Full Length Article

Effects of Combined or Along VFA, pH, Lipopolysaccharide and Histamine on the Rumen Epithelial Permeability of Dairy Goats *In Vitro*

YY Sun[1,2], M Gao[1], LW Song[1], M Xu[2], C Li[1,2], Y Li[1,2], LQ Chen[1,2], HL Hu[1*] and LS Jiang[3]

[1]*Institute of Animal Nutrition and Feed, Inner Mongolia Academy of Agricultural & Animal Husbandry Sciences, Hohhot, Inner Mongolia, 010031, P. R. China*

[2]*College of Animal Science, Inner Mongolia Agricultural University, Hohhot, Inner Mongolia, 010018, P. R. China*

[3]*Beijing Key Laboratory of Cow Nutrition, College of Animal Science and Technology, Beijing University of Agriculture, Beijing, 102206, P. R. China*

[*]For correspondence: honglianhu2010@163.com

Abstract

This study investigated whether concurrent presence of lipopolysaccharide (LPS) and histamine (HIS) have the potential to increase permeability of the ruminal epithelium at physiological pH and acidotic ruminal pH. Nine 2.5-year-old female lactating Saanen dairy goats (42.79 ± 5.61 kg of BW; Mean $\pm$ SD) were used as a ruminant model. ruminal epithelium of goats were collected and mounted in Ussing chambers on their mucosal side in different gradient buffer solutions (pH 7.4, 5.5 and 5.2) containing LPS (0, 30 and 60 KEU·mL^{-1}) or HIS (0, 0.5 and 10 ng·mL^{-1}). The rumen epithelial electrophysiological indexes, such as short-circuit (I_{sc}), tissue conductance (G_t) and the permeability of marker molecules of different sizes (horseradish peroxidase, HRP and fluorescein 5(6)-isothiocyanate, FITC) from the mucosal to the serosal side, were measured. Both I_{sc} and G_t were increased, accompanied by enhanced flux of FITC, with a decrease of mucosal pH ($P < 0.05$). The addition of LPS at mucosal pH 5.2 significantly increased I_{sc}, G_t and FITC flux rates and decreased potential difference (PD) ($P < 0.05$). Additionally, the concurrent presence of LPS and HIS at both physiological and acidotic ruminal pH also significantly increased the permeability of ruminal epithelium as evidenced by increasing I_{sc}, G_t and FITC flux rates and decreasing PD. In summary, our results have shown that concurrent presence of LPS 60 KEU·mL^{-1} and HIS 10 ng·mL^{-1} at mucosal pH 5.5 can increase the permeability of ruminal epithelium. The combination of low pH and both high LPS and HIS may increase vulnerability to aggravated rumen epithelial barrier dysfunction. © 2021 Friends Science Publishers

Introduction

Subacute ruminal acidosis (SARA) is a common nutritional metabolic disease involved in ruminant production. It has a great impact on the long-term health and production efficiency of animals (Danscher *et al.* 2015). In recent years, in order to improve the production efficiency of ruminants and the quality of animal products, researchers have conducted extensive research on the adverse effects of SARA on intensive ruminant production systems. Reports indicate that ruminants fed rapidly fermentable carbohydrates for a long time will develop an excessive accumulation of organic acids in the rumen, and a dramatic decline of rumen pH, further producing a variety of abnormal metabolites such as HIS and LPS (Liu *et al.* 2013). These toxic and harmful substances can be absorbed into the blood, which in turn causes a systemic inflammatory response (Sun 2017) and ultimately induces SARA with

loss of appetite, laminitis and diarrhea (Plaizier *et al.* 2012). Evidence suggests that rumen LPS is produced by Gram-negative bacteria (Khafipour *et al.* 2009; Wang *et al.* 2015). When ruminants suffer from SARA, Gram-negative bacteria in the rumen rupture and cell lysis releases a large amount of LPS, which can compromise rumen epithelial barrier function (Liu *et al.* 2013; Sato 2016). The free LPS are then translocated from the rumen into the blood across the rumen epithelial barrier, increasing the concentration of blood LPS, further activating the inflammatory and acute phase responses (Dong *et al.* 2011). Therefore, the accumulation and translocation of LPS might cause disruption of epithelial barrier integrity in the gastrointestinal tract (Tao *et al.* 2014), which results in an increase in the permeability of LPS. Many researchers accept that the increase of ruminal LPS is often accompanied by the grain-induced SARA challenge (Gozho *et al.* 2007). HIS is an important bioactive substance and

also an important mediator of the inflammatory response and immune challenge (Khafipour *et al.* 2009). Aschenbach *et al.* (1998) were the first to show that application of HIS in relevant dosages (10 and 100 μm) impaired differentiation of rumen epithelial barrier integrity and function. A recent *in vitro* study indicated HIS could activate the inflammatory pathway of cultured rumen epithelial cells *via* NF-κB (Sun 2017), which has consequences for rumen epithelial integrity and function (Aschenbach *et al.* 2019). Taken together, SARA is known to be characterized by an increased VFA concentration, low pH, hyperosmolarity and elevated LPS and HIS concentrations in the rumen, and these variables have some detrimental effects on the ability of the rumen epithelium to facilitate the translocation of toxic compounds such LPS and HIS (Penner *et al.* 2011).

Several studies conducted in cow and goat reported that SARA increased ruminal epithelial permeability and compromised rumen epithelial barrier function (Sun *et al.* 2018b). Previous studies have investigated the effects of low pH (Gaebel *et al.* 1989; Penner *et al.* 2011), hyperosmolarity (Lodemann and Martens 2006), or an exposure to toxins (Emmanuel *et al.* 2007) on ruminal epithelial barrier function *in vitro*. Greco *et al.* (2018) and Meissner *et al.* (2017) used chambers to demonstrate that a low pH in combination with SCFA induces damage to the rumen epithelial barrier function. While any one or a combination of these factors may affect epithelial barrier function, the extent to which LPS and HIS contribute to disruption of rumen epithelial permeability at low ruminal pH has not been systematically investigated. Therefore, the present study was designed to elucidate the effects of LPS and HIS on the permeability of the ruminal epithelium at physiological and acidotic luminal pH values, with a special focus on determining whether the co-presence of LPS and HIS can aggravate the damage of the rumen epithelial barrier elicited by low pH.

Materials and Methods

The animal experiment protocols were approved by the Animal Care and Use Committee of The Inner Mongolia Academy of Agricultural & Animal Husbandry Sciences and were in accordance with relevant guidelines formulated by the Ministry of Agriculture of the People's Republic of China.

Animals, experimental design and treatments

Nine 2.5-year-old female lactating Saanen dairy goats (42.79 ± 5.61 kg of BW; Mean ± SD) were placed in individual stalls with free access to water. Goats were fed a diet containing a non-fiber carbohydrate to neutral detergent fiber ratio (NFC/NDF) of 1.40 (NRC 2007). The nutrient compositions of the diets are presented in Table 1. The diet (800 g dry matter per animal per day) was provided in equal amounts at 0830 h and 1830 h daily for 30 days.

Rumen tissue sampling

The dairy goats were killed by exsanguination, and ruminal tissue from the ventral sac was harvested for subsequent Ussing chamber experiments. Six ruminal epithelial tissues were collected from each goat, and every set of three ruminal epithelial tissues were included in one treatment group.

Ussing Chamber Measurements

The electrophysiological properties and permeability of the ruminal epithelium were determined for the intact ruminal epithelium using the Ussing chamber technique (Physiologic Instruments, America). Firstly, for preparation of the electrode, 2 g of Agarose was weighed and inserted into 50 mL centrifuge tubes, then KCl (150 mL, 3 mol/L) solution was added, and the centrifugal tubes were placed into 100°C water for 90 min, until the liquid had a consistency of transparently sticky and there were no bubbles. The KCl-Agar solution was drawn with a 5 mL syringe, and a 0.5 ~ 1 cm length of KCl-Agar was injected into the tip of the electrode sleeve, and then placed into KCl (3 mol/L) solution (Fig. 1).

A piece of ruminal epithelial tissue from the ventral sac (~100 cm^2) was rinsed by immersion in the buffer solution (Table 2). The time from the goat slaughter to mounting the epithelium was 30~45 min. The ruminal epithelium was removed from the muscle layer, placed quickly in a buffer solution kept at 37°C, gassed with 95% O_2 and 5% CO_2 and then cut into squares (about 1 cm × 0.5 cm) and mounted in the Ussing chamber (EM-CSYS-6). The aperture area of sliders in the Ussing chamber was 0.5 cm^2, which provided sufficient contact area for the ruminal epithelium and buffer. Both halves of the chambers were immediately filled with buffer solution (Table 2) and gassed with 95% O_2/5% CO_2 at 37°C. Glucose was added to the serosal and mucosal sides for a final concentration of 10 mmol/L. The buffer temperature was kept constant at 37°C throughout the measurement.

Chemicals and reagent

Six rumen epithelial tissues were collected from each goat and treated in two groups with 3 replicates per group. Finally, 18 groups were completed with different mucosal incubation solutions as follows: pH (7.4, 5.5 and 5.2), HIS (0, 0.5 and 10 ng·mL^{-1}) and LPS (0, 30 and 60 KEU·mL^{-1}), each alone or in combination. The different mucosal pH values were adjusted by adding VFA and lactic acid according to Table 3, and then using HCl to adjust the final pH value. In total, repeated measurements were made on three different incubation chambers per group. After a 20 min equilibration period, the 8 μL FITC (final concentration 0.2 mmol/L) and 8 μL HRP (final concentration 2 μmol/L) were added to the mucosal side of each chamber. After a 20

Table 1: Composition and nutrient levels of experimental diets

Ingredients, %		Nutrient levels[2], DM bases	
Alfalfa	30.72	NE_L, MJ/kg	7.12
Hay	18.57	ME, MJ/kg	9.68
Corn	37.88	CP, %	12.45
Soybean meal	1.47	NFC[3], %	44.45
Wheat bran	8.20	NDF, %	31.78
NaCl	0.46	ADF, %	21.33
Limestone	0.19	Ca, %	0.54
Premix[1]	2.51	P, %	0.32
Concentrate: forage	51:49	Ca: P	1.68
Total	100.00	NFC/NDF ratio	1.40

Legend:[1] One kilogram of Premix contained the following: $MnSO_4 \cdot 5H_2O$ 1560 mg, $FeSO_4 \cdot 7H_2O$ 6240 mg, $ZnSO_4 \cdot 7H_2O$ 3500 mg, KI 17 mg, $NaSeO_3$ 130 mg, $Co_2Cl \cdot 6H_2O$ 206 mg, $CuSO_4 \cdot 5H_2O$ 300 mg, $VA_1$620 000 IU, VD_3 324 000 IU, VE 540 IU, VB_{12} 0.9 mg, VB_5 450 mg, VK_3 150 mg, folic acid 15 mg/kg, calcium pantothenate 750 mg/kg
[2] Ca and P were tested values and NFC, DM and ME were calculated values
[3] NFC (%) =1-NDF-CP-EE-Ash

Table 2: Composition of the buffer solution used in the Ussing Chamber

Component	Content (mmol/L)
NaCl	80.0
KCl	5.0
$NaH_2PO_4 \times H_2O$	0.40
$Na_2HPO_4 \times 2H_2O$	2.4
$C_3H_5NaO_2$	10.0
$C_2H_3NaO_2 \times 3H_2O$	25.0
$C_4H_7NaO_2$	5.0
$MgCl_2 \times 6H_2O$	1.2
$CaCl_2 \times 2H_2O$	1.2
$NaHCO_3$	25.0

Table 3: Compounds with different pH values of VFA and lactic acid mixture

Item	pH = 7.4	pH = 5.5	pH = 5.2
Acetate, mM	30	60	90
Propionate, mM	30	60	90
Butyrate, mM	10	20	30
Lactate, mM	0.5	1.0	1.5
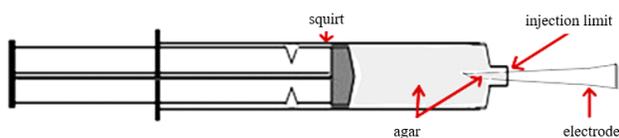
Fig. 1: Effect of SARA on expression levels of intracellular junction proteins in the epithelium of dairy goats

min equilibration period, transepithelial conductance (G_t, as a measure for passive ion permeability) and short-circuit current (I_{sc}, as a measure for active electrogenic electrolyte transport) data were continuously collected with the aid of a computer-controlled voltage-clamp device (voltage/current clamp) (Wang *et al*. 2021). Mucosal-to-serosal fluxes of HRP and FITC were measured by sampling 200 μL of solution from the serosal side at 20-min intervals over a 100-min period. The volume from the serosal side was replenished with 200 μL of fresh standard buffered solution to maintain a constant volume. The concentrations of HRP

and FITC in the serosal samples were measured as described previously (Cheng 2016).

Statistical Analysis

Each replicate served as an experimental unit. Data for pH $\times$ LPS and pH $\times$ HIS were obtained for the analysis in the double factors MIXED model in S.A.S. Version 9.3 (S.A.S. Institute Inc., Cary, NC). There are three levels of pH factor, LPS factor and HIS factor. Data for pH $\times$ LPS $\times$ HIS were analyzed by one way-ANONA for a single-factor variance analysis. Duncan's test was used to test the significance of multiple differences, and the data are presented as means $\pm$ SD. $P < 0.05$ was considered the level of significance.

Results

Interaction of pH and LPS on rumen epithelial permeability

As shown in Table 4, rumen epithelial I_{sc} and G_t values were greatest at mucosal pH 5.2 and lowest ($P < 0.05$) at mucosal pH 7.4. In the pH $\times$ LPS group, the effect of treatment was significant ($P < 0.05$). With LPS as the main factor, Isc and Gt of ruminal epithelium incabated at different pH levels in combination with LPS 60 were greater ($P < 0.05$) than those in LPS 30. When pH as the main factor, I_{sc} and G_t of ruminal epithelium incubated with mucosal addition of LPS-containing solution were the highest ($P < 0.05$) at pH 5.2, and PD at both pH 5.5 and pH 5.2 were lower ($P < 0.05$) than that at pH 7.4. Overall, the Isc and Gt of incubated ruminal epithelium were the highest ($P < 0.05$), while the PD value was lowest in the pH 5.2-LPS 60 group, which indicated the highest permeability of the ruminal epithelium.

Table 5 summarizes data for the mucosal-to-serosal fluxes of FITC and HRP. The fluxes of FITC and HRP through the ruminal epithelium at mucosal pH 5.2 were greater than those at mucosal pH 7.4. At mucosal pH 5.2, the HRP flow rate was higher than that at mucosal pH 5.5, while FITC flow rate was lower ($P < 0.05$). The effect of treatment was significant ($P < 0.05$) for the FITC flow rate. When LPS as the main factor, the flow rates of HRP and FITC ($P < 0.05$) at different pH values in combination with LPS 60 were greater than those in LPS 30; in addition, the concentration of LPS in the serosal side at different pH levels in combination with LPS 60 was greatest ($P < 0.05$). When pH as the main factor, the FITC flow rate of ruminal epithelium incubated with mucosal addition of LPS-containing solution was greatest ($P < 0.05$) at mucosal pH 5.2. The mucosal-to-serosal fluxes of HRP and FITC of ruminal epithelium incubated at the mucosal pH 5.2-LPS 60 were greatest ($P < 0.05$) (Table 5).

Interaction of pH and HIS on rumen epithelial permeability

As shown in Table 6, the interaction between pH and HIS had significant effects on I_{sc}, G_t and PD of incubated rumen

Table 4: Effects of different pH × LPS treatments on rumen epithelial electrophysiological parameters in dairy goats (n = 3)

LPS content/KEU·mL⁻¹	pH value	I_{sc}/mA (cm²·h)⁻¹	G_t/mS (cm²·h)⁻¹	PD/mV (cm²·h)⁻¹
0	7.4	0.05[d]	3.70[d]	1.15[d]
	5.5	0.16[c]	4.05[bc]	1.08[d]
	5.2	0.46[b']	4.14[bc]	6.66[a]
30	7.4	0.03[d]	2.71[e]	1.74[c]
	5.5	0.13[cd]	4.41[bc]	1.24[cd]
	5.2	0.16[c]	5.05[b]	2.54[b]
60	7.4	0.09[d]	3.87[bc]	2.75[b]
	5.5	0.15[c]	4.17[b]	2.44[bc]
	5.2	0.65[a]	5.87[a]	1.26[d]
SEM		0.043	0.228	0.125
Main effects				
LPS	0	0.22[B]	3.96[C]	2.96[A]
	30	0.11[C]	4.06[B]	1.84[C]
	60	0.30[A]	4.64[A]	2.16[B]
pH	7.4	0.06[C]	3.43[C]	1.88[B]
	5.5	0.15[B]	4.21[B]	1.58[C]
	5.2	0.42[A]	5.02[A]	3.49[A]
P-value	pH	<.0001	<.0001	<.0001
	LPS	<.0001	<.0001	<.0001
	pH×LPS	<.0001	<.0001	<.0001

Means with different lowercase letters are significantly different; means with different (*P* < 0.05). Uppercase letters within the same column are significantly different

Table 6: Effects of different pH×HIS treatments on rumen epithelial electrophysiological parameters in dairy goats

HIS content/ng·mL⁻¹	pH value	I_{sc}/Ma (cm²·h)⁻¹	G_t/mS (cm²·h)⁻¹	PD/mV (cm²·h)⁻¹
0	7.4	0.05[ef]	3.70[d]	1.15[d]
	5.5	0.06[ef]	4.05[c]	1.08[d]
	5.2	0.31[b]	4.14[c]	6.66[a]
0.5	7.4	0.02[g]	3.70[d]	2.92[c]
	5.5	0.08[e]	4.35[b]	0.73[de]
	5.2	0.13[d]	4.84[a]	0.86[de]
10	7.4	0.16[d]	3.46[c]	3.94[b]
	5.5	0.24[bc]	5.93[a]	0.24[f]
	5.2	0.46[a]	4.41[b]	3.43[b]
SEM		0.022	0.165	0.290
Main effects				
HIS	0	0.14[B]	3.96[C]	2.96[A]
	0.5	0.08[B]	4.30[B]	1.50[C]
	10	0.29[A]	4.60[A]	2.54[B]
pH	7.4	0.08[C]	3.62[C]	2.67[B]
	5.5	0.13[B]	4.78[A]	0.68[C]
	5.2	0.30[A]	4.46[B]	3.65[A]
P-value	pH	<.0001	<.0001	<.0001
	HIS	0.001	<.0001	<.0001
	pH×HIS	<.0001	<.0001	<.0001

Means with different lowercase letters are significantly different; means with different (*P* < 0.05). Uppercase letters within the same column are significantly different

Table 5: Effects of different pH × LPS treatments on rumen epithelial HRP and FITC flows and LPS content on the serosal side in dairy goats

LPS content/KEU·mL⁻¹	pH value	FITC/mmol(cm²·h)⁻¹	HRP/mol (cm²·h)⁻¹	LPS content/KEU·mL⁻¹
0	7.4	0.15[c]	0.03[b]	-
	5.5	0.15[c]	0.12[ab]	-
	5.2	0.21[bc]	0.10[ab]	-
30	7.4	0.11[d]	0.04[b]	25.92
	5.5	0.12[cd]	0.05[b]	21.94
	5.2	0.38[ab]	0.05[b]	27.91
60	7.4	0.15[c]	0.05[b]	29.11
	5.5	0.18[c]	0.06[b]	35.75
	5.2	0.48[a]	0.16[a]	26.36
SEM		0.056	0.042	3.730
Main effects				
LPS	0	0.17[C]	0.08	-
	30	0.20[B]	0.05	25.26[B]
	60	0.27[A]	0.09	30.41[A]
pH	7.4	0.14[B]	0.04	27.52[B]
	5.5	0.15[B]	0.08	28.85[A]
	5.2	0.36[A]	0.10	27.14[B]
P-value	pH	<.0001	0.099	0.041
	LPS	0.007	0.101	0.048
	pH×LPS	0.036	0.047	0.472

Means with different lowercase letters are significantly different; means with different (*P* < 0.05). Uppercase letters within the same column are significantly different

Table 7: Effects of different pH × HIS treatments on rumen epithelial HRP and FITC flows and HIS content in the serosal side in dairy goats

HIS content /ng·mL⁻¹	pH value	FITC/mmol (cm²·h)⁻¹	HRP/mol (cm²·h)⁻¹	HIS content/ng·mL⁻¹
0	7.4	0.15[bc]	0.03[c]	-
	5.5	0.15[bc]	0.14[a]	-
	5.2	0.21[a]	0.10[b]	-
0.5	7.4	0.17[ab]	0.09[b]	0.15
	5.5	0.13[cd]	0.09[b]	0.12
	5.2	0.12[cd]	0.08[b]	0.12
10	7.4	0.14[bc]	0.12[ab]	0.13
	5.5	0.13[cd]	0.02[c]	0.13
	5.2	0.09[d]	0.14[a]	0.14
SEM		0.013	0.026	0.009
Main effects				
HIS	0	0.17[A]	0.09	-
	0.5	0.14[B]	0.09	0.13
	10	0.12[C]	0.09	0.14
pH	7.4	0.16[A]	0.10	0.14
	5.5	0.13[B]	0.08	0.13
	5.2	0.14[B]	0.11	0.13
P-value	pH	0.001	0.052	0.275
	HIS	0.021	0.710	0.253
	pH×HIS	0.451	0.050	0.097

Means with different lowercase letters are significantly different; means with different (*P* < 0.05). Uppercase letters within the same column are significantly different

epithelium. Compared with mucosal pH-HIS 0.5 groups, I_{sc} and G_t were significantly increased (*P* < 0.05) in mucosal pH-HIS 10 groups with an HIS-based effect, while PD was reduced (*P* < 0.05). When pH was the main factor, I_{sc} of ruminal epithelium incubated with mucosal addition of HIS-containing solution was greatest (*P* < 0.05) at mucosal pH 5.2, G_t was greatest (*P* < 0.05) at mucosal pH 5.5, and PD was lowest (*P* < 0.05) at mucosal pH 5.5. The interaction between pH and HIS showed that I_{sc} and G_t of incubated ruminal epithelium were the highest (*P* < 0.05) at mucosal pH 5.2-HIS 10 and at mucosal pH 5.5-HIS 10, respectively, and the PD was lowest (*P* < 0.05) at mucosal pH 5.5-HIS 10.

Table 7 summarizes data for the mucosal-to-serosal fluxes of FITC and HRP. When HIS was the main factor, the FITC flow rate from mucosal to serosal was greater (*P* < 0.05) in mucosal HIS 0.5 than that in mucosal HIS 10, while the HRP flow rate remained consistent. When pH was the main factor, the FITC flow rate of ruminal epithelium

Table 8: Effects of different pH × LPS × HIS treatments on rumen epithelial electrophysiological parameters in dairy goats

pH × LPS × HIS treatments	I_{sc}/Ma (cm²·h)⁻¹	G_t/mS (cm²·h)⁻¹	PD/mV (cm²·h)⁻¹
7.4×60 KEU·mL⁻¹×10 ng·mL⁻¹	0.31ᶜ	4.16ᵇ	0.36ᵇ
5.5×60 KEU·mL⁻¹×10 ng·mL⁻¹	0.76ᵃ	3.79ᶜ	0.28ᶜ
5.2×60 KEU·mL⁻¹×10 ng·mL⁻¹	0.52ᵇ	5.03ᵃ	1.49ᵃ
SEM	0.004	0.108	0.114
P-value	< .0001	< .0001	< .0001

ᵃ⁻ᶜ Means with different superscript letters differ significantly (*P* < 0.05)
[1] I_{sc} = short-circuit current
[2] G_t = tissue conductance
[3] HRP = horseradish peroxidase
[4] FITC = fluorescein isothiocyanate

Table 9: Effects of different pH × LPS × HIS treatments on rumen epithelial HRP and FITC flows and LPS and HIS contents in the serosal side in dairy goats

pH × LPS × HIS treatments	FITC/mmol (cm²·h)⁻¹	HRP/mol (cm²·h)⁻¹	LPS/KE U·mL⁻¹	HIS/ng· mL⁻¹
7.4×60 KEU·mL⁻¹×10 ng·mL⁻¹	0.62ᵇ	0.18	41.94	0.29
5.5×60 KEU·mL⁻¹×10 ng·mL⁻¹	0.68ᵃ	0.25	35.83	0.19
5.2×60 KEU·mL⁻¹×10 ng·mL⁻¹	0.60ᵇ	0.39	41.18	0.19
SEM	0.020	0.094	2.242	0.045
P-value	0.012	0.275	0.09	0.241

Means with different lowercase letters are significantly different, means with different (*P* < 0.05). Uppercase letters within the same column are significantly different

incubated with mucosal addition of HIS-containing solution at mucosal pH 5.2 was lowest (*P* < 0.05), while the HRP flow rate was highest (*P* < 0.05). The interaction between pH and HIS showed that the mucosal-to-serosal flux of FITC at mucosal pH 5.5-HIS 10 was increased compared with mucosal pH 5.2-HIS 10, whereas the HRP flow rate was decreased, and the concentration of HIS in the serosal side showed no significant change (Table 7).

Interactions of pH, LPS and HIS on rumen epithelial permeability

As seen in Table 8, 9 the co-presence of pH, LPS and HIS had a significant effect on I_{sc}, G_t and PD of the incubated ruminal epithelium. I_{sc} was the highest in pH 5.5-LPS 60-HIS 10, whereas PD was the lowest, and the difference between the treatments was significant (*P* < 0.05). G_t reached the highest level (*P* < 0.05) in pH 5.2-LPS 60-HIS10.

The FITC flow rate of the incubated rumen epithelium was greatest (*P* < 0.05) in pH 5.5-LPS 60-HIS 10. The HRP flow rate was greater at mucosal pH 5.2 than for the other groups. No significant differences among the treatments were observed for the concentrations of LPS and HIS in the serosal side (Table 9).

Discussion

Previous reports have clearly shown that SARA can compromise the rumen epithelial barrier and increase rumen epithelial permeability (Steele *et al.* 2011; Klevenhusen *et al.*

2013; Meissner *et al.* 2017; Sun *et al.* 2018a). One of our previous studies from the same experiment demonstrated that pH interacts with both HIS and LPS to decrease the abundance of mRNA for genes involved in tight junction protein of the ruminal epithelium, which are probably related to increases in the permeability of the ruminal epithelium (Sun *et al.* 2018b). In the present study, we provide evidence that concurrent presence of low pH with excessive LPS and HIS in the rumen might the main trigger for increased rumen epithelial permeability.

The ruminal epithelium is a stratified squamous epithelium consisting of four distinct strata with a junctional complex that forms a barrier between the luminal contents and the internal milieu. As a permeable barrier, its role is to facilitate absorption of ions, water and nutrients, while at the same time preventing paracellular permeation of microorganisms and toxic compounds including LPS (Amaral *et al.* 2007; Liu *et al.* 2013). The Ussing chamber could indicate the permeability of the ruminal epithelium by the measurement of electrophysiological parameters (Vidyasagar and MacGregor 2016). Increased I_{sc} indicates an increase in the transport capacity of ions through the epithelium, and significantly increased G_t after rumen mucosal acidification indicates impaired rumen epithelial barrier function and increased epithelial permeability. Since the PD value is positively proportional to the epithelial resistance, the epithelial resistance can represent the tight junction of the epithelial intercellular and paracellular permeability and can also be used to monitor the rumen epithelial activity of ruminants (Ussing and Zerahn 1951). In the present study, the Ussing chamber technique was used to monitor rumen epithelial permeability in terms of I_{sc}, G_t and PD of the incubated ruminal epithelium and the fluxes of HRP and FITC. Our results indicated that the I_{sc}, as a measure of active electrogenic electrolyte transport, as well as the G_t, as a measure of passive ion permeability, significantly increased in the concurrent presence of low mucosal pH with excessive addition of LPS and HIS, which indicated that the rumen epithelial barrier functions were profoundly compromised.

Ruminal pH, VFA, osmolality and LPS concentration have been suggested as triggers for impairment of the rumen epithelial barrier, because they are known to be detrimental for the rumen epithelial barrier (Penner *et al.* 2011; Greco *et al.* 2018). Of these, the ruminal pH clearly plays a crucial role and impairs epithelial barrier function as indicated by increased permeability of the ruminal epithelium (Penner *et al.* 2010). An *in vivo* study conducted by Klevenhusen *et al.* (2013) demonstrated that low pH is a primary event preceding LPS release and LPS translocation across the rumen epithelial barrier during SARA (Enemark *et al.* 2002). In this study, pH, LPS and HIS were applied to healthy ruminal epithelium *in vitro* on a short-term basis. As a result of the single factor of pH, rumen epithelial permeability increased, and tissue activity decreased with a decrease of mucosal pH. Moreover, low mucosal pH in combination

with 60 KEU·mL^{-1} LPS induced a higher permeability of the ruminal epithelium, again suggesting that the influence of both pH and LPS on the rumen epithelial permeability was greater than that of pH alone. This may indicate that the combined effect of LPS and pH in the rumen of goats suffering from SARA aggravated the destruction of the ruminal epithelium. The ruminal LPS concentration has been found to increase to 26,915 EU/mL when SARA was induced (Gozho *et al*. 2006). SARA was the main cause of rumen epithelial barrier dysfunction, which is associated with low pH and high osmotic pressure (Aschenbach *et al*. 1998; Dong *et al*. 2013). In addition, HIS or inflammatory responses during acidosis can also impair the barrier function of the ruminal epithelium.

HIS is produced by the decarboxylation of histidine catalyzed by histidine decarboxylase. Under normal physiological conditions, the body generally contains HIS, but in very small amounts (Slyter 1976; Martens *et al*. 1987). Trace amounts of HIS can be involved in the collective regulation of a variety of physiological functions, such as nerve, endocrine, gastrointestinal and circulatory regulation (Klingspor *et al.* 2013). The notion that SARA is accompanied by the increase of abnormal metabolites, such as HIS, is widely accepted. When there is so much HIS in the rumen that it exceeds the normal metabolic capacity of the body, HIS will be transported into the blood circulation through the damaged ruminal epithelium and cause inflammation. This further aggravates the SARA and causes the further destruction of the ruminal epithelium (Gozho *et al*. 2007).

Cheng (2016) showed that in dairy goats the concentrations of LPS and HIS in plasma and rumen were significantly increased during grain-induced SARA. The increased HIS or LPS translocating from the gastrointestinal tract into the blood can down-regulate the expression of gastrointestinal tight junction protein and embedded protein, and increase the apoptosis rate of epithelial cells, resulting in further damage of the epithelial barrier (Pilachai *et al.* 2012). Aschenbach *et al*. (1998) showed that HIS-induced apoptosis increased cell shedding and interfered with nuclear division and cell maturation. This might mean that HIS could interfere with the regeneration of epithelial cells during SARA, thus causing cell damage and triggering an inflammatory reaction. In the present study, our data showed that the rumen epithelial permeability was significantly increased, and tissue activity was reduced in mucosal pH 5.2-HIS 10. This suggests that lower mucosal pH with excessive HIS induced more severe barrier dysfunction. Our data are similar with the results of Penner *et al*. (2010) and Meissner *et al*. (2017), which indicated a low pH of 5.2 has only moderate effects on the ruminal epithelial barrier, whereas concurrent presence of low pH with high SCFA concentrations can trigger a profound impairment of epithelial barrier function (Hu 2008; Hu *et al*. 2015). Therefore, we concluded that the disruption of the rumen epithelial barrier function was not caused only by pH,

and the concurrent presence of pH-HIS or pH-LPS might contribute to the more obvious increases of rumen epithelial permeability in the present study.

Additionally, the permeability of marker molecules of different sizes (HRP as a large marker, FITC as a small marker) was also measured in the present study. Compared with mucosal pH alone or concurrent presence of pH-LPS and pH-HIS, significant increases in mucosal-to-serosal fluxes of HRP and FITC coupled with enhanced I_{sc} and G_t were observed in the concurrent presence of mucosal pH with LPS and HIS. An increased flux of FITC or HRP reflects increased paracellular permeability and impaired ruminal barrier. Our results further suggested that the combined treatment of pH, LPS and HIS contributes to triggering higher epithelial permeability and more profound barrier dysfunction. Thus, subacute rumen acidosis is a process involving pH, LPS, HIS and their synergistic interactions. One of our previous studies in dairy goats reported that a concurrent increase of both HRP and FITC mucosal-to-serosal flux rates were observed during SARA (Sun *et al.* 2018b), which is somewhat inconsistent with the results of the present study. In the short-term pH×LPS×HIS cross-treatment, the small molecule (FITC) permeability of the incubated ruminal epithelium was increased, but the permeability to large molecules (HRP) was not increased significantly. This observation may also be related to the different absorption mechanisms of large and small molecular markers by ruminal epithelium in the short term. In order to ensure the health of the animal, the ruminal epithelium may normally prevent penetration of large molecule toxic substances and only allow small molecules, such as amino acids and water, to pass through (Oba *et al.* 2005).

Conclusion

Our results have shown an increased rumen epithelial permeability during SARA is caused by the combined action of low pH with high LPS and high HIS concentrations, which is critical for the impairment of the rumen epithelial barrier. Our study also showed that concurrent presence of LPS 60 KEU·mL^{-1} and HIS 10 ng·mL^{-1} at mucosal pH 5.5 can aggravate rumen epithelial barrier dysfunction.

Acknowledgments

Conflict of Interest

The authors declare no conflict of interest in this study

Data Availability

All data presented in this study are available upon request

Ethics Approval

The experimental design and procedures were approved by the Animal Care and Use Committee of the Inner Mongolia Academy of Agricultural and Animal Husbandry Sciences and were performed in accordance with relevant guidelines formulated by the Ministry of Agriculture of the People's Republic of China.

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