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1 # Buffering Agent Induced Lactose Content Increases via Growth
2 # Hormone-Mediated Activation of Gluconeogenesis in Lactating
3 # Goats

4 ## Authorship

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24 ## Abstract:

25 Dairy goats are often fed a high-concentrate (HC) diet to meet lactation demands;
26 however, long-term concentrate feeding is unhealthy and decreases milk yield and
27 lactose content. Therefore, we tested whether a buffering agent increases the output of
28 glucose in the liver and influences of lactose synthesis. In this study, sixteen lactating
29 goats were randomly assigned to two groups: one group received a HC diets
30 (Concentrate : Forage = 6:4, HG), and the other group received the same diet with a
31 buffering agent added (0.2% NaHCO3, 0.1% MgO, BG) as a treatment for 19-weeks
32 experimental period. The results showed that the total volatile fatty acids and
33 lipopolysaccharide (LPS) declined in the rumen leading to the rumen pH was
34 stabilized in the BG group. Milk yield and lactose content increased. The alanine
35 aminotransferase, aspartate transaminase, alkaline phosphatase, pro-inflammatory
36 cytokines, LPS and lactate content in the plasma was significantly decreased, whereas
37 prolactin and growth hormone levels were increased. The hepatic vein content of
38 glucose was increased. In addition, the expression of pyruvate carboxylase (PC),

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39 phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6PC) in
40 the liver was significantly up-regulated. In mammary gland, the glucose transporter
41 type-1, 8, 12 and sodium-glucose cotransporter-1 levels were increased. Cumulatively,
42 the buffering agent treatment increased blood concentrations of glucose via the
43 gluconeogenes and promoting their synthesis in the liver. It may contribute to
44 the increase in milk yield and lactose synthesis of lactating goats.

45 **Keywords**: Magnesium oxide, Sodium bicarbonate, high-concentrate diet, Hepatic
46 gluconeogenesis

47 Introduction

48 In the dairy industry, it is currently common practice to feed a high-concentrate (HC)
49 diet to lactating cows or goats to meet their energy requirements to support high milk
50 production. However, long-term high concentrate feeding is harmful to the health of
51 ruminants and leads to a decrease in milk yield [1]. It was reported that feeding of HC
52 diets to lactating cows causes a decline in the rumen pH if organic acids, such as
53 volatile fatty acid (VFA) and lactic acid, accumulate in the rumen[2]. Digestion of a
54 HC diets results in less production of saliva and bicarbonate, and reduced buffering
55 capacity coupled with greater accumulation of organic acids has been reported to
56 increase the incidence of subacute ruminal acidosis (SARA)[3]. A rumen pH of less
57 than 5.8 for over 4 h per day is used as a parameter to determine the occurrence of
58 SARA[4]. In addition, decreased rumen pH results in the release of
59 lipopolysaccharide (LPS), which originate from the cell-wall component of

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60 gram-negative bacteria[5]. Previous studies have shown that LPS can translocate into
61 the bloodstream from the digestive tract under conditions of high permeability, and
62 after injury to the liver tissue [6].

63 In the ruminant, lactose constitutes about 40% of total solids in milk composition. As
64 lactose maintains the osmolarity of milk, the rate of lactose synthesis serves as a
65 major control of the volume of milk yield [7]. Glucose is the main precursor of lactose
66 synthesis in the epithelial cell of the mammary gland, however, the mammary gland
67 cannot synthesize glucose from other precursors due to the lack of glucose
68 6-phosphatase (G6PC)[8]. Therefore, the mammary gland is dependent on the blood
69 supply for its glucose needs and as a consequence, mammary glucose uptake is a
70 rate-limiting factor for milk yield[9]. Liver glycometabolism of ruminants is different
71 from monogastric animals. In the lactating dairy cows, glucose is supplied primarily
72 by hepatic gluconeogenesis to maintain stable blood glucose[10]. Dairy cows
73 experience an increased demand for glucose to support whole body glucose
74 metabolism and to supply glucose for lactose synthesis[11]. Therefore, liver
75 gluconeogenesis plays an important role in the lactose synthesis of mammary gland.

76 Buffering agent could enhance the acid base buffer capacity and has been used to
77 prevent ruminant rumen SARA and improve the production performance. Previous
78 studies indicated that the addition of sodium bicarbonate (NaHCO3), magnesium
79 oxide (MgO) to a diet could be given to lactating cows to increase the content of
80 lactose, as well as milk yield[12]. It is well documented that dietary addition of 2%
81 NaHCO3 could increase the buffering capacity and prevent the acidosis in rumen[13].

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82 However, at present, the research of buffering agent is focused on the composition
83 and production of milk from dairy cows. Furthermore, little is known regarding the
84 mechanism of how a buffering agent improves milk yield and lactose content in goats.
85 In this study, we created a buffering agent consisting of (0.2% $NaHCO3$, 0.1% MgO)
86 and mixed it with a high-concentrate diet source that was fed to lactating goats. We
87 then investigated the effect of this buffering agent on the development of SARA and
88 milk yield and lactose content to elucidate potential mechanisms for this phenomenon.

89 Materials and Methods

90 Ethical approval

91 The Institutional Animal Care and Use Committee of Nanjing Agricultural University
92 (Nanjing, People's Republic of China) approved all of the procedures (surgical
93 procedures and care of goats). The protocol of this study was reviewed and approved
94 specifically, with the project number 2011CB100802. The slaughter and sampling
95 procedures strictly followed the 'Guidelines on Ethical Treatment of Experimental
96 Animals'(2006) no. 398 set by the Ministry of Science and Technology, China and the
97 'Regulation regarding the Management and Treatment of Experimental Animals'
98 (2008) no. 45 set by the Jiangsu Provincial People's Government.

99 Animals and experimental procedures

100 Sixteen healthy multiparous mid-lactating saanen goats (body weight, 39 ± 7 kg,
101 mean ± SEM, 3-4 weeks post-partum) at the age of 2-4 years were used in
102 experiments. They were housed in individual stalls in a standard animal feeding house

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103 at Nanjing Agricultural University (Nanjing, China). All goats were randomly divided
104 into two groups: one group recieved a high-concentrate diet (Concentrate : Forage =
105 6:4, HG, n=8), and the other group received the same diet with a buffering agent
106 added (0.2% NaHCO3, 0.1% MgO, purchased from Nanjing Jiancheng
107 Bioengineering Institute, China, BG, n=8) as a treatment. The ingredients and
108 nutritional composition of the diets are presented in Table 1. The animals were fed the
109 respective diets for 19 weeks, and had free access to water during the experimental
110 period.

111 Prior to the initiation of the experiment, all of the goats were installed rumen fistula
112 and hepatic catheters. Specific steps are as follows: First, all goats were installed
113 rumen cannula, and indwelt with hepatic and portal vein catheters, under general
114 anaesthesia by inhalation of isoflurane (2.5% in 1:1 mixture of oxygen and air; Abbott
115 Scandinavia AB, Solna, Sweden). Reflexes (pupillary and palpebral) and respiratory
116 rate were monitored before and during the surgery to verify anaesthetic depth. Next, a
117 small cannula constructed from polyvinylchloride was inserted into the rumen of each
118 goat[14]. After rumen fistula surgery, all goats were ruminally cannulated, fitted with
119 indwelling catheters in the portal vein and hepatic vein[15]. At the end of these
120 procedures the goats received an intramuscular injection of flunixin meglumine [2 mg
121 (kg body weight)$^{-1}$], an anti-inflammatory and analgesic (Banamine; Mantecorp Ind.
122 Quím. e Farm. Ltda, Rio de Janeiro, Brazil) and their respiratory movements were
123 monitored until they regained consciousness. Goats were continuously monitored for
124 1 h after the surgery, and all animals used in the experiments on the day after

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125 presented in a good and stable clinical condition with no signs of inflammation or
126 pain problems. After surgery, goats were observed for 2 weeks during recovery from
127 the surgery. Sterilized heparin saline (500 IU/mL, 0.3 mL/time) was administered at
128 6-hour intervals every day until the end of the experiment to prevent catheters from
129 becoming blocked.

130 **Rumen fluid collection and analysis**

131 Fifteen minutes prior to feed delivery and 0, 2, 4, 6, 8 and 10 h after feed delivery on 7
132 consecutive days during week 19, 20 mL rumen fluids was collected with a nylon bag
133 and the pH value was measured immediately with pH-meter.

134 The rumen fluid was collected and each sample was transferred into a 50-mL sterile
135 tube and kept on ice until transported to the laboratory for the initial processing before
136 LPS determination. Another part of each rumen fluid sample was centrifuged at 3,200
137 × g for 10 min at 4°C immediately after collection and the supernatant was collected.
138 To analyze VFA in ruminal fluid, a 5-mL aliquot was deproteinized with 1 mL of 25%
139 metaphosphoric acid. These samples were stored at -20°C until analysis.

140 The concentration of LPS in rumen fluid was measured by a Chromogenic End-point
141 Tachypleus Amebocyte Lysate Assay Kit (Chinese Horseshoe Crab Reagent
142 Manufactory Co. Ltd, Xiamen, China). Pretreated rumen fluid samples were diluted
143 until their LPS concentrations were in the range of 0.1-1.0 endotoxin units (EU)/mL
144 relative to the reference endotoxin.

145 VFA were measured using capillary column gas chromatography (GC-14B, Shimadzu,

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146 Japan; Capillary Column: 30 m × 0.32 mm × 0.25 mm film thickness; Column
147 temperature = 110°C, injector temperature = 180°C, detector temperature = 180°C).

148 **Plasma biochemical parameters analysis**

149 In the 19th week, blood samples were collected from the jugular vein, hepatic vein and
150 portal vein blood in 10 mL vacuum tubes containing sodium heparin. Blood was
151 centrifuged at 3000 x *g* for 15 min to separate the plasma, which was then stored at
152 -20°C until analysis. The plasma glucose content was quantified using a Beckman Kurt
153 AU5800 series automatic biochemical analyzer (Beckman Kurt, USA) at the General
154 Hospital of Nanjing Military Region (Nanjing, China).

155 The growth hormone (GH), tumor necrosis factor-a (TNF-a), interleukin 1β (IL-1β)
156 concentration in the plasma were measured by radioimmunoassay with commercially
157 available human radioimmunoassay kits purchased from Beijing North Institute of
158 Biological Technology. The detection range of radioimmunoassay kits for GH (rabbit,
159 B12PZA), TNF-a (rabbit, C06PZA) and IL-1β (rabbit, C09PDA) were 0.1-50 ng/mL,
160 1-10 ng/mL and 0.1-8.1 ng/mL, respectively. All of the procedures were performed
161 according to the manufacturer's instructions.

162 The analyses for the prolactin, glucocorticoids, histamine and lactate were performed
163 using Enzyme-Linked Immunosorbent Assay (ELISA) kit (Shanghai Enzyme-linked
164 Biotechnology Co. Ltd, Shanghai, China) according to the manufacturer's
165 instructions. The detected range of ELISA kits for prolactin, glucocorticoids,
166 histamine and lactate were 5-2000 pg/mL, 0-80 ng/mL, 2-600 ng/mL and 0.1-30

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167 mmol/mL, respectively. The LPS concentration were determined using a chromogenic
168 endpoint assay (CE64406, Chinese Horseshoe Crab Reagent Manufactory Co., Ltd.,
169 Xiamen, China) with a minimum detection limit of 0.01 EU/mL. The procedures were
170 performed according to the manufacturer's instructions.

171 Milk composition analysis

172 Goats were milked at 8:30 h and 18:30 h, and the milk yield was recorded daily. A
173 50-mL milk sample was taken to determine the lactose content once a week
174 (Milk-Testing™ Milkoscan 4000, FOSS, Denmark) at the Animal Experiment Center
175 of College of Animal Science and Technology at the Nanjing Agricultural University.

176 Sample collection

177 In the 19th week, mammary gland tissues were obtained by biopsy after 4 h after the
178 morning feeding. Local anesthesia (2% lidocaine hydrochloride) was administered
179 into breast skin in a circular pattern surrounding the incision site, then a 2cm incision
180 was made and mammary gland tissue was dissected. Tissue samples (500-800 mg)
181 were rinsed with 0.9% saline, snap frozen in liquid nitrogen and were used for RNA
182 extraction. Goats were slaughtered after overnight fasting. Incisions were sutured, and
183 antibiotics were administered intramuscularly to avoid infection.

184 After 19 weeks, all goats were killed with neck vein injections of xylazine [0.5 mg
185 (kg body weight)$^{-1}$; Xylosol; Ogris Pharme, Wels, Austria] and pentobarbital [50 mg
186 (kg body weight)$^{-1}$; Release; WDT, Garbsen, Germany]. After slaughter, liver tissue
187 was collected and washed twice with cold physiological saline (0.9% NaCl) to

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

RNA isolation, cDNA synthesis and real-time PCR

Total RNA was extracted from liver samples using TRIzol reagent (15596026, Invitrogen, USA) and converted to cDNA using commercial kits (Vazyme, Nanjing, China). All PCR primers were synthesized by Generay Company (Shanghai, China); 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 a reference for normalization. The $2^{-\Delta\Delta Ct}$ method was used to analyse the real-time PCR results, and each gene mRNA level is expressed as the fold change relative to the mean value of the control group.

Western blot analysis

Total protein was extracted from frozen liver samples, and the concentration was determined using a bicinchoninic acid (BCA) assay kit (Pierce, Rockford, IL, USA). We isolated 30 μg of total protein from each sample, which was subjected to electrophoresis on a 10% SEMS-PAGE gel. The separated proteins were transferred onto nitrocellulose membranes (Bio Trace, Pall Co., USA). The blots were incubated with the following primary antibodies for overnight at 4°C at dilutions of 1:1000 in block: rb-anti-phosphoenolpyruvate carboxykinase (rb-anti-PEPCK, #12940, CST),

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209 rb-anti-glucose transporter type 1 (rb-anti-GLUT1, ab14683, Abacm) and
210 rb-anti-glucose transporter type 12 (rb-anti-GLUT12, ab100993, Abacm). A
211 rb-anti-GAPDH primary antibody (A531, Bioworld, China, 1:10,000) was also
212 incubated with the blots to provide a reference for normalization. After washing the
213 membranes, an incubation with HRP-conjugated secondary antibody was performed
214 for 2 h at room temperature. Finally, the blots were washed, and the signal was
215 detected by enhanced chemiluminescence (ECL) using the LumiGlo substrate (Super
216 Signal West Pico Trial Kit, Pierce, USA). The ECL signal was recorded using an
217 imaging system (Bio-Rad, USA) and analyzed with Quantity One software (Bio-Rad,
218 USA).

219 Statistical analysis

220 The results were expressed as mean ± SEM. The data of ruminal pH and glucose in
221 plasma of hepatic vein, portal vein and jugular vein were analyzed for differences due
222 to diet, feeding time, and their interactions by Univariate using the General Linear
223 Models of SPSS 11.0 for Windows (StatSoft Inc, Tulsa, OK, USA). The differences in
224 milk yield, lactose content, plasma biochemical index, mRNA and protein expression
225 between two groups were analyzed by Independent-Samples T test using the Compare
226 Means of SPASS 11.0 for Windows (StaSoft Inc, Tulsa, OK, USA). Data were
227 considered statistically significant if $P < 0.05$, $P < 0.01$. The numbers of replicates
228 used for statistics are noted in the Tables and Figures.

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230 # Results

231 **The buffering agent treatment increased daily milk yield and lactose content in**
232 **lactating goats**

233 We quantified the daily milk yield and lactose content in the milk of the two
234 experimental groups from 1 to 19 weeks of treatment. During week 1 to week 2,
235 there was no significant difference in average daily milk yield and lactose content
236 between BG goats and HG goats. However, the average daily milk yield ($P < 0.05$)
237 and lactose content ($P < 0.01$) increased significantly in the BG group from 3-19
238 weeks of treatment compared to the HG group (Fig. 1).

239 **The buffering agent treatment stabilized ruminal fluid pH in lactating goats fed a**
240 **high-concentrate diet**

241 After feeding 19 weeks, the dynamic pH curve in the BG group was higher than that
242 in the HG group during the long-term experiment. It showed that a pH value under 5.8
243 lasted for 6 h in the HG group, which indicated that SARA was successfully induced.
244 The pH value of the BG group was significantly increased in comparison to those in
245 the HG group ($P < 0.05$). However, ruminal pH was affected significantly by
246 digesting time, while there was no interaction of digesting time and diet on ruminal
247 pH ($P > 0.05$; Fig. 2).

248 **VFA and LPS concentrations in rumen fluid**

249 As shown in Table 3, BG goats showed significantly lower level of LPS concentration

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250 in rumen fluid than HG goats. Concentrations of total VFA, propionate, butyrate in
251 rumen fluid were significantly decreased in BG goats compared to HG ($P < 0.05$).
252 However, the ratio of propionate to butyrate in the rumen was significantly elevated in
253 the BG group ($P < 0.05$).

254 **The buffering agent treatment changed plasma hormone, enzyme, primary**
255 **pro-inflammatory cytokines and metabolic produced in the lactating goats**

256 As shown in Table 4, the plasma content of alanine aminotransferase (ALT), aspartate
257 transaminase (AST) and alkaline phosphatase (AKP) were significantly lower in the
258 BG group compared to the HG group ($P < 0.05$). Although the plasma content of
259 lactic dehydrogenase (LDH) was declined, there was no significant difference
260 between BG and HG groups. The pro-inflammatory cytokines including TNF-a and
261 IL-1β in the BG were significantly lower than that in the HG goats ($P < 0.05$).
262 Meanwhile, we found that the metabolism products of LPS, histamine and lactate
263 content were also lower in the BG goats compared to the HG goats. Among them, LPS
264 and lactate were significant different ($P < 0.05$). Moreover, the BG goats showed
265 significantly higher levels of GH and prolactin concentration in plasma than HG goats,
266 while there was no significant difference of glucocorticoids concentration in plasma
267 between BG and HG goats.

268 **The buffering agent treatment regulated enzymes required for glucose transfer in**
269 **the mammary gland of lactating goats**

270 We found that the mRNA expression of glucose transporter type 1 (GLUT1), glucose

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271 transporter type 8 (GLUT 8), glucose transporter type 12 (GLUT12) and
272 sodium-glucose cotransporter 1 (SGLT1) were also higher in the BG goats compared
273 to expression in the HG goats. In particular, expression of GLUT1 and SGLT1 were
274 significantly higher than that in the HG goats ($P < 0.05$). The level of GLUT1 protein
275 expression in the mammary gland was significantly up-regulated in BG goats
276 compared to HG ($P < 0.05$). Additionally, there was a tendency increase in protein
277 expression of GLUT12 in BG goats (Fig. 3).

278 **The buffering agent treatment increased production of glucose in the liver**

279 We next examined glucose in plasma obtained from the jugular vein, hepatic vein and
280 portal vein of both treatment groups. After 19 weeks feeding, the jugular and hepatic
281 vein content of glucose was significantly increased in the BG group compared to the
282 HG group ($P < 0.01$). The portal vein content of glucose was increased, but there was
283 no significant difference between BG and HG groups. Compared to HG group, we
284 found that the glucose content of BG group was significantly higher in the hepatic
285 vein compared to the portal vein ($P < 0.05$, Table 5). This indicates that more glucose
286 is produced in the liver. It is possible that the synthesis of glucose was activated
287 following treatment with the buffering agent.

288 **The buffering agent treatment regulated enzymes required for gluconeogenesis**
289 **and GHR in the livers of lactating goats**

290 We found that the mRNA expression of phosphoenolpyruvate carboxykinase (PEPCK)
291 and pyruvate carboxylase (PC), glucose-6-phosphatase (G6PC) was higher in the BG

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292 goats compared to expression in the HG goats. In particular, expression of PEPCK and
293 G6PC were significantly higher than that in the HG goats ($P < 0.05$). The level of
294 PEPCK protein expression in the liver was significantly up-regulated in BG goats
295 compared to HG ($P < 0.05$). This is consistent with our previous observation that
296 PEPCK mRNA expression increases in BG goats (Fig. 4). Taken together, these results
297 suggested that treatment with the buffering agent promoted the gluconeogenesis in the
298 liver. The level of GHR expression in the liver was significantly up-regulated in BG
299 goats compared to HG ($P < 0.05$, Fig. 5).

300 **Discussion**

301 In recent years, dairy goats are often fed HC diets to meet the energy demand for high
302 milk yields. However, the consumption of a HC diets is harmful to the health of dairy
303 goats [16,17]. It's well documented that feeding HC diets to ruminants results in
304 subacute ruminal acidosis (SARA), a common metabolic disease especially occurred
305 in high-producing animals. The root cause is that excessive amounts of rapidly
306 fermentable nonstructural carbohydrates increase the accumulation of organic acids
307 and shift of microbial population in gastrointestinal tract in ruminants [18]. Moreover,
308 an increased amount of fermentable carbohydrates, such as starch, pass through the
309 forestomach to the intestinal tract in acidosis, which accelerates intestinal tract
310 fermentation[19]. It ultimately affects the intestinal absorption of nutrients.
311 Importantly, in accordance with previous research, long-term feeding a
312 high-concentrate diet could induce the depression of the content of lactose and milk
313 yield[20].

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314 The NaHCO3 could increase the buffering capacity and prevent acidosis in the rumen.
315 It was reported that the rumen pH profile improved and a higher yield of milk and
316 milk solids when NaHCO3 was supplemented to a high-concentrate diet [21].
317 Previous studies indicated that the addition of NaHCO3 and MgO to
318 restricted-roughage rations for goats could increase the content of lactose and milk
319 yield [22]. Prolactin is involved in the development of the mammary gland, and the
320 start and continuation of lactation by influencing lactogenesis. It is responsible for the
321 synthesis of the lactose and milk production found in milk [23]. In our experiment, the
322 duration of a rumen pH less than 5.8 lasted for 4h in the goats fed a high-concentrate
323 diet. According to the definition of experimental SARA, HG goats were suffering
324 from SARA disease. However, after feeding 19 weeks, the buffering agent added to
325 the high-concentrate diet stabilized ruminal pH and prevented the occurrence of
326 SARA. Meanwhile, a increase in the milk yield and lactose content was observed in
327 the BG group. The concentrations of prolactin in blood was also markedly increased.
328 Therefore, its increased levels in the blood are associated with milk yield and lactose
329 content improvement.

330 It is well known that the feeding of HC diets leads to the translocation of LPS from
331 gram-negative bacteria in the gastrointestinal tract into the circulating blood. Other
332 studies showed that feeding a diet containing 60% concentrate to lactating goats
333 elevated blood LPS concentrations[24]. The increased levels of circulating LPS also
334 can elevated the concentration of blood pro-inflammatory cytokines IL-1β and TNF-α
335 and activation of liver inflammatory responses [25,26]. The biochemical parameters

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336 ALT, AST and AKP in peripheral blood are common indicators used to assess the
337 status of liver function[27]. In particular, ALT is a specific parameter that reflects
338 hepatocyte damage. In the present study, we observed that feeding of a HC diets
339 induced the massive release of LPS in the rumen, which could trigger a local or
340 systemic inflammatory response after the translocation of LPS into the bloodstream.
341 Furthermore, our data demonstrated that the feeding of a HC diets significantly
342 increased the concentrations of LPS, TNF-α and IL-1β in the plasma. The increase in
343 blood pro-inflammatory cytokines is consistent with the translocation of LPS and
344 activation of inflammatory responses. In addition, the concentrations of ALT, ALP and
345 AKP in peripheral blood were also higher in the HG goats compared to the BG goats.
346 These results provide insight into feeding the HC diets resulted in the breach of
347 hepatocytes releasing these enzymes into the circulation. Importantly, the results
348 showed that those pro-inflammatory cytokines, LPS, TNF-α and IL-1β in the plasma
349 of the BG goats were significantly lower than that in the HG goats. Therefore, we
350 hypothesized that the buffering agent added to the high-concentrate diet reduced the
351 release of the rumen LPS and stabilized the body health of lactating goats.

352 Compared to monogastric animals, glucose is supplied primarily by hepatic
353 gluconeogenesis to maintain stable blood glucose content in the ruminants[10].
354 Therefore, the liver plays a crucial physiological role in the body, and is responsible
355 for glucose metabolism. Our study documented that feeding HC diet to lactating goats
356 for a long time leads to the LPS-cytokines-induced the inflammatory response, and
357 increases the consumption and catabolism of glucose in liver [28]. GH is a polypeptide

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358 hormone synthesized and secreted by the anterior pituitary gland, which plays a key
359 role in regulating ruminant mammary gland development and lactation [29]. It is
360 important for regulating glycometabolism, in part through its promotion of
361 gluconeogenesis in the liver [30]. The body's health is essential to the normal
362 production of hormones. However, the increased translocation of LPS may into the
363 brain via the blood enhances the inflammatory response, which might be ultimately
364 affect on the levels of the growth hormone. PEPCK and G6PC are two key hepatic
365 gluconeogenic enzymes the expression and activity of which are increased hepatic
366 glucose output [31]. PC is the first regulatory enzyme in gluconeogenic pathway that
367 converts pyruvate to oxaloacetate in gluconeogenesis [32]. Major glucose precursors
368 of ruminant liver include propionate, amino acids and lactate. It has been documented
369 that the increased proportion of propionate may be related to glycogenesis in ruminant.
370 Because most VFA emerge in the portal vein after absorption from the digestive tract
371 [33], alterations in proportion of propionate influence the gluconeogenesis in the liver.
372 Therefore, the liver gluconeogenesis plays a crucial physiological role in maintaining
373 the body blood sugar levels, as it is the main organ for glucose storage, in the form of
374 glycogen, as well as endogenous glucose production [34]. Our results indicated that
375 the buffering agent added to the high-concentrate diet significantly decreased the total
376 VFA, propionate and butyrate levels in the ruminal fluid. However, the ratio of
377 propionate to butyrate was increased in the BG group. We also observed that the
378 buffering agent treatment promoted the expression of PEPCK, PC and G6PC,
379 indicating that gluconeogenesis in the liver was increased. In addition, the BG diets

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380 increased glucose content in hepatic vein. The plasma GH and GHR levels were also
381 increased in the BG goats: elevated GH increases the glucose content and activity of
382 gluconeogenesis in the liver. Meanwhile, the buffering agent added to the HC diet
383 inhibited the consumption of glucose and stabilized the liver health of lactating goats.
384 Taken together, these findings suggest that the feeding of BG diets can promote liver
385 gluconeogenesis by the increased proportion of propionate in the rumen, and the
386 increased entry of glucose into the blood through the hepatic vein.

387 In lactating animals, providing glucose for the mammary gland is a metabolic priority
388 because glucose is the primary precursor for lactose synthesis in the mammary gland.
389 Once taken up by the lactating mammary epithelial cell, glucose is either used in the
390 synthesis of lactose or processed by glycolysis to provide energy. Lactose is
391 synthesized from free glucose and uridine diphosphate (UDP)-galactose by lactose
392 synthase catalysis [35]. The mammary gland itself cannot synthesize glucose from
393 other precursors because of the lack of glucose-6-phosphatase [8,36]. Therefore, the
394 mammary gland is dependent on the blood supply for its glucose needs. In addition,
395 lactose maintains the osmolarity of milk, the rate of lactose synthesis serves as a major
396 factor influencing milk yield. It is also indicated that lactose synthesis and milk yield
397 show a linear or positive correlation with glucose uptake in the mammary gland of
398 goats and cows [37,38]. Glucose uptake in the mammary gland is increased
399 dramatically during lactation. As is known, glucose transport across the plasma
400 membranes of mammalian cells is carried out by 2 distinct processes: facilitative
401 transport, mediated by a family of facilitative glucose transporters (GLUT); and

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402 sodium-dependent transport, mediated by the Na+/glucose cotransporters (SGLT) [39].
403 An early study demonstrated that facilitated GLUT 1, GLUT 8, GLUT 12 and SGLT1
404 have different expression in mammary gland [40]. The GLUT1 is ubiquitously
405 expressed in lactating cow tissues, being most abundant in the mammary gland and
406 kidney and lowest in the omental fat and skeletal muscle [41]. SGLT1 play a
407 important role in glucose transport of Golgi membrane [42]. In our experiment, we
408 found that the glucose content in the plasma of the jugular vein was increased in the
409 BG group compared to the HG group. GLUT 1, GLUT 8, GLUT 12 and SGLT1
410 expression in mammary gland were also elevated in the BG goats. Moreover, the level
411 of GLUT 1 protein was significantly enhanced in mammary gland of BG goats. Taken
412 together, it is indicated that the buffering agent added to the high-concentrate diet led
413 to the translocation of more glucose from the peripheral blood into the mammary
414 epithelial cells and thereby increased the milk yield and lactose content.

415 **Conclusions**

416 In summary, we systematically investigated the effects of the buffering agent on milk
417 quality of lactating goats and found milk yield and lactose content were increased.
418 Furthermore, the blood GH and prolactin levels were increased in the BG goats:
419 elevated GH increases the hepatic gluconeogenesis and activity. Activated
420 gluconeogenesis promotes levels of blood glucose released from the liver (Figure 9).
421 Thus, the increased glucose in the hepatic vein during of goats fed a BG diets may be
422 play a key role in increasing the milk yield and lactose synthesis of lactating goats.
423 However, the GLUT1, 8, 12 and SGLT1 in mammary gland were also elevated in BG

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424 goats. It is possible that the buffering agent added to the HC diet inhibited the release
425 of inflammatory cytokine and stabilized the mammary gland health of lactating goats.
426 Then it causes to the increase in glucose transporters in the mammary gland and
427 prolactin levels in the blood, which could also increase the lactose content in milk.
428 Therefore, further research is needed to elucidate the underlying mechanism.

429 Author contributions

430 L.L. performed the experiment and drafted the manuscript. M.H., and Y.L.
431 performed the experiment and analysed the data. Y.Z. contributed to experimental
432 design and manuscript revision. L.L., and Y.Z. conceived the idea, designed the
433 experiment and finalized the manuscript. All authors read and approved the final
434 manuscript.

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438 Compliance with Ethical Standards

439 The study was approved by the ethical committee of Nanjing agricultural university.

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444 Jiangsu Higher Education Institutions (PAPD).

445 **Competing interests**

446 The authors declare that they have no financial, personal or professional interests that
447 would have influenced the content of the paper or interfered with their objective
448 assessment of the manuscript.

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571

572

573 **Table**

574 **Table 1. Composition and nutrient levels of experimental diets**

Concentrate : Forage ratio 6 : 4	
Ingredient (%)	
Leymus chinensis	27.00
Alfalfa silage	13.00
Corn	23.24
Wheat bran	20.77
Soybean meal	13.67
Limestone	1.42
NacL	0.30
Premix[a]	0.60

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Total	100.00
Nutrient levels[b]	
Net energy/(MJ.kg-1)	6.71
Crude protein/%	16.92
Neutral detergent fiber/%	31.45
Acid detergent fiber/%	17.56
Calcium/%	0.89
Phosphorus/%	0.46

575 a. Providedper kg of diet:VA 6000IU/kg, VD 2500IU/kg, VE 80mg/kg, Cu 6.25 mg/kg, Fe 62.5

576 mg/kg, Zn 62.5 mg/kg, Mn 50mg/kg, I 0.125 mg/kg, Co 0.125 mg/kg.

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

578 **Table 2. Primer sequences and product size**

Target genes	Primer sequences (5'-3')	Products/bp
G6PC	CCCACAGCTTCAACAAACTCTT	230
	GATGTCCATGCCATTCTCCTT	
PEPCK	CCCTACTCTCCCGGGATGGAAAGT	306
	GCCCTCCGAAGATGATGCCCTCAA	
PC	CCCACAGCTTCAACAAACTCTT	352
	GATGTCCATGCCATTCTCCTT	
GLUT1	AGACACCTGAGGAGCTGTTC	233
	GACATCACTGCTGGCTGAAG	
GLUT8	TGGCATCTACAAGCCCTTCA	244
	ACCATGACCACACCTGACAA	

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GLUT12	ACGTGACCATGGTACCTGTT	321
	TCCCAAGTTCATACCCCACC	
SGLT1	GCAAGAGAGTCAATGAGCCG	235
	ATGGCCAGGATGACGATGAT	
GHR	TTGGAATACTTGGGCTAACA	262
	GACCCTTCAGTCTTCTCATCG	
GAPDH	GGGTCATCATCTCTGCACCT	177
	GGTCATAAGTCCCTCCACGA	

579 Abbreviations: PC, pyruvate carboxylase; PEPCK, phosphoenolpyruvate carboxykinase; G6PC,
580 glucose-6-phosphatase; GLUT1, glucose transporter type 1; GLUT8, glucose transporter type 8;
581 GLUT12, glucose transporter type 12; SGLT1, sodium-glucose cotransporter 1; GHR, growth
582 hormone receptor.

583

584 **Table 3. Effects of the buffering agent treatment on rumen fermentation**
585 **parameters in goats**

Item	BG	HG	P-alue
LPS, EU/mL	25201 ± 3398	43395 ± 4723	0.002**
Total VFA, mM	90.20 ± 3.55	116.37 ± 8.14	0.04*
Acetate, mM	58.28 ± 2.45	65.48 ± 5.45	0.39
Propionate, mM	17.01 ± 0.25	22.45 ± 1.51	0.03*
Butyrate, mM	12.65 ± 1.77	18.36 ± 1.79	0.02*
Acetate: Propionate	3.41 ± 0.58	2.9 ± 0.21	0.11

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Propionate: Butyrate	1.34 ± 0.05	1.22 ± 0.12	0.03*

586 Abbreviations: HG, high-concentrate diet group; BG, buffering agent group; LPS,
587 lipopolysaccharide; VFA, volatile fatty acid. Values are shown as means ± SEM, n = 8/group. **P*
588 < 0.05, ***P* < 0.01 compared with the HG group.

589 **Table 4. Effects of the buffering agent treatment on plasma enzyme, primary**
590 **pro-inflammatory cytokines, metabolic produced and hormone of lactating goats**

Item	BG	HG	P-alue
Plasma biochemical parameter			
ALT (IU/L)	40.33 ± 2.84	77.67 ± 10.44	0.03*
AST (IU/L)	43.33 ± 4.48	71.33 ± 8.67	0.04*
LDH (IU/L)	233.66 ± 16.45	243.66 ± 13.54	0.66
AKP (IU/L)	109.67 ± 17.07	213.5 ± 20.50	0.02*
TNF-a (ng/mL)	2.47 ± 0.30	4.61 ± 0.48*	0.03*
IL-1β (ng/mL)	0.74 ± 0.03	0.79 ± 0.05	0.04*
LPS (EU/mL)	1.82 ± 0.14	3.76 ± 1.13	0.04*
Histamine (ng/mL)	1.99 ± 0.06	2.11 ± 0.09	0.09
Lactate (mmol/L)	0.95 ± 0.05	1.39 ± 0.16*	0.04*
Hormone levels			
Prolactin (pg/mL)	436.57 ± 37.78	373.29 ± 30.59	0.04*
Glucocorticoids (ng/mL)	10.2 ± 1.67	9.8± 2.56	0.08
Growth hormone (ng/mL)	0.94 ± 0.08	0.67 ± 0.03	0.03*

591 Abbreviations: BG, buffering agent group; HG, high-concentrate diet group; ALT, alanine
592 aminotransferase; AST, aspartate transaminase; LDH, lactic dehydrogenase; AKP, alkaline

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593 phosphatase; TNF-a, tumor necrosis factor-a; IL-1β, interleukin 1β; LPS, lipopolysaccharide;

594 Values are shown as means ± SEM, n = 8/group. *$P < 0.05$ compared with the HG group.

595

596 **Table 5. The average concentrations of glucose in plasma of hepatic vein, portal**

597 **vein and jugular vein of lactating goats**

Glucose (mmol/L)	BG	HG	Effect, p-value		
			Diet	Time	Diet × Time
Hepatic vein					
0h	3.34 ± 0.37*	3.01 ± 0.18	0.003	0.292	0.636
4h	3.35 ± 0.37*	3.15 ± 0.18			
8h	3.44 ± 0.37*	3.07 ± 0.18			
Portal vein					
0h	3.27 ± 0.11	3.26 ± 0.13	0.102	0.902	0.494
4h	3.28 ± 0.12	3.27 ± 0.12			
8h	3.27 ± 0.09	3.25 ± 0.15			
Jugular vein					
0h	3.30 ± 0.05	3.27 ± 0.09	0.002	0.890	0.579
4h	3.33 ± 0.24	3.29 ± 0.12			
8h	3.34 ± 0.14	3.25 ± 0.04			

598 Abbreviations: HG, high-concentrate diet group; BG, buffering agent group. Values are shown as

599 means ± SEM, n = 8/group. *$P < 0.05$ compared with the HG group.

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601 **Figure 1. Comparison of the average weekly lactose content and milk yield**
602 **between the buffering agent (BG) and high-concentrate diet (HG) groups.** Values
603 are shown as means ± SEM, n = 8/group. **P* < 0.05 compared with the HG group.
604 **Figure 3. pH value in ruminal fluid after 19 weeks feeding regime.** Data were
605 analyzed for differences due to diet, time, and their interactions by Univariate using the General
606 Linear Models of SPSS 11.0 for Windows (StatSoft Inc, Tulsa, OK, USA). Values are mean ±
607 SEM, n = 8/group. **P* < 0.05 compared with the HG group.
608 **Figure 3. Effects of buffering agent treatment on the expression of mammary**
609 **gland glucose transfer genes in lactating goats.** The experiments were repeated three
610 times. Values are shown as means ± SEM, n = 3. **P* < 0.05 compared with the HG group.

611 **Figure 4. Effects of buffering agent treatment on the expression of liver**
612 **gluconeogenesis genes in lactating goats**. The experiments were repeated three times.
613 Values are shown as means ± SEM, n = 3. **P* < 0.05, compared with the HG group.

614 **Figure 5. Effects of buffering agent treatment on the expression of GHR in livers**
615 **of lactating goats.** The experiments were repeated three times. Values are shown as means ±
616 SEM, n = 3. **P* < 0.05 compared with the HG group.

617 **Figure 6. Growth hormone activates the gluconeogenesis to regulate glucose**
618 **synthesis in the liver.** Abbreviations: PEPCK, phosphoenolpyruvate Carboxykinase; PC,
619 pyruvate carboxylase; G6PC, glucose-6-phosphatase.

A

Lactose content(%)

4.0
3.5
3.0
2.5
2.0

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19

(week)

BG
HG

B

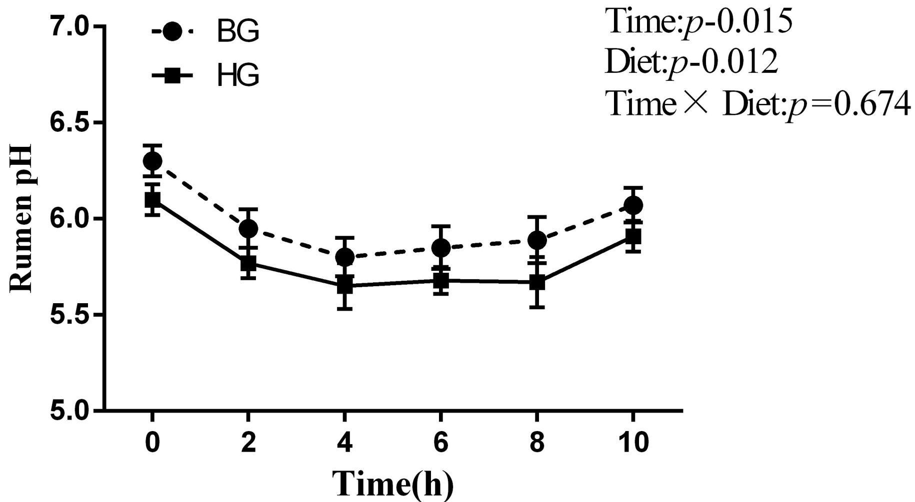
Milk yield (kg)

1.4
1.3
1.2
1.1
1.0

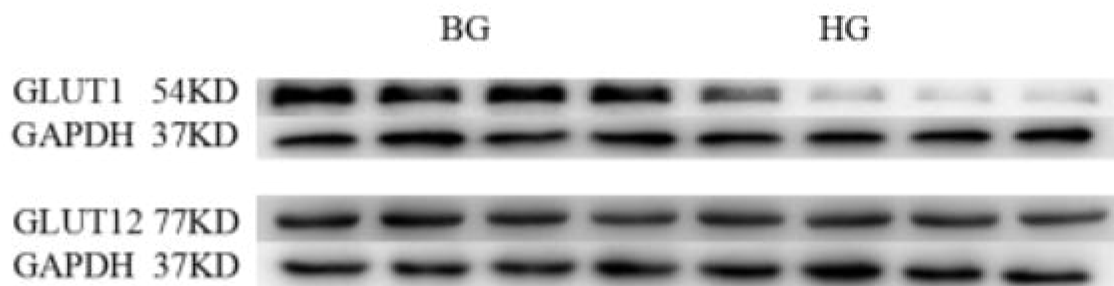
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19

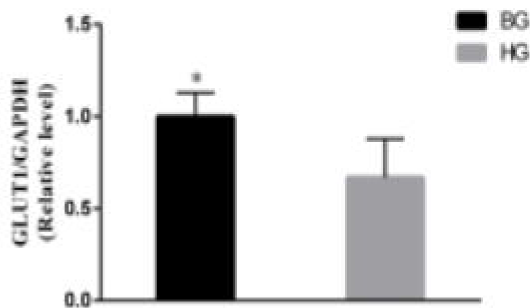
(week)

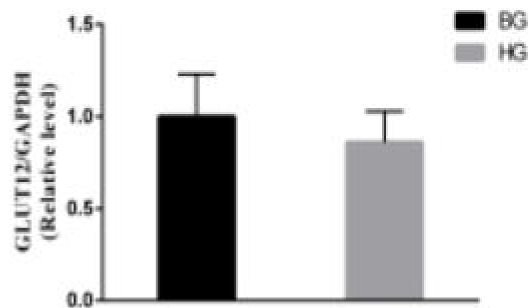
BG
HG


BG
HG
Time:p-0.015
Diet:p-0.012
Time × Diet:p=0.674
Rumen pH
7.0
6.5
6.0
5.5
5.0
0
2
4
6
8
10
Time(h)

Fold change of genes expression
2.0
1.5
1.0
0.5
0.0
GLUT1
GLUT8
GLUT12
SGLT1
BG
HG







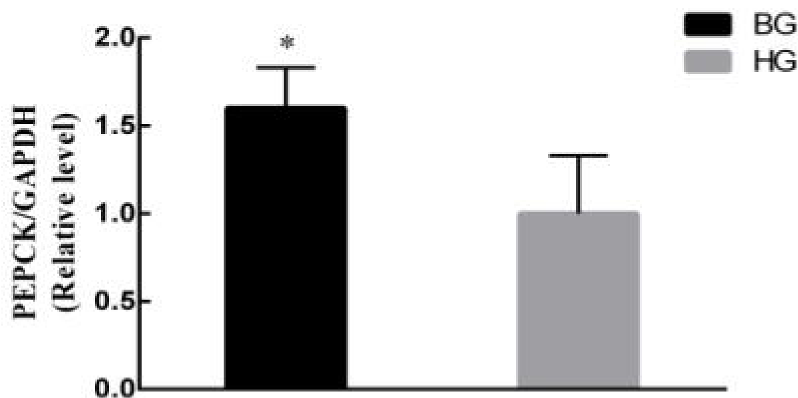
Fold change of genes expression

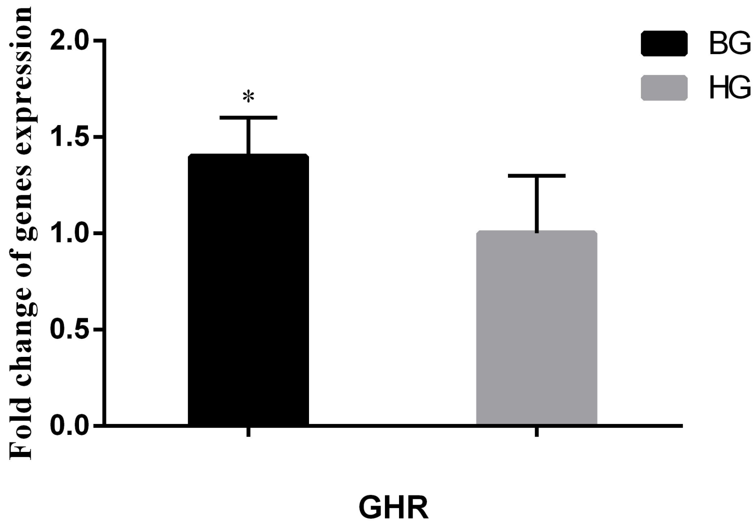
2.0
1.5
1.0
0.5
0.0

*
*

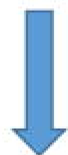
BG
HG

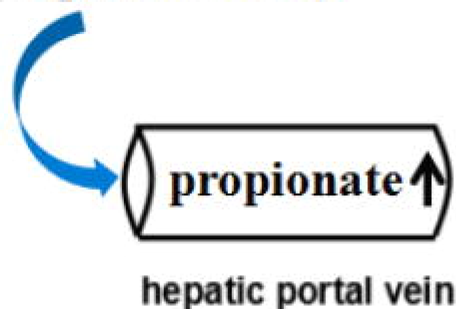
PEPCK PC G6PC





Fold change of genes expression
2.0
1.5
1.0
0.5
0.0
*
BG
HG
GHR





0.2% NaHCO3,
0.1% MgO
The proportion of propionate (Digestive tract) ↑
propionate ↑
hepatic portal vein
Growth hormone
GHR
Liver
Gluconeogenesis ↑
promote
The mRNA expression levels of PEPCK, PC, G6PC
Peripheral Blood
Glucose ↑
hepatic vein
Milk yield
Lactose