Buffering agent via insulin-mediated activation of PI3K/AKT signaling pathway to regulate lipid metabolism in lactating goats

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Short title: Buffering agent regulate lipid metabolism in lactating goats

Summary

Ruminants are often fed a high-concentrate (HC) diet to meet lactating demands, yet long-term concentrate feeding induces subacute ruminal acidosis (SARA) and leads to a decrease in milk fat. Buffering agent could enhance the acid base buffer capacity and has been used to prevent ruminant rumen SARA and improve the content of milk fat. Therefore, we tested whether a buffering agent increases lipid anabolism in the livers of goats and influences of milk fat synthesis. Twelve Saanen-lactating goats were randomly assigned to two groups: one group received a HC diet (Concentrate : Forage = 60:40, Control) and the other group received the same diet with a buffering agent added (10g sodium butyrate, $C_4H_7NaO_2$; 10g sodium bicarbonate, NaHCO3; BG) over a 20-week experimental period.Overall, milk fat increase (4.25 ± 0.08 *vs* 3.24 ± 0.10; $P < 0.05$), and lipopolysaccharide levels in the jugular (1.82 ± 0.14 *vs* 3.76 ± 0.33) and rumen fluid (23340 ± 134 *vs* 42550 ± 136) decreased in the buffering agent group ($P < 0.05$). Liver consumption and release of nonesterified fatty acid (NEFA) into the bloodstream increased ($P < 0.05$). Phosphatidylinositol 3-kinase (PI3K), protein kinase B (AKT) and ribosomal protein

S6 kinase (p70S6K) up-regulated significantly in the livers of the buffering agent group ($P < 0.05$). It also up-regulated expression of the transcription factor sterol regulatory element binding protein-1c (SREBP-1c) and its downstream targets involved in fatty acid synthetic, including fatty acid synthetase (FAS), stearoyl-CoA desaturase (SCD-1) and acetyl-CoA carboxylase 1 (ACC1) ($P < 0.05$). The BG diet increased insulin levels in blood (19.43 ± 0.18 *vs* 13.81 ± 0.10, $P < 0.05$), and insulin receptor was likewise elevated in the liver ($P < 0.05$). Cumulatively, the BG diet increased plasma concentrations of NEFA by INS-PI3K/AKT-SREBP-1c signaling pathway promoting their synthesis in the liver. The increased NEFA concentration in the blood during BG feeding may explain the up-regulated in the milk fat of lactating goats.

Key Words: High-concentrate diet • $C_4H_7NaO_2$ • $NaHCO_3$ • PI3K/AKT signaling pathway • Lipid metabolism.

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Introduction

Ruminants are often fed a high-concentrate (HC) diet to meet lactating requirements for high milk performance (Gozho *et al*. 2005). However, long-term feeding with a HC diet causes a decline in the rumen pH if organic acids, such as lactic acid and volatile fatty acids, accumulate in the rumen, and a chronic digestive

45 disorder known as subacute ruminal acidosis (SARA) may occur (Plaizier *et al*. 2008,
46 Chen and Oba 2012) In experimental study, ruminal pH value < 5.6 lasted more than 3
47 hours was considered as the critical value of SARA diagnosis (Gozho *et al*. 2005).
48 Moreover, previous studies have shown that lactating goats fed a HC diet induced a
49 SARA, which was characterized by inflammation and depressed milk fat (Khafipour
50 *et al*. 2009). It has been reported that the feeding of HC diets to lactating cows results
51 in the release of lipopolysaccharide (LPS) from the rumen or hindgut (Zebeli and
52 Ametaj 2009). Meanswhile, previous studies have shown that LPS can translocate into
53 the bloodstream from the digestive tract under conditions of high permeability and
54 after injury to the liver organ (Khafipour *et al*. 2016).

55 Milk fat is an important nutritional ingredient of milk that is beneficial to human
56 health. However, long-term feeding with a HC diet induces a reduction in milk fat
57 (Zebeli *et al*. 1999). Triglycerides (TG) are the main component of milk fat and are
58 synthesized using fatty acids and α-glycerophosphate in mammary epithelial cells
59 (Pennington *et al*. 1952). The uptake of nonesterified fatty acid (NEFA) components
60 by mammary glands is affected by their concentrations in the blood. Previous studies
61 have shown that with an increasing NEFA content in the blood, the absorbed quantity
62 applied to milk fat synthesis was also elevated in mammary cells (Bauman *et al*.
63 2011). Therefore, the substrate precursor of NEFA plays a crucial physiological role in
64 milk fat synthesis. Nutrients required for milk synthesis must be transported from the
65 rumen and gut to the liver to undergo metabolic conversion. In ruminants, the liver is
66 the major site of lipid metabolism and gluconeogenesis, which provides the substrate

67 precursors to the mammary gland for milk production. Liver lipidolysis and lipid
68 synthesis rely on the absorption and utilization of NEFA in the blood (Bell 1979,
69 Kristensen 2005). NEFA are transported through the hepatic portal vein into the liver,
70 where they are metabolized. Then, they exit the liver through the hepatic vein, where
71 they are taken up into the blood.

72 Buffering agent could enhance the acid base buffer capacity and has been used to
73 prevent ruminant rumen SARA and improve the production performance. It is well
74 documented that dietary addition of sodium butyrate ($C_4H_7NaO_2$) could enhance solid
75 feed intake, rumen development and health status of neonatal calves (Meng *et al*.
76 1999). In addition, dietary addition of 2% sodium bicarbonate ($NaHCO_3$) could
77 increase the buffering capacity and prevent the acidosis in rumen (Gorka *et al*. 2009).
78 Previous studies indicated that the addition of $NaHCO_3$ to a restricted-roughage
79 rations could be given to lactating cows to increase the content of milk fat (Islam *et al*.
80 2014). However, at present, the research of buffering agent is focused on the milk
81 production and composition of dairy cows. Furthermore, little is known regarding the
82 mechanism of how a buffering agent improves milk fat metabolism in goats. In this
83 study, we created a buffering agent consisting of $NaHCO_3$ and $C_4H_7NaO_2$ and mixed it
84 with a HC diet source that was fed to lactating goats. We then investigated the effect
85 of these buffering agent on the development of SARA and milk fat production to
86 elucidate potential mechanisms for this phenomenon.

87 **Methods**

88 *Ethical approval*

All animal procedures were approved by the Institutional Animal Care and Use Committee of Nanjing Agricultural University. The protocols were reviewed and approved, and the project number 2011CB100802 was assigned. The slaughter and sampling procedures strictly followed the '*Guidelines on Ethical Treatment of Experimental Animals*' (2006) no. 398 established by the Ministry of Science and Technology, China and the '*Regulation regarding the Management and Treatment of Experimental Animals*' (2008) no. 45 set by the Jiangsu Provincial People's Government.

Animal and experimental procedures

A total of twelve healthy multiparous mid-lactating goats (body weight, 38 ± 8 kg, mean ± SEM, 3-5 weeks post-partum) at the age of 2-3 years were used in experiments. They were housed in individual stalls in a standard animal feeding house at Nanjing Agricultural University (Nanjing, China). Goats were randomly divided into two groups: high-concentrate diet group (Control, concentrate: forage = 60:40) and buffering agent group (BG, concentrate: forage = 60:40 with 10g $C_4H_7NaO_2$ and 10g $NaHCO_3$), six in each group. Dietary $C_4H_7NaO_2$ and $NaHCO_3$ were obtained from Nanjing Jiangcheng Bioengineering Institute, China). The ingredients and nutritional composition of the diets are presented in Table 1. The goats were fitted with a rumen fistula and hepatic catheters two weeks before the experiment and were ensured that they recovered from the surgery. Animals were monitored for 2 weeks after surgery. Sterilized heparin saline (500 IU/ml, 0.3 ml/time) was administered at 8-hour intervals every day until the end of the experiment to prevent catheters from

becoming blocked. During the experimental period of 20 weeks, goats were fed two times daily at 8.00 and 18.00, had free access to fresh water, and the feed amount met or exceeded the animal's nutritional requirements. The Institutional Animal Care and Use Committee of Nanjing Agricultural University (Nanjing, People's Republic of China) approved all of the procedures (surgical procedures and care of goats).

Milk composition analysis

We collected 50-ml samples of fresh milk into vials with potassium dichromated every week, and the milk fat, protein, total solids and lactose concentrations in the samples was analyzed using the Integrated Milk-Testing™ Milkoscan 4000 (Foss Electric, Hillerod, Denmark) at the Animal Experiment Center of College of Animal Science and Technology at the Nanjing Agricultural University.

Rumen fluid collection and analysis

Rumen fluid was sampled at the last day of weeks 17, 18 and 19 through a cannulafrom the ventral sac of the rumen after mixing the content at 0 h, 2 h, 4 h, 6 h, 8 h, 10h and 12h after feeding, 20 ml rumen fluids was collected with a nylon bag and the pH value was measured immediately with pH-meter. The rumen fluid was collected and each sample was transferred into a 50-ml sterile tube and kept on ice until transported to the laboratory for the initial processing before LPS determination.

The concentration of LPS in rumen fluid was measured by a Chromogenic End-point Tachypleus Amebocyte Lysate Assay Kit (CE64406, Chinese Horseshoe Crab Reagent Manufactory Co., Ltd., Xiamen, China). Pretreated rumen fluid samples were diluted until their LPS concentrations were in the range of 0.1-1.0 endotoxin

units (EU)/ml relative to the reference endotoxin.

Measurement of plasma biochemical parameters

At the end of the experiment, plasma was sampled thirty minutes prior to feed delivery using EDTA-containing vacuum tubes from the jugular, hepatic and portal veins. Blood was centrifuged at 2500 × g for 10 min to separate the plasma. Plasma glucose, TG, NEFA and total cholesterol were quantified using a Beckman Kurt AU5800 series automatic biochemical analyzer (Beckman Kurt, USA) at the General Hospital of Nanjing Military Region (Nanjing, China). The concentration of INS and glucagon in the plasma were determined by ELISA kits (Shanghai Enzyme-linked Biotechnology Co. Ltd, Shanghai, China). The detected range of ELISA kits for insulin (INS) and glucagon were 0.1-40 mIU/l and 5-1000 pg/ml, respectively. The procedures were performed according to the manufacturer's instructions.

The LPS concentration were determined using a chromogenic endpoint assay (CE64406, Chinese Horseshoe Crab Reagent Manufactory Co., Ltd., Xiamen, China) The procedures were performed according to the manufacturer’s instructions.

Sample collection

After 20 weeks, goats were slaughtered after overnight fasting. All goats were killed with neck vein injections of xylazine [0.5 mg (kg body weight)$^{-1}$; Xylosol; Ogris Pharme, Wels, Austria] and pentobarbital [50 mg (kg body weight)$^{-1}$; Release; WDT, Garbsen, Germany]. After slaughter, liver tissue was collected and washed twice with cold physiological saline (0.9% NaCl) to remove blood and other

contaminants. Livers were then transferred into liquid nitrogen and used for RNA and protein extraction.

RNA extraction, cDNA synthesis and quantitative real-time PCR (qRT-PCR)

Relative mRNA expression in liver tissue was measured by qRT-PCR using the $2^{-\Delta\Delta Ct}$ method. Briefly, total RNA was extracted from liver samples using TRIzol reagent (Invitrogen, USA) and converted to cDNA using commercial kits (Vazyme, Nanjing, China). All PCR primers were synthesized by Generay Company (Shanghai, China), and the primer sequences are listed in Table 2. PCR was performed using the AceQ qPCR SYBR Green Master Mix kit (Vazyme, Nanjing, China) and the MyiQ2 Real-time PCR system (Bio-Rad, USA) with the following cycling conditions: 95°C for 2 min, 40 cycles of 95°C for 15 sec and 60°C for 30 sec. Glyeraldehyde 3-phosphate dehydrogenase (GAPDH) served as reference for normalization.

Western blotting

Total protein was extracted from frozen liver tissue, and the concentration was determined using the bicinchoninic acid (BCA) assay (Pierce, Rockford, IL, USA). We isolated 30 μg of protein from each sample, which was subjected to electrophoresis on a SDS-PAGE. The separated proteins were transferred onto nitrocellulose membranes (Bio Trace, Pall Co., USA). The blots were incubated with the following Cell Signaling Technology primary antibodies for overnight at 4^{o}C with a dilution of 1:1000 in block: rb-anti-phosphatidylinositol 3-kinase (rb-anti-PI3K, #4249S), rb-anti-protein kinase B (rb-anti-AKT, #9272S), rb-anti-Phosphorylated protein kinase B (rb-anti-P-AKT, #4060S), rb-anti-ribosomal protein S6 kinase

(rb-anti-p70S6K, #9204S), rb-anti-Phosphorylated ribosomal protein S6 kinase (rb-anti-P-p70S6K, #9202S), rb-anti-acetyl-CoA carboxylase 1 (rb-anti-ACC1, #3662S), rb-anti-Phosphorylated acetyl-CoA carboxylase 1 (rb-anti-P-ACC1, #3661S). A rb-anti-GAPDH primary antibody (a531, Bioworld, China, 1: 10,000) was also incubated with the blots to provide a reference for normalization. After washing the membranes, an incubation with HRP-conjugated secondary antibody was performed for 2 h at room temperature. Finally, the blots were washed and signal was detected by enhanced chemiluminescence (ECL) using the LumiGlo substrate (Super Signal West Pico Trial Kit, Pierce, USA). ECL signal was recorded using an imaging system (Bio-Rad, USA) and analyzed with Quantity One software (Bio-Rad, USA). The phosphorylation level of ACC1, AKT and p70S6K was determined by the ratio of P-ACC1 to total ACC1, P-AKT to total AKT and P-p70S6K to total p70S6K, respectively. The expression level of PI3K was determined by the ratio of PI3K to GAPDH.

Statistical analysis

Data are presented as the means ± SEM. Data were tested for normal distribution, and statistical significance was assessed by the independent sample t-test using SPSS version 11.0 for Windows (SPSS Inc., Chicago, IL, USA). Data were considered statistically significant if $P < 0.05$. The numbers of replicates used for statistics are noted in the Tables and Figures.

Results

The milk yield and milk composition of lactating goats from treatment and control

groups

Different diets had no influence on the dry matter intake (DMI) of goats. However, the milk protein and fat content in the BG goats were significantly higher than those of the control group ($P < 0.05$). In addition, within 20 weeks of treatment, the milk yield, lactose and total solids were higher in the BG goats than that in the control goats (Table 3).

Rumen pH, LPS content in the rumen and plasma

The dynamic pH curve in the BG group was higher than that in the control group during the long-term experiment. However, it showed that a pH value under 5.6 lasted for 3 h in the control group, which indicated that SARA was successfully induced (Figure 1). The LPS concentration in the rumen fluid and plasma was significantly lower in the BG group than that in the control group ($P < 0.05$, Table 4).

The plasma biochemical parameters of lactating goats from treatment and control groups

As shown in Table 4, the plasma content of NEFA and INS were significantly higher in the BG group compared to the control group ($P < 0.05$). Meanwhile, we found that the concentrations of plasma glucose, TG, glucagon, and total cholesterol were higher in the BG goats compared to the control goats, although the difference was not statistically significant.

BG diet increased the production of TG and NEFA in the liver of lactating goats

We next examined nutrition substances in the plasma obtained from the hepatic and portal veins of both treatment groups. We calculated the ratio of portal vein

220 levels:hepatic vein levels (H-P). If H-P > 0, it indicates that more nutrition substances
221 are produced in the liver than those that enter the blood. Conversely, if H-P < 0, it
222 indicates that nutrition substances are consumed in the liver, suggesting that NEFA is
223 catabolize there. Our measurements showed that NEFA was significantly higher in BG
224 goats when compared to control goats ($P < 0.05$, Table 5). This suggested that more
225 milk fat precursors were produced in the liver. In addition, the total cholesterol was
226 consumed in the livers of both BG and control goats.
227 *BG diet treatment regulated key enzymes required for lipid metabolism in the livers of*
228 *goats*
229 Sterol regulatory element binding protein-1c (SREBP-1c) is a key regulator of
230 intracellular lipid metabolism, including the uptake and synthesis in the liver. We
231 therefore examined expression of SREBP-1c mRNA and some of its known
232 downstream targets in BG and control goats (Figure. 2A). We found that SREBP-1c
233 expression in the BG goats was significantly higher than that in the control goats ($P <$
234 0.05). The expression of downstream targets of SREBP-1c, such as stearoyl-CoA
235 desaturase (SCD-1), ACC1, and fatty acid synthetase (FAS) were also increased by the
236 BG diet. In particular, FAS and ACC1 expression in the BG goats were significantly
237 higher than that in the control goats ($P < 0.05$). Peroxisome proliferator activated
238 receptors α (PPARα) is a key transcription factor that controls intracellular lipid
239 oxidation. It likely achieves this by regulating carnitine palmitoyltransferase-1
240 (CPT-1), carnitine palmitoyltransferase-2 (CPT-2), liver-fatty acid binding protein
241 (L-FABP) and acyl-CoA oxidase (ACO), which are enzymes required for lipid

oxidation in the liver. Here, we found that the mRNA expression of PPARα, CPT-1, CPT-2, L-FABP, and ACO were decreased in the BG goats compared to expression in the goats. In particular, expression of PPARα, CPT-1, L-FABP and ACO were significantly lower than that in the controls ($P < 0.05$; Figure. 2B). However, CPT-2 expression in the BG goats was not significantly different from that in the controls.

We also investigated the extent of P-ACC1 protein. We found that it was significantly lower in the BG goats compared to the levels in the control goats by Western blotting ($P < 0.05$, Figure. 3). It is indicated that ACC1 activity was significantly higher in the BG groups than in the control groups. This is consistent with our previous observation that ACC1 mRNA expression increases in BG goats.

The BG diet treatment modulated the PI3K/AKT-SREBP-1c signaling pathway

During the course of our earlier experiments, we observed that INS levels in the plasma were significantly higher in goats treated with the buffering agent. To further explore a potential mechanism for how the BG diet regulates expression of key liver enzymes, we next examined the activity of the PI3K/AKT signaling pathway. The results indicated that the mRNA expression of insulin receptor (INSR) and insulin receptor substrates (IRS) were significantly higher in the BG goats compared to the levels in the control goats ($P < 0.05$, Figure. 4). We also found that levels of PI3K, P-AKT, and P-p70S6K protein in the BG goats were significantly higher than those in the control goats by Western blotting ($P < 0.05$, Figure. 5A-C). This suggested that the INS-PI3K/AKT-SREBP-1c signaling pathway was activated following treatment with the BG diet of lactating goats.

Discussion

In recent years, intensive production systems for ruminants have encouraged the use of the HC diet or easily fermentable carbohydrate diet to support high milk yields or rapid weight gain. Although this feeding practice can enhance economic efficiency in the short-term, the feeding of HC diet leads to the translocation of LPS from the digestive system into the circulating blood. As a result, SARA often occurs during the periods of early and mid-lactation in dairy production herds. Due to the rapid fermentation and the accumulation of volatile fatty acids in the rumen, as well as the lactic acid, the value of rumen pH markedly decreased and prolonged for long time (Garrett *et al*. 1999). Moreover, previous studies have reported that feeding a high-grain diet could cause the SARA and induce the depression of milk fat (Xu *et al*. 2015).

The $NaHCO_3$ could increase the buffering capacity and prevent the acidosis in rumen. Diets with $NaHCO_3$ did not result in as great a drop of rumen pH, and rumen pH was more stable for the post feeding (Snyder *et al*. 1983). Previous studies indicated that the addition of $NaHCO_3$ to a restricted-roughage rations could be given to lactating cows to increase the content of milk fat (Emery *et al*. 1965). It is well documented that dietary addition of $C_4H_7NaO_2$ could promote development of the rumen mucosa and health status of young dairy cows (Sander *et al*. 1959). Previous studies indicated that the addition of $C_4H_7NaO_2$ to a HC diet could be given to lactating goats to decrease the content of LPS in rumen (Dai *et al*. 2017). In this study, the control goats fed a HC diet for 20 weeks exhibited a lower ruminal pH, which

decreased to <5.6 and persisted for more than 3 hours per day after feeding. According to the definition of experimental SARA, the control goats were suffered SARA disease. However, we found that the pH in the rumen fluid of BG-fed goats was much higher than that of control goats. The concentrations of LPS in the rumen and jugular were also markedly decreased, indicating that the BG diet stabilized ruminal pH and prevented the release of LPS. Moreover, the goats that consumed the HC diet with the added buffering agent displayed a higher milk fat content, which was consistent with the study conducted in dairy cows. However, the mechanism of milk fat up-regulated still requires further study.

NEFA is one of the most important precursors in milk fat. Previous studies have reported that feeding a high-concentrate diet could cause the depression of milk fat with the decline of NEFA in blood. It is well documented that with the increasing of NEFA content in blood, the absorbed quantity applied to milk fat synthesis was also elevated in mammary (Kadegowda *et al*. 2017, Li *et al*. 2017). Furthermore, we quantified the precursors for milk fat synthesis. The results showed that the levels of NEFA in the plasma of the BG goats were significantly higher than that in the control goats. In ruminants, the liver is the major site for gluconeogenesis and lipogenesis, which provides the substrate precursors to the mammary gland for milk production (Dorland *et al*. 2012). Therefore, the concentration of these precursors in the blood have an important influence on milk fat synthesis. In order to further study the changes observed in milk fat precursors, we examined the dynamics of NEFA production in the liver by assaying plasma obtained from the hepatic vein and portal

308 vein. The results suggested that more NEFA was produced from the livers of BG goats
309 compared to that in control goats. However, the relationship between increased plasma
310 NEFA and liver still warrants further investigation in this study.

311 Peroxisome proliferator-activated receptors (PPARs) involved in the transport of
312 TG in the blood, cellular fatty acid uptake, and mitochondrial beta oxidation (Pettinelli
313 *et al*. 2011). PPARs have three subtypes including PPARα, PPARβ, and PPARγ.
314 PPARα has an important role in the regulation of mitochondrial and peroxisomal fatty
315 acid oxidation in ruminants, including modulation of four downstream targets, ACO,
316 CPT-1, CPT-2 and L-FABP (Barger and Kelly 2000). Sterol regulatory element
317 binding proteins (SREBPs) are transcription factors that activate genes involved in
318 lipogenesis and fatty acid synthesis (Shimano, 2002). SREBP-1c is one member of
319 this family, and it may regulate many genes involved in lipid synthesis and deposition
320 (Horton *et al*. 2003), such as ACC1, SCD-1, and FAS, which are all required for fatty
321 acid synthesis in white adipose tissue, the liver, skeletal muscle, and other tissues (Li
322 *et al*. 2018). To explore the mechanisms by which the buffering agent treatment
323 improves milk fat synthesis, we analyzed the expression of key transcription factors
324 and enzymes required for lipid metabolism regulation in the liver. The results showed
325 that the buffering agent treatment inhibited mRNA expression of PPARα, CPT-1,
326 CPT-2, L-FABP and ACO. In contrast, the mRNA levels of SREBP-1c and its
327 downstream protein targets SCD-1, ACC1, and FAS were elevated. Taken together,
328 these results suggest that the buffering agent treatment promotes NEFA uptake and
329 synthesis, and inhibits NEFA catabolism by regulating the expression of key liver

enzymes found in lactating goats. Simply put, the buffering agent treatment reduces NEFA consumption while increasing its accumulation in liver. This may be useful for developing ways to generate more synthetic precursors for producing milk fat in the mammary gland, and could also explain why milk fat synthesis is increased by treatment with the buffering agent.

INS is a anabolic hormone secreted by the pancreatic beta cells, it is transported to the liver through the blood circulation, and binds to INSR on the liver to play the physiological effect by INS signaling pathway (McAtee and Trenkle 1971). Previous studies have also shown that INS promotes lactation and regulates liver lipid metabolism to increase the synthesis of NEFA in the liver while inhibiting NEFA catabolism through the PI3K signaling pathway (Shimomura *et al*. 2000, Hanssen *et al*. 2015). SREBP-1c is a major regulator of lipid production, it is induced by INS, and it can promote the expression of genes related to lipid synthesis through PI3K signaling pathway (Yoshikawa *et al*. 2000, Dong *et al*. 2010). Furthermore, the increase in expression of SREBP-1c was found to be achieved via modulation of the PI3K signaling pathway after adding INS to hepatic cells (Foretz *et al*. 1999). Lipid synthesis and metabolism likely increases as a result of SREBP-1c activity induced by the PI3K-AKT signaling pathway (Porstmann *et al*. 2005). To further explore the mechanisms by which the buffering agent treatment regulated liver enzyme expression, we studied the activity of the INS-PI3K/AKT-SREBP-1c signaling pathway. In the present study, the results showed that the buffering agent treatment increased protein expression of PI3K, AKT in liver. In addition, the plasma INS levels

in the BG group were also higher. Therefore, we verified that this pathway is activated in the livers of goats that received the buffering agent. Furthermore, the SREBP-1c pathway mediates the observed effects on NEFA metabolism by enhancing fatty acid synthesis and inhibiting oxidation in the livers of lactating goats.

In summary, we systematically investigated the effects of a buffering agent on the metabolism of lipid in the livers of lactating goats fed with a high-concentrate diet and found that NEFA precursors were produced in the liver and increased in plasma. Furthermore, the plasma INS levels were also increased in the BG goats: elevated INS increases PI3K/AKT phosphorylation and activity. Activated PI3K/AKT promotes the expression and transcriptional activity of SREBP-1c, thereby down-regulating the expression of the lipid synthesis genes and promoting lipid synthesis. Thus, long-term BG diet feeding may lead to the up-regulated expression of lipid synthesis genes and a increase in the NEFA content in the blood via the INS-PI3K/AKT-SREBP-1c signalling pathway. Meanwhile, the increased NEFA concentration in the blood of goats fed a BG diet may explain the up-regulated in milk fat in these lactating goats.

Competing interests

There is no conflict of interest.

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Table 1. Ingredients and nutritional composition of the diets.

Concentrate: Forage ratio 60:40			
Ingredient (%)		Nutrient levels[b]	
Leymus chinensis	27.00	Net energy/(MJ.kg^{-1})	6.71
Alfalfa silage	13.00	Crude protein/%	16.92
Corn	23.24	Neutral detergent fiber/%	31.45
Wheat bran	20.77	Acid detergent fiber/%	17.56
Soybean meal	13.67	Calcium/%	0.89
Limestone	1.42	Phosphorus/%	0.46
NaCl	0.30		
Premix[a]	0.60		
Total	100.00		

a. Provided per kg of diet: VA 6000IU/kg, VD 2500IU/kg, VE 80mg/kg, Cu 6.25 mg/kg, Fe 62.5 mg/kg, Zn 62.5 mg/kg, Mn 50mg/kg, I 0.125 mg/kg, Co 0.125 mg/kg.

b. Nutrient levels were according to National Research Council (NRC,2001).

477 **Table 2.** Primer sequences used for qRT-PCR analysis of target genes in lactating goats.

Target genes	Primer sequences (5'-3')	Products/bp
CPT-1	CCCATGTCCTTGTAATGAGCCAG AGACTTCGCTGAGCAGTGCCA	230
CPT-2	ACGCCGTGAAGTATAACCCT CCAAAAATCGCTTGTCCCTT	119
L-FABP	AATACCAAGTCCAGACCCAG CACGATTTCCGACACCC	110
ACO	TAAGCCTTTGCCAGGTATT ATGGTCCCGTAGGTCAG	189
PPARα	GGAGGTCCGCATCTTCCACT GCAGCAAATGATAGCAGCCACA	352
FAS	GCACTACCACAACCCAAACCC CGTTGGAGCCACCGAAGC	161
ACC1	ACGCAGGCATCAGAAGATTA GAGGGTTCAGTTCCAGAAAGTA	179
SCD-1	CCGCCCTGAAATGAGAGATG AGGGCTCCCAAGTGTAACAGAC	154
SREBP-1c	CGACTACATCCGCTTCCTTCA ACTTCCACCGCTGCTACTG	259
IRS	GGCAGTCCTGTGAGTCCTA AAGGCGAGCAGCGAGAA	124

INSR	CACACAGCCACTGCCAGAAAGGG AGAAACCGAGTGCGGACCGC	151
GAPDH	GGGTCATCATCTCTGCACCT GGTCATAAGTCCCTCCACGA	177

478

479 **Table 3.** Dry matter intake (DMI), milk yield, and milk composition from the two groups of

480 lactating goats.

Item	Treatment		p-value
	Control	BG	
DMI, kg/d	1.80 ± 0.13	1.98 ± 0.02	0.82
Milk			
Yield, kg/d	1.00 ± 0.02	1.38 ± 0.03	0.10
Fat content, %	3.24 ± 0.10	4.25 ± 0.08	0.03*
Protein, %	2.82 ± 0.05	3.80 ± 0.01	0.05*
Lactose, %	4.32 ± 0.38	4.75 ± 0.29	0.81
Total solids, %	12.10 ± 0.23	13.23±0.22	0.77

481 Data are presented as the means ± SEM (n = 6/group). *$p < 0.05$ indicates statistically significant

482 differences when compared with the control group.

483

484 **Table 4.** Effects of the BG diet on plasma indicators of lactating goats.

Item	Control	BG	P-value
Glucose (mmol/l)	3.34 ± 0.25	3.44 ± 0.29	0.56

Nonesterified fatty acid (nmol/l)	1.34 ± 0.10	1.98 ± 0.09	0.04*
Triacylglycerol (mmol/l)	0.34 ± 0.02	0.41 ± 0.04	0.67
Insulin (mIU/l)	13.81 ± 0.10	19.43 ± 0.18	0.03*
Glucagon (pg/ml)	457.43 ± 45.96	525.81 ± 38.23	0.20
Total cholesterol (mmol/l)	0.94 ± 0.05	0.96 ± 0.06	0.32
LPS (EU/ml)			
Rumen fluid	42550 ± 136	23340 ± 134	0.02*
Jugular vein	3.76 ± 0.33	1.82 ± 0.14	0.02*

485 Data are presented as the means ± SEM (n = 6/group). *$p < 0.05$ indicates statistically significant
486 differences when compared with the control group.

487

488 **Table 5.** Effect of the BG diet on plasma indicators in lactating goats.

	Treatment		p-value
	Control	BG	
Hepatic vein (H)			
Triglyceride (mmol/l)	0.16 ± 0.03	0.15 ± 0.03	0.15
Nonesterified fatty acid (mmol/l)	1.42 ± 0.03	1.78 ± 0.02	0.03*
Total cholesterol (mmol/l)	0.61 ± 0.09	0.55 ± 0.03	0.21
Portal vein (P)			
Triglyceride (mmol/l)	0.16 ± 0.01	0.13 ± 0.01	0.41
Nonesterified fatty acid (mmol/l)	1.67 ± 0.04	1.73 ± 0.03	0.80
Total cholesterol (mmol/l)	0.86 ± 0.05	0.84 ± 0.03	0.33

(H-P)			
Triglyceride (mmol/l)	0.00 ± 0.01	0.02 ± 0.02	0.16
Nonesterified fatty acid (mmol/l)	-0.25 ± 0.02[1]	0.05 ± 0.01[2]	0.02*
Total cholesterol (mmol/l)	-0.25 ± 0.03	-0.29 ± 0.03	0.17

1 H-P < 0 represents a higher nutritional substance concentration in the portal vein blood but a lower nutritional substance concentration in the hepatic vein blood, which indicates that the nutritional substances were consumed by the liver. 2 H-P > 0 represents a lower nutritional substance concentration in the portal vein blood but a higher nutritional substance concentration in the hepatic vein blood, which indicates that the nutritional substances were produced in the liver. Data are presented as the means ± SEM (n = 6/group), *p < 0.05 indicates statistically significant differences when compared with the control group.

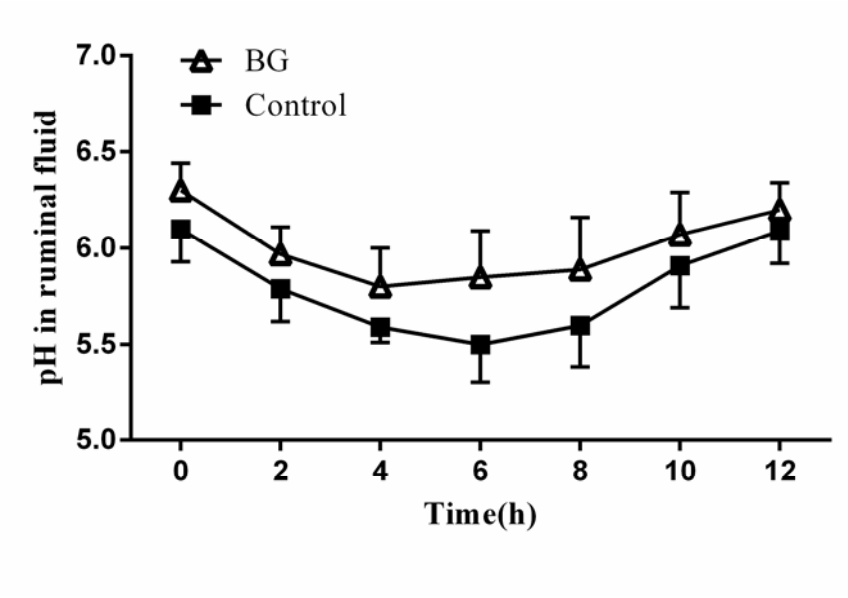
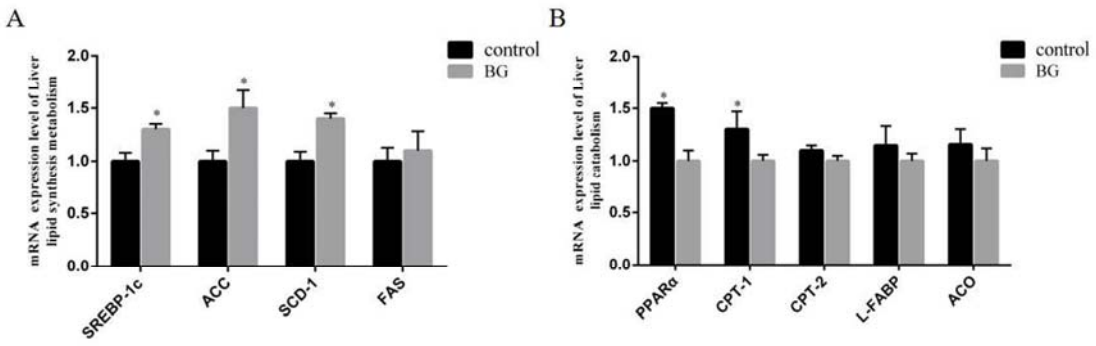


Figure 1. pH value in ruminal fluid after 20 weeks feeding regime. Data are presented as the means ± SEM (n=6/group). *p < 0.05 indicates statistically significant differences when compared with the control group.



500

501 **Figure 2.** Effects of the BG diet on the expression of liver lipid metabolism in lactating goats.

502 (A) The lipid synthesis genes involved in sterol regulatory element-binding protein-1c (SREBP-1c),

503 fatty acid synthetase (FAS), acetyl-CoA carboxylase 1 (ACC1), and stearoyl-CoA desaturase 1

504 (SCD-1) were measured in the liver tissue. (B) The lipid catabolism genes involved in peroxisome

505 proliferator-activated receptor α (PPARα), carnitine palmitoyl transferase-1 (CPT-1), carnitine

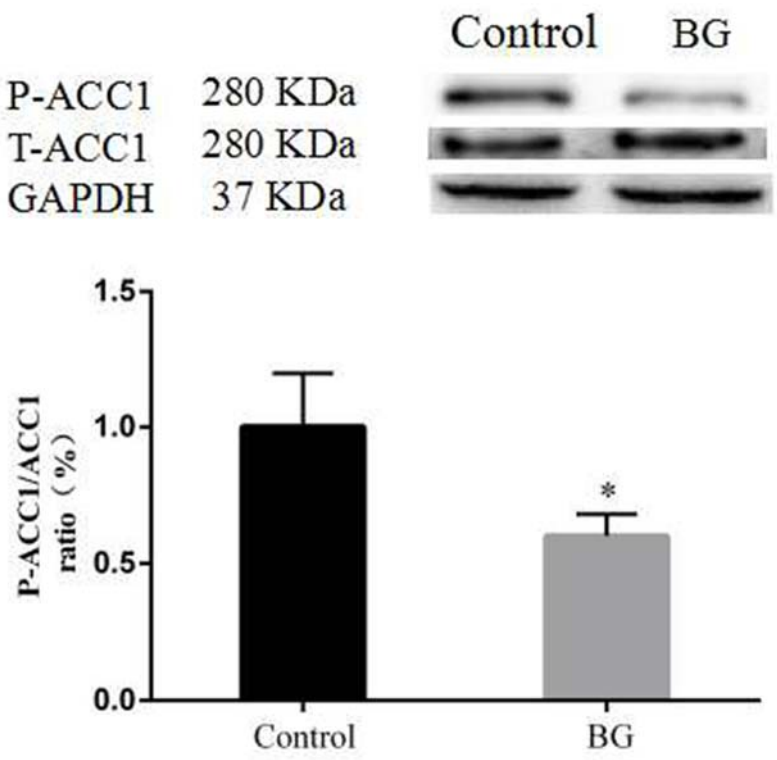
506 palmitoyl transferase-2 (CPT-2), liver-fatty acid-binding protein (L-FABP) and acyl-CoA oxidase

507 (ACO) were measured in the liver tissue. GAPDH was used as the control. The experiments were

508 repeated three times. Data are presented as the means ± SEM (n=6/group). *$p < 0.05$ indicates

509 statistically significant differences when compared with the control group.

510



511

512 **Figure 3.** Effects of the BG diet on the expression of acetyl-CoA carboxylase 1 (ACC1) protein in

513 the liver of lactating goats.The experiments were repeated three times. Data are presented as the

514 means ± SEM (n=6/group). *$p < 0.05$ indicates statistically significant differences when compared

515 with the control group.

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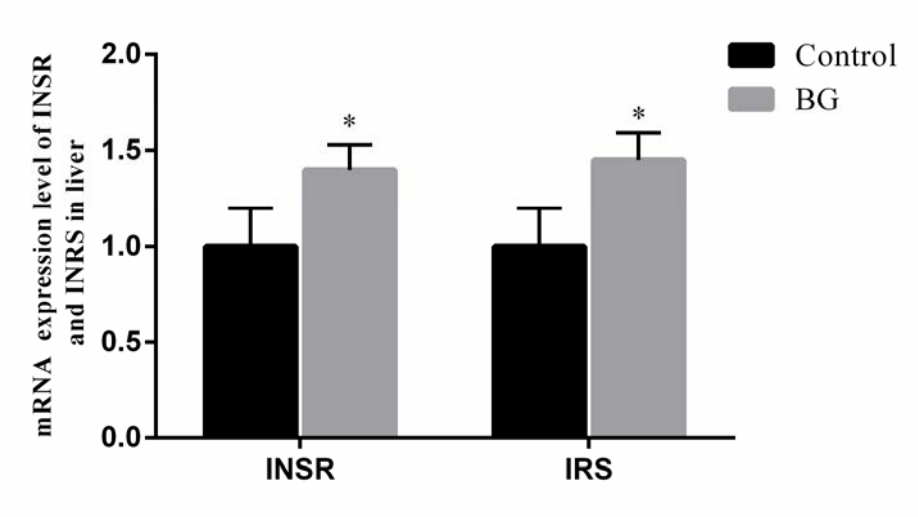

519

520 **Figure 4.** Effects of the BG diet on the expression of liver insulin receptor (INSR) and insulin
521 receptor substrates (IRS) genes in lactating goats.The experiments were repeated three times. Data
522 are presented as the means ± SEM (n=6/group). *p < 0.05 indicates statistically significant
523 differences when compared with the control group.

524

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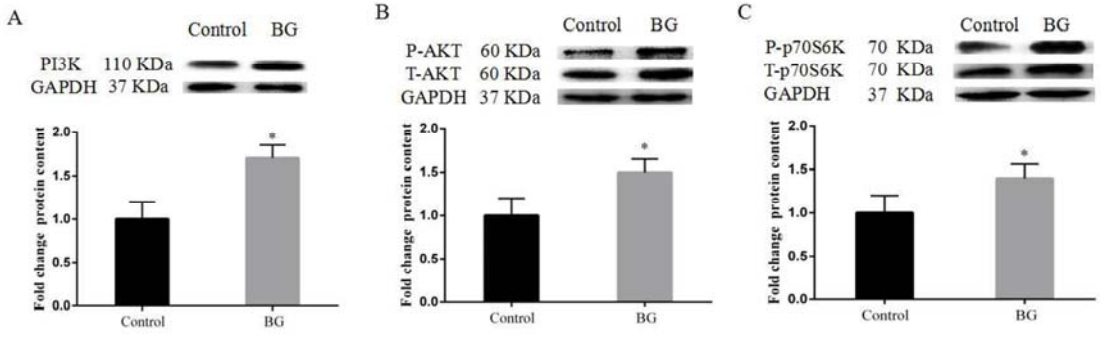

526

527 **Figure 5.** Effects of the BG diet on the expression of phosphatidylinositol 3-kinase (PI3K), protein
528 kinase B (AKT) and ribosomal protein S6 kinase (p70S6K) proteins in the liver of lactating goats.
529 The experiments were repeated three times. Data are presented as the means ± SEM (n=6/group).
530 *p < 0.05 indicates statistically significant differences when compared with the control group.

531