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RESEARCH ARTICLE

Effect of Sodium Butyrate in Combating the Negative Effects of Sub-Acute Ruminal Acidosis Induced Lipopolysaccharides in the Uteri of Lactating Goats

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ABSTRACT

Sub-acute ruminal acidosis (SARA) is known to enhance the systemic inflammatory response that most likely occurs due to the translocation of lipopolysaccharide (LPS) from the digestive tract into the blood stream. The objective of the present study was to explore whether sodium butyrate could minimize the negative effects of high-grain diet-induced inflammation in the goat uterus and to explore the differences between high-concentrate diet alone and high-concentrate diet supplemented with sodium butyrate. Eighteen mid-lactating goats surgically installed with a rumen fistula were divided into two groups: a group receiving a high-concentrate diet (HC) (60% concentrate + 40% forage) as a control and a group receiving a high-concentrate diet with sodium butyrate (HC+Na B) (60% concentrate+40% forage+sodium butyrate) as the treatment group. The LPS concentrations in the rumen fluid were 234653 EU/ml and 431564 EU/ml in the HC+Na B and HC groups, respectively, whereas in peripheral blood, LPS concentrations were 0.013 EU/ml and 0.039 EU/ml in the HC+Na B and HC groups, respectively, indicating a significant decrease in LPS in the HC+Na B group. The concentrations of the pro-inflammatory cytokines IL-1β and IL-6 were significantly lower, with concomitant down-regulation of the expression of the inflammatory genes TLR4, MyD-88, NF-κB, TRAF-6, IL-6 and IL-1β in the uteri of HC +Na B group. Therefore, supplementing sodium butyrate with a high-concentrate diet reduced the degree of uterine inflammation and played a vital role in combating the inflammatory effects of LPS migration from the digestive tract.

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INTRODUCTION

A high-grain diet is commonly fed to dairy animals to increase milk production. Highly fermentable forages result in the production and accumulation of high levels of acidic substances in the rumen, which result in the poor development of rumen papilla due to slower absorption of short chain fatty acids; consequently, the buffering of acidic materials is reduced (Jing *et al*., 2014). Gozho *et al*. (2007) proposed that SARA induced by the consumption of high-grain diet causes the lysis of gram-negative bacteria, resulting in the release of large amounts of lipopolysaccharide (LPS) in the rumen. Intestinal epithelial cells play a key role in normalizing inflammatory conditions and producing a barrier against invading pathogens in the intestine (Furrie *et al*., 2005), but LPS impairs ruminal epithelial barrier function. Furthermore, LPS might be translocated into the circulatory system of the body and consequently promote the production of pro-inflammatory cytokines (Plaizier *et al*., 2012).

Endometrial infection, which is generally due to gram-negative microorganisms, is a cause of uterine disease (Cronin *et al*., 2012), that results in impaired endometrial tissues (Sheldon *et al*., 2009). The endometrial epithelia exclusively participate in clearing the microorganisms by recognizing pathogen-associated molecular patterns (PAMPs) via pattern recognition receptors (PRR). Toll-like receptor4 (TLR4) is responsible for activating the innate immune response in

the body and identifies LPS most effectively with the help of LPS-binding protein (LBP) and cluster of differentiation 14 (CD14) (Sohn *et al*., 2008). After LPS interacts with TLR4 on the cell surface of the host, it activates myeloid differentiating factor 88 (MyD88) (Ju *et al*., 2014). MyD88 triggers tumour necrosis factor (TNF) receptor-associated factor 6 (TRAF6), which induces the IκB kinase (IκK) complex. As a result, the nuclear transcription factor NF-κB is translocated from the cytoplasm to the nucleus (Turner *et al*., 2014), which triggers the dependent cascade of pro-inflammatory cytokines, comprising TNF-α, IL-1β, IL-6, and other inflammatory mediators (Cronin *et al*., 2012).

It is well documented that animals fed a buffered diet have a higher ruminal pH. Sodium butyrate (Na B) is a type of short chain fatty acid that has been shown to stimulate the growth of the rumen epithelium when given orally (Mentschel *et al*., 2001). Moreover, dietary sodium butyrate decreases gastrointestinal tract (GIT) permeability, facilitates substantial alterations in the intestinal microbe population (Huang *et al*., 2015) and reduces colonic inflammation (Wirtz and Neurath, 2007). Basically, sodium butyrate acts as an anti-inflammatory and anti-oxidative agent (Ferreira *et al*., 2012) that can also alter the activation of pro-inflammatory genes such as IL-1β, TNF-α and IL-6 (Bailon *et al*., 2010) and inhibit the activation of NF-κB (Segain *et al*., 2000). Hence, sodium butyrate acts as an anti-inflammatory agent to combat SARA. Therefore, the present study focused on whether the addition of sodium butyrate to a high-concentrate diet can decrease SARA-induced inflammation in the uteri of lactating goats by modulating the inflammatory TLR-4 pathways.

MATERIALS AND METHODS

Ethical approval: The experimental design and sampling procedures were approved by the Animal Ethics Committee, Nanjing Agricultural University, Nanjing, China, before the beginning of the experiment.

Animals, diets plan and experimental design: Eighteen mid-lactating goats (38.86±1.47 kg) with rumen fistulae were divided into two groups. A high-concentrate powdered diet (HC) (60% concentrate+40% forage) was given to one group (n=9), as the control group. The other group (n=9) was given a high-concentrate, uniformly mixed, palletized diet with sodium butyrate (HC+Na B) (60% concentrate+40% forage+sodium butyrate) as the treatment group. The diets were offered to their respective groups for 24 weeks. The feed was offered twice daily at 08:00 am and 06:00 pm. The details of the ingredients of diets as well as their nutritive compositions are shown in Tables 1 and 2.

Sample collection and analysis: The rumen fluid was collected in a sterile glass tube on the 23rd week. The ruminal fluid pH was measured by using a pH metre (Sartorius, Basic pH metre PB-10, PB-21, and Goettingen, Germany) at 2 hours-interval up to 10 hours after feeding. At 72 hours prior to slaughtering, blood samples from all the goats were collected from the jugular vein using 5-ml sterile vacuum tubes containing heparin sulphate. At the end of the experiment, the goats were slaughtered after overnight fasting, and uterine tissues from all the goats were taken immediately frozen in liquid nitrogen and then stored at -70°C.

Table 1: Ingredients and nutritional composition of the diets

Items	Ingredients	Quantity
Concentrate	Corn (g/100g)	23.1
	Bran (g/100g)	28.0
	Soybean meal (g/100g)	2.00
	Rape seed meal (g/100g)	3.70
	Limes stone meal (g/100g)	1.43
	Calcium hydrophosphate (g/100g)	0.60
	Premix* (g/100g)	0.50
	Salt (g/100g)	0.40
Coarse material		
	Oat grass (g/100g)	32.0
	Alfalfa (g/100g)	8.00
Nutritional index		
	Net energy (MJ/kg)	17.6
	Fat (%)	3.60
	Crude protein (%)	16.8
	Neutral detergent fiber (%)	42.6
	Acid detergent fiber (%)	7.22
	Calcium (%)	1.55
	Phosphorus (%)	0.76

*Premix analysis/kg diet: Vitamin A: 6.00×10^3 U; Vitamin D: 2.5×10^3 U. Vitamin E, Cu, Fe, Zn, Mn, I, Co and Mo: 80.0, 6.25, 62.5, 62.5, 50.0, 0.125, 0.125 and 0.125mg, respectively.

Detection of lipopolysaccharide (LPS) in the plasma and ruminal fluid: The concentrations of LPS in the plasma and rumen fluid were measured by using Chromogenic Endpoint Limulus Amebocyte Lysate assay kits (CE64406 & CE80545, Chinese Horseshoe Crab Reagent Manufactory Co., Ltd., Xiamen, China), respectively, as reported by Dong *et al*. (2013).

Radioimmunoassay: Radioimmunoassay (RIA) was used to determine the concentration of the pro-inflammatory cytokines IL-1β and IL-6 in peripheral plasma with radioimmunoassay kits (IL-1β, C09DJB; and IL-6, C12DJB), respectively (Beijing North Institute of Biological Technology, Beijing, China).

RNA extraction and cDNA synthesis: Total RNA from 100 mg of uterine tissue was extracted using RNA iso Plus™ reagent (Takara Co., Otsu, Japan). The concentration of RNA in each sample was determined by using a spectrophotometer (Eppendorf Biotechnology, Hamburg, Germany) at 260 nm. First-strand cDNA was prepared by using a pre-quantified total RNA template (250 ng/µl) and a Prime Script RT Master Mix Perfect Real-Time kit (Takara Co., Otsu, Japan).

Table 2: Formulation of diets for the high concentrate group (HC) and the high concentrate with sodium butyrate group (HC +Na B)

Animals	Concentrate powered diet(gm)	Concentrate pelletized diet(gm)	Roughage (gm)		Buffer agent (gm)
			Alfalfa	Oat grass	Sodium butyrate
(HC) group	600	-	133	267	-
(HC+ Na B) group	-	600	133	267	10

Quantitative real-time PCR (qRT-PCR): Primers for the genes TLR4, NF-κB, MyD88, IL-1β, IL-6 and β-actin were designed using Premier 6.0 (Premier Biosoft International, USA) (Table 3). The reaction conditions consisted of 0.4 µM primers and 2 µl of cDNA in a total volume of 20 µl of super mix. The thermal cycling parameters were as follows: initial denaturation at 95°C for 15 s followed by 40 cycles of annealing at 95°C for 5 seconds and primer extension at 60°C for 31 s. All the reactions were performed in triplicate. Each cDNA sample was amplified using SYBR Green (Takara Co., Otsu, Japan) on an ABI 7300 Fast Real-Time PCR System (Applied Biosystems, USA). The data were normalized to the mean of the internal reference β-actin gene to control for randomness in the expression levels and were calculated using the equation $R= 2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001).

Statistical analysis: The data from the present study were analysed by applying independent *t*-tests to calculate the mean±SEM differences between the groups as integrated using the statistical software SPSS version 16.0 (SPSS Inc., Chicago, IL, USA). P<0.05 was recognized statistically significant.

RESULTS

Rumen pH and LPS content of rumen fluid and blood plasma: The pH values of the rumen fluid are presented in Fig. 1. Long-term consumption of a high-grain diet linearly reduced the active pH curve in the HC group compared to the HC+Na B group during the experiment. The results demonstrated that a pH value <5.8 was sustained in the HC group for more than 3 hours at different times throughout the experiment, indicating that SARA was induced effectively. The dynamic pH curve revealed that the pH in the HC+Na B group was significantly higher compared with HC group throughout the duration of this study (P<0.05). This result indicated that a high-concentrate diet with sodium butyrate (buffered diet) improved the rumen pH and alleviated acidosis.

The concentration of LPS in the rumen fluid of the HC group was 431564 EU/ml, while the LPS concentration in the HC+Na B group was 234653 EU/ml, indicating a significant decrease in the LPS levels in the HC+Na B group (P<0.02) versus HC group. Similarly, the LPS level in the peripheral plasma of the HC+Na B group 0.013 EU/ml, (P<0.002) was significantly decreased than that in the HC group 0.039 EU/ml (Table 4).

Levels of pro-inflammatory cytokines in the blood: The concentrations of the pro-inflammatory cytokines IL-1β and IL-6 were measured by radioimmunoassay; the results are represented in Fig. 2. The concentrations of IL-1β were 0.1700 ng/ml and 0.2167 ng/ml in HC+Na B group and HC group, respectively (P<0.02) (Fig. 2A). The levels of IL-6 detected in the peripheral blood of goats were 401.5 pg/ml in the HC+Na B group and 607.3 pg/ml in the HC group (P<0.02) (Fig. 2B). The concentration of pro-inflammatory cytokines in the peripheral blood was significantly lower in the HC +Na B group compared to that in the HC group (P<0.05).

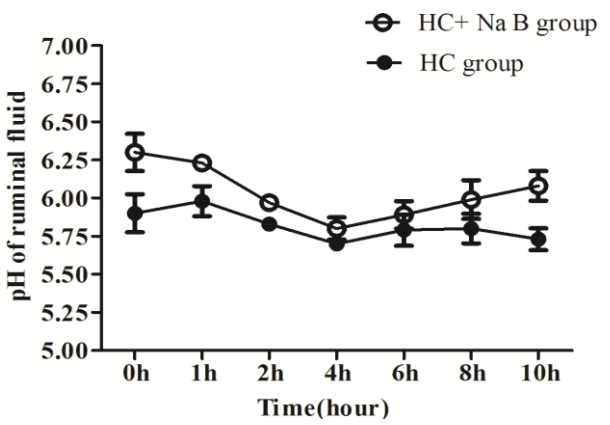

Fig. 1 Comparison of the pH values in the ruminal fluid between the high-concentrate (HC) control group and the high-concentrate diet with sodium butyrate treatment (HC+Na B) group. The data were collected during the 23rd week using ruminal fluid samples collected between 0 to 10 hours (as shown on the x-axis). Student's *t*-test was used to evaluate the significance of the treatment; significant variations were found across all the sampling times (P<0.05).

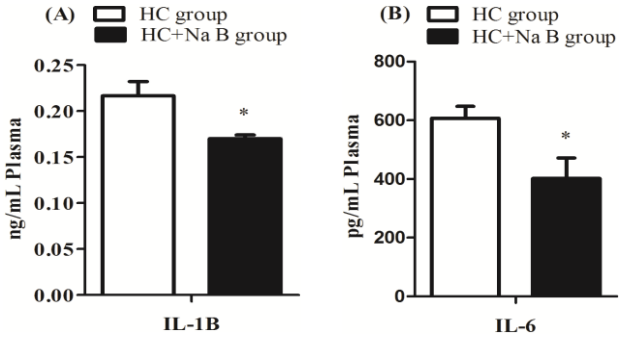

Fig. 2 (A) IL-1β and (B) IL-6 levels in peripheral plasma. The data are expressed as the mean ± SEM; asterisks show differences between the high-concentrate (HC) control group and the high-concentrate diet with sodium butyrate treatment (HC+Na B) group (*P<0.05, n=18).

Table 3: Primer sequences for real-time polymerase chain reaction (qRT-PCR) amplification of mRNA

Target gene	Gen Bank accession no.	primer sequence (5′–3′)	
TLR-4	NM_ 001285574.1	Forward:	CTGAGAACCGAGAGCTGGGAC
		Reverse:	TTCGCATCTGGATAAATCCAGC
MyD88	XM_ 005695671.1	Forward:	TGGACACATACAAGCCCACT
		Reverse:	AGTGTCCCATCTTGTCCAGG
TRAF-6	XM_ 005690091.1	Forward:	TTTCAGAGACCCACCATCCC
		Reverse:	GAAAACCCTCCCTCCGAAGA
NF-KB	XM_ 00569893.1	Forward:	AGGTGGCGATCGTTGTTCTA
		Reverse:	TTGCCTTTGTTCTTCCTGCC
IL-6	JF432019.1	Forward:	CGAAGCTCTCATTAAGCACATC
		Reverse:	CCAGGTATATCTGATACTCCAG
β-actin	JQ409458.1	Forward:	CTTGATGTCACGGACGATTT
		Reverse:	CACGGCATTGTCACCAACT
IL-1β	XM_ 005686747.1	Forward:	CCGTGATGATGACCTGAGGAG
		Reverse:	CAAGACAGGTATAGATTCTTGC

Table 4: Lipopolysaccharide concentration in the rumen and plasma of HC group and HC+Na B group

LPS Concentration (EU/mL)	HC group		HC +Na B group		P value
	Mean	SEM	Mean	SEM	
Ruminal LPS	431564	24305.6	234653	37316.2	0.02
Plasma LPS	0.039	0.003	0.013	0.003	0.002

SEM=Standard error of mean between the two groups. The LPS data were compared using Student's *t*-test. LPS=Lipopolysaccharide; HC=High concentrate; HC+Na B=High concentrate with sodium butyrate.

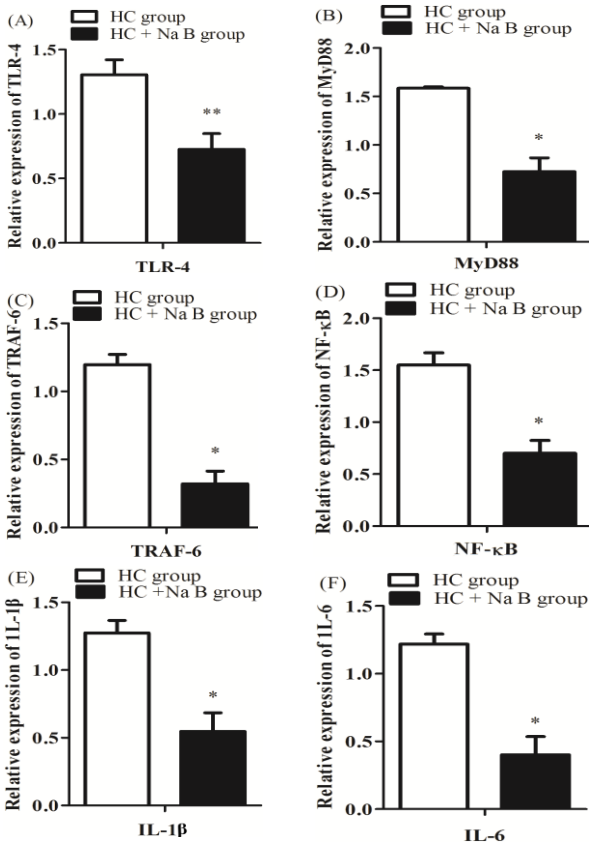

Fig. 3 qRT-PCR analysis of the expression profile of uterine genes. The effect of sodium butyrate treatment on the mRNA levels of (A) TLR-4, (B) MyD-88, (C) TRAF-6, (D) NF-κB, (E) IL-1β and (F) IL-6. Genes involved in transcriptional regulation were determined in the uterine tissues of dairy goats. The error bars demonstrate the standard error of the mean, and the asterisks represent significance at *P<0.01 and **P<0.05.

The gene expression levels (mRNA) in uterine tissues: Real-time PCR was implemented to assess the gene expression levels of the cytokines responsible for inflammation (e.g., TLR-4, MyD88, NF-κB, TRAF-6, IL-1β, and IL-6) in the uterine tissues of goats from both groups. The results revealed that the mRNA expression levels of TLR4 (P<0.008), MyD88 (P<0.02), TRAF-6 (P<0.01), NF-κB (P<0.02), IL-1β (P<0.04), and IL-6 (P<0.03) in the uterine tissues of lactating goats were significantly decreased in the HC +Na B group compared to those of the lactating goats in the HC group (Fig. 3 A-F).

DISCUSSION

In the current study, we revealed the effect of sodium butyrate on combating the negative effects of LPS-induced SARA in the uteri of lactating goats. It has been documented that the consumption of high-concentrate diets resulted in digestive disturbance, particularly SARA (Khafipour *et al*., 2009), which is characterized by decreased feed intake, low milk fat and inflammation (Xu *et al*., 2015). The key indicator of SARA in many animals is the low pH value of the rumen fluids (Xu *et al*., 2015). The present study showed that the dynamic pH curve of the rumen fluid in the HC+Na B group was higher compared to that in the HC group, illustrating that SARA did not develop in the goats from the HC+Na B group, whereas SARA was induced in the HC group. These findings are in accordance with the results of Ghorbani *et al*. (2002), who suggested that an average pH value below 5.8 in the HC group for more than three hours confirmed the presence of SARA. In contrast to our results, Larrman *et al*. (2013) found no difference in the ruminal pH profile in the control and butyrate treatment groups and demonstrated ruminal acidosis in both groups (Laarman *et al*., 2013). Low pH values in the rumen resulted in the lysis of gram-negative bacteria, at which point LPS is free to enter the circulation (Andersen, 2000). Due to epithelial impairment and modifications in GIT permeability, LPS may translocate into the blood circulation, and this increase in LPS produces an immune response, which is in agreement with earlier studies (Dong *et al*., 2011). This observed immune response is why many studies on dairy animals recommend a suitably coarse fibre-buffered diet for good rumination and increased saliva secretions to neutralize ruminal acidosis (Calitz, 2009). Clinical data from previous studies showed that the addition of buffering agents helped in reducing the onset of SARA by maintaining optimal pH levels in the rumen (Dörffel *et al*., 1999).

The anti-inflammatory properties of sodium butyrate in different cell lines were studied under conditions of ulcerative colitis and in macrophages during atherogenesis in previous studies (Ferreira *et al*., 2012). Moreover, favouring our results, earlier clinical evidence indicated that the supplementation of buffering agent such as sodium butyrate is important in increasing the ruminal pH of affected animals (Erdman, 1988). In accordance with a previous report (Liang *et al*., 2013), our results also demonstrate lower concentrations of the pro-inflammatory cytokines IL-6 and IL-1β in the blood sera of the dairy goats in the HC+Na B group compared with the HC group. This result may be due to the anti-inflammatory properties of sodium butyrate. We found low expression of immune-related genes in the HC+Na B group relative to the HC group. Data published by Segain *et al*. (2000) also supported our results illustrating that butyrate decreased pro-inflammatory cytokine expression through inhibition of NF-κ B activation. These anti-inflammatory properties described the immunomodulatory effects of butyrate (Segain *et al*., 2000). Moreover, Hu *et al*. (2014) observed significant down-regulation of TNF-α and IL-6 expression in rats treated with sodium butyrate (Hu *et al*., 2014). Furthermore, previous reports have shown that sodium butyrate can decrease IL-6, IL-1β and TNF-α release by reducing NF-κB activation (Murray, 2005). Therefore, the down-regulation of cytokine mRNA expression in the HC+Na B group confirmed the effect of a sodium butyrate buffer on NF-κB (Baldwin, 1996). Another study also suggested that butyrate molecules in bovine milk fat possess unique anti-inflammatory and antimicrobial characteristics and the results revealed that sodium butyrate could efficiently modulate the innate immune response against microbial infection in the mammary gland (Ochoa-Zarzosa *et al*., 2009).

In short, LPS increased the concentrations of IL-1β and IL-6 in the HC group while sodium butyrate significantly inhibited the concentrations of IL-1β and IL-6 in the goats in the HC+Na B group.

Conclusions: The supplementation of sodium butyrate in a high-concentrate diet increased the ruminal pH and decreased the uterine accumulation of LPS, which translocated from the digestive tract into the peripheral

blood. The present research specifies innovative evidence for the critical role of the anti-inflammatory properties of sodium butyrate in the uterine tissues of lactating goats during SARA. Moreover, we have further demonstrated for the first time that sodium butyrate buffer inhibits inflammatory responses by inhibiting NF-κB activation in the uterine tissues of lactating goats during SARA.

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Authors contribution: XS and MSB generated plan and designed the research. MSB, ZA, XT, JAA, HD, JY, XL, MM, UW, and XL conducted the majority of measurements of parameters in this work and drafted the manuscript. MSB, TX, and HD contributed to the statistical analyses. All authors read and approved the final manuscript.

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