et al., 2019). In the current experiment, the addition of SB increased the protein expression of GPR41 and stimulated the phosphorylation of Akt and mTOR. Further verification is needed to determine whether the phosphorylation of Akt and mTOR was stimulated by SB addition via the activation of GPR41. These findings suggest that the activation of the Akt-mTOR signalling pathway might stimulate the expression of proliferative markers and inhibit the expression of apoptotic markers. Considering that the net growth of mammary gland during lactation is expected to be minimal, the AKT activated by butyrate in the mammary epithelial cells during (early) lactation would be more involved in the control of metabolism, especially milk protein and lipid synthesis.

5. Conclusion

Supplementation of SB in dairy cow diets improved the milk yield and feed efficiency by stimulating nutrient digestion and ruminal fermentation. Moreover, the addition of SB increased the protein expression of GPR41, stimulated the phosphorylation of Akt and mTOR, promoted the expression of proliferative markers, and attenuated the expression of apoptotic markers.

Author contributions

Jing Zhang and **Qiang Liu** designed the research; **Lijun Bu**, **Yapeng Liu** and **Wenjie Huo** conducted the research; **Chengqiang Xia** and **Caixia Pei** analyzed the data; **Jing Zhang**, **Lijun Bu** and **Yapeng Liu** wrote the original draft; **Jing Zhang** and **Qiang Liu** had primary responsibility for the final content. All authors read and approved the final manuscript.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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