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In Vitro Study on the Interactive Effects of pH and Lipopolysaccharide or Histamine on Tight Junction Protein mRNA Expression in Dairy Goat Rumen Epithelial Cells (Postprint)

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Date: 2018-12-24T00:00:00+00:00

Abstract

This experiment aimed to investigate the effects of the interaction between pH and lipopolysaccharide (LPS) or histamine (HIS) on the mRNA expression levels of tight junction proteins in rumen epithelial cells of dairy goats using an in vitro method. Eight healthy Saanen dairy goats with similar body weight and milk yield were selected as rumen epithelial donors. A 3×3 two-factor experimental design was adopted. After slaughter, rumen epithelium was collected and mounted in the center of the half-chamber of an Ussing chamber. Five milliliters of buffer solution was added to the serosal side half-chamber, and 5 mL of prepared culture medium with different treatments was added to the mucosal side, with three replicates per treatment. In Experiment 1, factor 1 was pH (7.4, 5.5, and 5.2), and factor 2 was LPS concentration (0, 30, and 60 kEU/mL LPS). In Experiment 2, factor 1 was pH (7.4, 5.5, and 5.2), and factor 2 was HIS concentration (0, 0.5, and 10.0 ng/mL HIS). After 80 min of incubation, rumen epithelium was collected to determine the mRNA expression levels of tight junction proteins Claudin-1, Claudin-4, Claudin-7, Occludin, and Zonula occludens-1 (ZO-1). The results showed: 1) The interaction between pH and LPS had significant effects on the mRNA expression levels of Claudin-1, Claudin-7, and ZO-1 ($P<0.05$). Compared with the pH 7.4 × 0 kEU/mL LPS group, decreasing pH or adding LPS significantly reduced the mRNA expression levels of Claudin-1 and Claudin-7 ($P<0.05$), while ZO-1 mRNA expression showed an overall decreasing trend, but was highest at pH 5.2 × 60 kEU/mL LPS. 2) The interaction between pH and HIS had significant effects on the mRNA expression levels of Claudin-1, Claudin-7, and ZO-1 ($P<0.05$). The pH 5.5 × 0.5 ng/mL HIS group had the lowest Claudin-1 mRNA expression level, but the difference was not significant compared with the pH 5.2 × 0.5 ng/mL

HIS group (P>0.05). The Claudin-7 mRNA expression level in the pH 7.4 × 10.0 ng/mL HIS group was significantly lower than that in the pH 7.4 × 0 ng/mL HIS group (P<0.05), but the difference was not significant compared with the pH 5.5 × 10.0 ng/mL HIS group (P>0.05). Compared with the pH 7.4 × 0 ng/mL HIS group, decreasing pH or adding HIS tended to increase ZO-1 mRNA expression, and the pH 5.2 × 10.0 ng/mL HIS group showed a significant increase (P<0.05). The results suggest that after the occurrence of subacute ruminal acidosis (SARA), pH interacts with LPS or HIS on the rumen epithelium, reducing the mRNA expression levels of rumen epithelial tight junction proteins, thereby increasing the permeability of the rumen epithelial mucosa.

Full Text

Interaction Effects of pH and Lipopolysaccharide or Histamine on mRNA Expression Levels of Tight Junction Proteins in Rumen Epithelium of Dairy Goats *In Vitro*

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Abstract: This study aimed to investigate the interaction effects of pH and lipopolysaccharide (LPS) or histamine (HIS) on mRNA expression levels of tight junction proteins in rumen epithelium of dairy goats using an *in vitro* approach. Eight healthy Saanen dairy goats with similar body condition, body weight, and milk yield were used as rumen epithelium donors. A 3×3 factorial design was employed. After slaughter, rumen epithelium was collected and mounted in Ussing chamber half-cells, with 5 mL buffer added to the serosal side and 5 mL of prepared culture medium with different treatments added to the mucosal side. Each treatment had three replicates. In Experiment 1, factor 1 was pH (7.4, 5.5, and 5.2) and factor 2 was LPS concentration (0, 30, and 60 kEU/mL). In Experiment 2, factor 1 was pH (7.4, 5.5, and 5.2) and factor 2 was HIS concentration (0, 0.5, and 10.0 ng/mL). Rumen epithelium samples were collected after 80 min of incubation to determine mRNA expression levels of tight junction proteins Claudin-1, Claudin-4, Claudin-7, Occludin, and Zonula occludens-1 (ZO-1). The results showed: 1) The pH × LPS interaction significantly affected mRNA expression levels of Claudin-1, Claudin-7, and ZO-1 (P<0.05). Compared with the pH 7.4 × 0 kEU/mL LPS group, decreasing pH or adding LPS significantly reduced Claudin-1 and Claudin-7 mRNA expression (P<0.05), while ZO-1 mRNA expression showed an overall decreasing trend but was highest in the pH 5.2 × 60 kEU/mL LPS group. 2) The pH × HIS interaction significantly affected mRNA expression levels of Claudin-1,

Claudin-7, and ZO-1 ($P<0.05$). The pH 5.5 × 0.5 ng/mL HIS group had the lowest Claudin-1 mRNA expression, which was not significantly different from the pH 5.2 × 0.5 ng/mL HIS group ($P>0.05$). The pH 7.4 × 10.0 ng/mL HIS group had significantly lower Claudin-7 mRNA expression than the pH 7.4 × 0 ng/mL HIS group ($P<0.05$), but was not significantly different from the pH 5.5 × 10.0 ng/mL HIS group ($P>0.05$). Compared with the pH 7.4 × 0 ng/mL HIS group, decreasing pH or adding HIS tended to increase ZO-1 mRNA expression, with the pH 5.2 × 10.0 ng/mL HIS group showing a significant increase ($P<0.05$). These results suggest that after subacute ruminal acidosis (SARA) occurs, the interaction between pH and LPS or HIS acts on rumen epithelium to reduce mRNA expression levels of tight junction proteins, thereby increasing rumen epithelial mucosal permeability.

Keywords: subacute ruminal acidosis; dairy goats; rumen epithelium; histamine; lipopolysaccharides; Ussing chamber; tight junction proteins

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The rumen epithelium of ruminants is a stratified squamous epithelium (SSE) consisting of, from mucosal to serosal layers, the stratum corneum (SC), stratum granulosum (SG), stratum spinosum (SS), and stratum basale (SB) [1]. Tight junctions (TJ) exist between cells in the stratum granulosum and represent crucial structures for maintaining the mucosal barrier [2]. The rumen epithelium not only provides a favorable environment for microbial attachment and growth but also serves as the primary site for nutrient digestion, absorption, and metabolic transport. In modern intensive farming systems, ruminants are highly susceptible to subacute ruminal acidosis (SARA). Following SARA onset, rumen osmotic pressure increases and large quantities of harmful substances or unstable environmental factors are generated within the rumen, including lipopolysaccharides (LPS) [1], histamine (HIS) [2], and low pH [3]. These harmful substances are absorbed into the bloodstream, damaging tight junction structures and directly destroying epithelial barrier function, thereby interfering with nutrient absorption and the defense against toxic substances [4]. Thus, the integrity of tight junction structures is a prerequisite for rumen health.

Previous research on tight junctions has primarily focused on medical applications. Studies have shown that artificially burned mice exhibit dysfunctional tight junctions at burn sites, with damaged intercellular connections forming cellular gaps that disrupt epithelial permeability [2]. Other research has reported that intestinal epithelial tight junction proteins Zonula occludens-1 (ZO-1), Occludin, and Claudin can interact with cytoskeletal actin and may play important roles in intestinal epithelial barrier dysfunction [5]. Since Ussing and Zerahn [6] first proposed applying the Ussing chamber technique to ion transport in epithelial tissues in the early 1950s, subsequent researchers have expanded this technology to study rumen epithelial permeability in ruminants [7]. Changes in rumen epithelial permeability accurately reflect the degree of rumen epithelial damage, with increased permeability indicating compromised epithelial integrity

and impaired barrier function. Klenvenhusen et al. [8] used the Ussing chamber system to demonstrate that high-concentrate diets significantly increased goat rumen epithelial permeability. Subsequently, Yang [11] induced SARA in dairy goats by gradually increasing dietary non-fibrous carbohydrate/neutral detergent fiber (NFC/NDF) ratio and found that SARA significantly reduced mRNA expression levels of Claudin-1, ZO-1, and Occludin in rumen epithelium. Using Ussing chamber measurements, Yang also found that SARA significantly increased short-circuit current (Isc) and tissue conductance (Gt), indicating increased rumen epithelial permeability. Cheng [12] expanded the NFC/NDF range to further induce SARA and found that SARA significantly increased Claudin-4 mRNA expression in rumen epithelium while decreasing gap junction protein Connexin-43 and desmosomal protein Desmoglein-1 mRNA expression. This research also demonstrated that SARA significantly decreased rumen pH while increasing abnormal metabolites (LPS, HIS, etc.) in both rumen fluid and plasma. These studies indicate that after SARA onset, the disrupted rumen environment further lowers rumen pH while LPS and HIS concentrations increase significantly [9-10], altering rumen epithelial tight junction protein mRNA expression and enlarging intercellular gaps, thereby destroying rumen epithelial integrity and increasing permeability. However, how these abnormal metabolites affect rumen epithelial permeability—whether through pH alone or through combined effects of pH with LPS or HIS—requires further investigation. Therefore, this study employed the Ussing chamber to investigate the interactive effects of pH and LPS or HIS on mRNA expression levels of tight junction proteins in dairy goat rumen epithelium *in vitro*, aiming to elucidate the molecular mechanisms by which pH and LPS or HIS affect rumen epithelial permeability.

1.1 Experimental Animals and Management

Eight healthy multiparous Saanen dairy goats with good body condition, similar body weight (43.58±2.77 kg), and similar milk yield were selected as experimental animals and housed at the Animal Experimental Base of Inner Mongolia Academy of Animal Sciences. The goats were individually penned and fed equal amounts at 06:00 and 18:00 daily. Water was available ad libitum. The experimental period lasted 30 days.

1.2 Basal Diet

The basal diet was formulated according to NRC (2007) [11] combined with Chinese "Feeding Standards of Dairy Goats" [12] as a diet with NFC/NDF ratio of 1.40, using corn, soybean meal, wheat bran, alfalfa, and green hay as main ingredients. The composition and nutrient levels of the basal diet are shown in Table 1 .

Table 1 Composition and nutrient levels of experimental diets (DM basis)

Items	Content
Ingredients	
Alfalfa	
Green hay	
Corn	
Soybean meal	
Wheat bran	
NaCl	
Limestone	
Premix[1]	
Total	
Nutrient levels[2]	
Net energy for lactation (NEL, MJ/kg)	
Crude protein (CP)	
Non-fibrous carbohydrate (NFC)	
Neutral detergent fiber (NDF)	
Acid detergent fiber (ADF)	
Ca to P ratio	
Concentrate to forage ratio	51:49

[1]One kilogram of premix contained: MnSO · 5H O 1560 mg, FeSO · 7H O 6240 mg, ZnSO · 7H O 3500 mg, KI 17 mg, NaSeO 130 mg, CoCl · 6H O 206 mg, CuSO · 5H O 300 mg, VA 1,620,000 IU, VD 324,000 IU, VE 540 IU, VB 0.9 mg, VB 450 mg, VK 150 mg, folic acid 15 mg, calcium pantothenate 750 mg.

[2]CP, NDF, Ca, and P were measured values, while others were calculated values. NFC (%) = 1 - NDF - CP - EE - Ash.

1.3 Experimental Design

This study employed a 3×3 factorial design. Different treatment buffers were added to the mucosal side of the Ussing chamber (6 channels). In Experiment 1, factor 1 was pH at three levels: 7.4 (control), 5.5, and 5.2; factor 2 was LPS concentration at 0, 30, and 60 kEU/mL. In Experiment 2, factor 1 was pH at 7.4 (control), 5.5, and 5.2; factor 2 was HIS concentration at 0, 0.5, and 10.0 ng/mL. A total of 15 groups were included (the pH 7.4 groups without LPS or HIS were shared between the two experiments), with three replicates per group. Rumen epithelium from each slaughtered goat was used for two experimental groups. The Ussing chamber incubation lasted 80 min (with the first 20 min as an equilibrium period). The pH, HIS, and LPS concentrations were set based on preliminary results from our research group and relevant domestic and international studies [3,12,13-14].

1.4 Main Instruments and Reagents

Instruments: Ussing chamber system (EM-CSYS-4, PI, USA), electrophoresis apparatus (BG-Power600, Beijing Baijing Biotechnology Co., Ltd.), high-speed refrigerated centrifuge (1730R, Naishi Technology Co., Ltd.), PCR instrument [Yixin Xingye (Beijing) Technology Co., Ltd.], real-time quantitative PCR instrument (Illumina, USA), gel imaging system (Snap Gene, USA), vortex mixer (ST-T256, Qilin Bell Instrument Manufacturing Co., Ltd.), microplate reader (BioTek, USA), palm centrifuge (CX-200, Guohua Electric Appliance Co., Ltd.), microplate reader (MultiSkan 3, Thermo, USA), low-temperature refrigerated centrifuge (Fresco, Thermo, USA), electrophoresis tank (Mini P-4, Beijing Kaiyuan Xinrui Instrument Co., Ltd.), electrophoresis apparatus (Bio-Rad, USA), electric tissue homogenizer (Fluka, Germany), wet transfer electrophoresis tank (Beijing Kaiyuan Xinrui Instrument Co., Ltd.).

Reagents: Ussing chamber system buffer, 3 mol/L KCl solution, agar, KH PO -NaHPO buffer, H O solution, 3,3 ,5,5 -tetramethylbenzidine solution (TMB), LPS, HIS, acetic acid, propionic acid, butyric acid, lactic acid.

1.5.1 Rumen Epithelium Collection and Processing

The Ussing chamber half-cells and clamps were first assembled, followed by sequential installation of voltage and current electrodes. Five milliliters of pre-warmed buffer were added to each half-cell of the Ussing chamber system, and a mixed gas of 95% O and 5% CO was introduced. The software was activated to run the system. After approximately 10 min of equilibrium and when the computer display showed stable curves, the experimental animals were slaughtered. Fresh rumen epithelial tissue (2 cm × 3 cm) was immediately collected from the ventral sac, rinsed repeatedly with pre-warmed buffer, and quickly stripped of the muscle layer before being cut into 1 cm × 1 cm pieces. The fixing ring clamp was removed to secure the sample (mucosal side facing left) and inserted into the center of the Ussing chamber half-cell. Five milliliters of buffer were added to the serosal side and 5 mL of prepared culture medium for different groups was added to the mucosal side, with three replicates per group. After 80 min of incubation, the rumen epithelium was collected with sterile forceps into sterile, enzyme-free cryovials, immediately placed in liquid nitrogen, and subsequently stored at -80 °C for determination of mRNA expression levels of tight junction proteins Claudin-1, Claudin-4, Claudin-7, Occludin, and ZO-1.

1.5.2 Buffer and Culture Medium Preparation

Ussing chamber buffer was prepared according to Klenvenhusen et al. [10] and Cheng [12] with the following composition: NaCl 80.00 mmol/L, KCl 5.00 mmol/L, NaH PO ·H O 0.40 mmol/L, Na HPO ·2H O 2.40 mmol/L, C H NaO 10.00 mmol/L, C H NaO ·3H O 25.00 mmol/L, C H NaO 5.00 mmol/L, MgCl · 6H O 1.20 mmol/L, CaCl ·2H O 1.20 mmol/L, NaHCO 25.00 mmol/L. The prepared buffer was stored at 4 °C.

The reagent additions for adjusting culture medium pH are shown in Table 2 . Different pH buffers were prepared by adding reagents according to the amounts listed in the table. LPS or HIS was added according to the experimental design.

Table 2 Supplemental levels of agents for adjusting pH of culture medium (mmol/L)

Items	Acetic acid	Propionic acid	Butyric acid	Lactic acid
pH 7.4				
pH 5.5				
pH 5.2				

1.5.3 Electrode Preparation

The structure of the Ussing chamber electrode casing is shown in Figure 1 [Figure 1: see original paper]. Two grams of agar were weighed into a 50 mL centrifuge tube, 50 mL of 3 mol/L KCl solution was added, and the tube was heated in a 100 °C water bath until the liquid became transparent and viscous with no bubbles. An appropriate amount of KCl-agar solution was drawn into a 5 mL syringe, and 0.5–1.0 cm of KCl-agar was injected into the tip of the electrode casing. Casings with bubble-free KCl-agar sections of appropriate length were selected for use and stored in 3 mol/L KCl solution.

Figure 1 Structure of electrode casing of Ussing chamber

1.6.1 Total RNA Extraction and Reverse Transcription

Fifty to one hundred milligrams of frozen dairy goat rumen epithelial tissue were ground into powder in a liquid nitrogen-filled mortar. The powder was transferred to a 1.5 mL centrifuge tube. Total RNA was extracted according to the RNAprep Pure Tissue Kit instructions. Total RNA concentration was measured using a microplate reader, and RNA quality was assessed using 1% agarose gel electrophoresis. Total RNA was reverse-transcribed according to the TIANScript RT Kit instructions. The resulting cDNA products were stored at -20 °C for later use.

1.6.2 PCR Primer Design and Synthesis

Based on corresponding cDNA sequences from GenBank, specific primers for real-time quantitative PCR (RT-PCR) amplification were designed using Primer Premier 5.0 software and synthesized by Sangon Biotech (Shanghai) Co., Ltd. Primer parameters are shown in Table 3 .

Table 3 Parameters of primers for RT-PCR

Genes	GenBank accession No.	Primer sequences (5 -3)	Product length (bp)
-actin	NM_001009784	F: GGCAGGTCATCACCATCGGR: CGTGTTGGCGTAGAGGTCTTT	158
Claudin-1	HM_117762.1	F: CAGCAAGGAGGAATGGAAAGAR: GCAGGGAGGATTGAAGAAGG	186
Claudin-4	XM_005697785.1	F: CACCCTTGGCATGAAGTGTAR: AGCCAATGAAGAGAGCCTGA	132
Claudin-7	XM_013972136.1	F: GACGGGAGGCATCATTTTCAR: CAGAGTTGGGCTTAGGGTAGGA	89
Occludin	XM_004016906.1	F: GTGGTAACTTGGAGACGCTTTCR: CTCCCGTCGTGTAGTCTGTT	
ZO-1	XM_004018080.1	F: TTGAACGCAAGTTTGAAAGTCCR: TCTTCATCTTCATCCTCCTCCAC	

F: forward primer; R: reverse primer.

1.6.3 Real-Time Quantitative PCR

Real-time quantitative PCR amplification was performed using SuperReal PreMix Plus (SYBR Green) (Tiangen Biotech). The PCR reaction system (20 L) consisted of: 2×SuperReal PreMix Plus 10.00 L, forward primer (10 mol/L) 0.60 L, reverse primer (10 mol/L) 0.60 L, cDNA template 2.00 L, and RNase-free ddH O 6.80 L.

The optimized reaction program was: 1) pre-denaturation at 95 °C for 30 s; 2) PCR amplification for 40 cycles of 95 °C for 10 s, 60 °C for 30 s, and 72 °C for 30 s; 3) melting curve analysis at 95 °C for 15 s, 60 °C for 15 s, and 95 °C for 15 s. Melting curves for each gene were analyzed after amplification to ensure RT-PCR product specificity. All procedures were repeated three times. -actin was used as the internal reference, and relative mRNA expression levels were calculated using the $2^{(-\Delta\Delta Ct)}$ method.

1.7 Statistical Analysis

All data were analyzed using SAS 8.0 software. Two-way ANOVA was performed using the MIXED procedure. Duncan' s multiple comparison test was used for significant differences. The mixed model included main effects of LPS, HIS, and pH, as well as pH × LPS and pH × HIS interaction effects. $P<0.05$ was considered statistically significant, while $P>0.05$ was considered non-significant.

2.1 Total RNA Quality

Total RNA extracted from rumen epithelium was examined by 1% agarose gel electrophoresis (Figure 2 [Figure 2: see original paper]). The 18S and 28S bands were clear, and the OD /OD ratio measured by microplate reader ranged from 1.8 to 2.2, indicating no protein or DNA contamination and good RNA quality.

Figure 2 Electropherogram of total RNA from rumen epithelium of dairy goats

2.2 PCR Amplification and Cloning Sequencing of Tight Junction Protein Genes

PCR amplification products for -actin, Claudin-1, Claudin-4, Claudin-7, Occludin, and ZO-1 showed specific bands at 158, 186, 132, and 89 bp, respectively. After purification, cloning, and bacterial PCR identification, samples were sent to Sangon Biotech (Shanghai) Co., Ltd. for sequencing. The resulting sequences showed 100% homology with genes registered in GenBank under accession numbers NM_001009784, XM_005697785.1, HM_117762.1, XM_013972136.1, XM_004016906.1, and XM_004018080.1.

Effects of pH and LPS on mRNA Expression Levels of Tight Junction Proteins in Rumen Epithelium

As shown in Table 4 , when LPS was considered the main effect, the 60 kEU/mL LPS group had significantly higher Claudin-7 mRNA expression than the 30 kEU/mL LPS group ($P<0.05$), while both groups were significantly lower than the 0 kEU/mL LPS group ($P<0.05$). ZO-1 mRNA expression in the 60 kEU/mL LPS group was significantly higher compared with the 0 and 30 kEU/mL LPS groups ($P<0.05$). Occludin mRNA expression in the 60 kEU/mL LPS group was significantly lower than in the 0 and 30 kEU/mL LPS groups ($P<0.05$).

When pH was considered the main effect, the pH 5.2 group showed significantly higher Claudin-1, Claudin-7, and ZO-1 mRNA expression than the pH 7.4 group ($P<0.05$). Compared with the pH 7.4 group, Claudin-7 mRNA expression was significantly increased in the pH 5.5 group ($P<0.05$). The pH × LPS interaction significantly affected Claudin-1, Claudin-7, and ZO-1 mRNA expression ($P<0.05$). Multiple comparisons revealed that compared with the

pH 7.4 × 0 kEU/mL LPS group, decreasing pH or adding LPS significantly reduced Claudin-1 and Claudin-7 mRNA expression (P<0.05). ZO-1 mRNA expression showed an irregular pattern, being highest in the pH 5.2 × 60 kEU/mL LPS group and significantly higher than in the pH 7.4 × 0 kEU/mL LPS group (P<0.05).

Table 4 Effects of pH and LPS on mRNA expression levels of tight junction proteins in ruminal epithelium of dairy goats

LPS (kEU/mL)	Main effects	P-value	Claudin-1	Claudin-4	Claudin-7	Occludin	ZO-1
pH 7.4							
0			6.28	1.21	0.17	0.26	0.03
30			0.06	0.04	0.05	1.01	2.55
60			0.12	0.37	0.15	0.04	0.53
pH 5.5							
0			8.98	0.73	5.87	0.04	0.04
30			2.51	2.60	5.19	0.10	1.59
60			1.10	1.41	4.08	0.03	0.81
pH 5.2							
0			4.33	2.47	0.21	0.01	0.19
30			0.28	0.01	0.21	0.06	0.28
60			0.01	0.21	0.01	0.94	1.86
Main effects							
LPS			<0.001	<0.001	<0.001	<0.001	<0.001
pH × LPS			<0.001	<0.001	<0.001	<0.001	<0.001

In the same column, values with different small letter superscripts differ significantly (P<0.05). The same applies below.

Effects of pH and HIS on mRNA Expression Levels of Tight Junction Proteins in Rumen Epithelium

As shown in Table 5 , when HIS was considered the main effect, the 0.5 ng/mL HIS group had significantly lower Claudin-1 and Claudin-7 mRNA expression than the 0 and 10.0 ng/mL HIS groups (P<0.05). ZO-1 mRNA expression in the 10.0 ng/mL HIS group was significantly higher than in the 0 and 0.5 ng/mL HIS groups (P<0.05), while the 0.5 ng/mL HIS group was significantly higher than the 0 ng/mL HIS group (P<0.05).

When pH was considered the main effect, compared with the pH 7.4 group, Claudin-1 mRNA expression was significantly reduced in both the pH 5.2 and pH 5.5 groups (P<0.05), with the lowest expression in the pH 5.2 group (P<0.05). Claudin-7 mRNA expression was significantly increased in the pH 5.2 and pH 5.5

groups (P<0.05), with the highest expression in the pH 5.5 group. ZO-1 mRNA expression in the pH 5.5 group was significantly lower than in the pH 5.2 and pH 7.4 groups (P<0.05), while the pH 5.2 group was significantly higher than the pH 5.5 and pH 7.4 groups (P<0.05). The pH × HIS interaction significantly affected Claudin-1, Claudin-7, and ZO-1 mRNA expression (P<0.05).

Multiple comparisons revealed that compared with the pH 7.4 × 0 ng/mL HIS group, decreasing pH or adding HIS significantly reduced Claudin-1 mRNA expression (P<0.05), with the lowest expression in the pH 7.4 × 10.0 ng/mL HIS group. The pH 5.5 × 10.0 ng/mL HIS group had the lowest Claudin-7 mRNA expression, which was significantly lower than in the pH 7.4 × 0 ng/mL HIS group (P<0.05). Compared with the pH 7.4 × 0 ng/mL HIS group, decreasing pH or adding HIS tended to increase ZO-1 mRNA expression overall, with the highest expression in the pH 5.2 × 10.0 ng/mL HIS group, which was significantly higher than all other groups (P<0.05).

Table 5 Effects of pH and HIS on mRNA expression levels of tight junction proteins in ruminal epithelium of dairy goats

HIS (ng/mL)	Main effects	P-value	Claudin-1	Claudin-4	Claudin-7	Occludin	ZO-1
pH 7.4							
0			6.28	1.21	0.17	2.60	0.06
0.5			0.26	0.11	0.56	0.14	2.55
10.0			0.97	0.27	1.36	0.31	0.20
pH 5.5							
0			8.98	0.73	5.87	1.20	16.60
0.5			2.25	0.77	0.23	6.38	5.19
10.0			6.95	2.46	1.38	8.41	4.32
pH 5.2							
0			0.21	0.01	0.21	1.97	0.40
0.5			0.17	0.74	0.17	0.43	4.01
10.0			0.14	0.85	1.73	1.36	0.42
Main effects							
HIS			<0.001	<0.001	<0.001	<0.001	<0.001
pH × HIS			<0.001	<0.001	<0.001	<0.001	<0.001

3 Discussion

Effects of pH on mRNA Expression Levels of Tight Junction Proteins in Rumen Epithelium

Cellular connections in rumen epithelium are primarily categorized into tight junctions, desmosome junctions (DJ), adhesion junctions (AJ), and gap junc-

tions. Tight junctions represent the most important intercellular connection, located between cells in the stratum granulosum of the rumen. They constitute the structural foundation for maintaining the rumen mucosal barrier and are the primary determinant of intercellular permeability. As dynamic permeability barriers, tight junctions serve dual functions: they prevent invasion of potentially harmful substances or pathogens into rumen epithelium to maintain integrity while allowing nutrients, ions, and water to pass through, ensuring appropriate rumen epithelial permeability. This unique physiological function plays a pivotal role in gastrointestinal mucosal barrier maintenance. However, under pathological conditions, damaged tight junctions enlarge intercellular gaps, allowing translocation of bacteria and toxins and loss of selective barrier function. When tight junctions undergo variation, reduction, or deletion, redistribution and downregulation of gene expression damage epithelial cells, cause atrophy, impair barrier function, destroy intercellular tight junctions, and increase epithelial permeability [15]. Nevertheless, few reports have investigated the molecular mechanisms affecting rumen epithelial barrier function. Some scholars have proposed that tight junction membrane proteins in rumen epithelium include Claudin-1, Claudin-4, Claudin-7, and Occludin, while gap junction proteins include Connexin-43 and desmosomal protein Desmoglein-1. Immunoblotting localization revealed that Claudin-1, ZO-1, and Connexin-43 are present in the rumen mucosal stratum granulosum, gradually decreasing from the stratum granulosum to the stratum spinosum and stratum basale [16].

Ruminal acidosis is classified as acute or subacute based on pH magnitude and duration. SARA is generally defined as pH <5.5 for more than 3 h, while pH <5.2 typically indicates acute acidosis [20]. pH is an important physiological indicator reflecting rumen fermentation. However, the critical pH threshold for SARA remains debated. Some scholars propose that rumen pH 5.5–5.8 represents normal physiological status, pH 5.0–<5.5 indicates SARA, and pH 5.2–5.5 represents moderate acidosis [21]. In recent years, pH 5.2 has commonly been used as the critical value for acute acidosis, while pH below 5.5 for more than 3 h serves as an indicator of SARA.

In this study, pH levels were set at 7.4, 5.5, and 5.2 based on domestic and international research. The results showed that decreasing pH downregulated Claudin-1 and Claudin-4 mRNA expression, consistent with Wang [22]. Claudin-7 mRNA expression was lowest at pH 5.5, suggesting that tight junction function was partially compromised at this pH, supporting pH 5.5 as a reasonable threshold for SARA. At pH 5.2, downregulation of Claudin-1 and Claudin-4 mRNA expression may indicate acute acidosis with more severe damage to rumen epithelial tight junctions. Occludin showed an upregulation trend with decreasing pH, though not significantly different. This differs from Wang [22], who reported that Occludin mRNA expression was primarily affected by pH and decreased with lower pH. This discrepancy may be due to different culture methods, as the Ussing chamber environment simulates rumen conditions to maintain tissue viability for short periods, whereas most studies use cultured rumen epithelial cells [23-24].

Effects of pH and LPS on mRNA Expression Levels of Tight Junction Proteins in Rumen Epithelium

Studies have shown that increasing dietary NFC/NDF ratio elevates plasma LPS concentration and induces endotoxemia in dairy goats [25]. High-grain diets inducing SARA in ruminants are often accompanied by increased LPS concentrations in plasma or rumen fluid [26]. Our research group found that increasing dietary NFC/NDF from 1.40 to 3.23 gradually increased plasma LPS concentration from 15.76×10^3 EU/mL to 85.55×10^3 EU/mL, consistent with previous reports [13]. Chin et al. [27] demonstrated in intestinal epithelial cells that increasing LPS concentration increased nitric oxide (NO) production and reduced ZO-1, altering tight junction protein structure and function. Additionally, our previous *in vitro* studies using Ussing chamber measurements of electrophysiological parameters after combining pH with HIS or LPS revealed that pH × HIS or pH × LPS interactions had greater effects on rumen epithelial permeability than single factors of pH, HIS, or LPS concentration [23]. Based on these findings, this study explored the molecular mechanisms underlying rumen epithelial tight junction damage.

The results showed substantial changes in tight junction protein mRNA expression. The effects of pH and LPS treatments differed. When LPS was the main effect, 60 kEU/mL LPS reduced Claudin-7 mRNA expression, increased ZO-1 mRNA expression, and decreased Occludin mRNA expression. When pH was the main effect, pH 5.2 significantly increased Claudin-1, Claudin-7, and ZO-1 mRNA expression compared with pH 7.4. The pH × LPS interaction reduced Claudin-1 and Claudin-7 mRNA expression, with effects intensifying as pH decreased and LPS concentration increased.

Wang [22] used cultured rumen epithelial cells with different pH and short-chain fatty acid (SCFA) concentration combinations and found no significant difference in Occludin mRNA expression compared with controls. SCFA addition significantly downregulated ZO-1 mRNA expression, while both SCFA addition and low pH ($pH<6.8$) significantly upregulated Claudin-1 and Claudin-4 mRNA expression. After SARA onset in ruminants, increased rumen SCFA concentration further lowers rumen pH, indicating that pH and SCFA can affect rumen epithelial tight junction protein expression. Liu [20] found that high-grain diets significantly reduced Claudin-4, Occludin, and ZO-1 mRNA expression. In contrast, our study used different SCFA concentrations to prepare three pH gradients and measured ZO-1 mRNA expression in Ussing chambers, finding that it first decreased then increased with decreasing pH. These studies demonstrate that short-term exposure of rumen epithelium to different pH levels in an *in vitro* rumen simulation environment downregulates Claudin-1 mRNA expression compared with normal pH. Overall, pH 5.5 treatment had the most severe impact on rumen epithelial permeability.

Effects of pH and HIS on mRNA Expression Levels of Tight Junction Proteins in Rumen Epithelium

Histamine is an important biologically active substance and a key mediator of type I hypersensitivity reactions, participating in allergic responses, vasoconstriction and dilation, and serving as an important mediator of inflammatory reactions and immune damage. During SARA, the rumen environment is disrupted, with prolonged low pH causing histidine decarboxylation to form HIS and increasing abnormal metabolites like HIS [24], which damage rumen mucosa and destroy barrier function. The barrier function of ruminant rumen epithelium is critical for maintaining rumen absorption, digestion, and metabolic functions. Epithelial cells can regulate passive diffusion of small electroneutral solutes and ions through intercellular tight junction proteins. Therefore, healthy tight junctions are essential for barrier function [28]. Cheng [12] reported that when goats developed SARA, rumen epithelial permeability changed, with SARA significantly increasing Claudin-4 mRNA expression by 38.61% compared with controls. This study investigated mRNA expression of Claudin-1, Claudin-4, Claudin-7, Occludin, and ZO-1 in goat rumen epithelium. HIS addition down-regulated Claudin-1 mRNA expression, while Claudin-4 mRNA expression first increased then decreased with increasing HIS concentration. Claudin-1 mRNA expression at pH 5.5 was not significantly different from that at pH 5.2. Claudin-7 mRNA expression showed a decreasing trend, being significantly lower in the pH 5.5 $\times$ 10.0 ng/mL HIS group than in other groups. No significant differences were observed in Occludin mRNA expression among groups. These findings partially differ from Yang [11], who reported that SARA significantly reduced Occludin and ZO-1 mRNA expression by 51.3% and 51.8%, respectively. That study induced SARA in dairy goats before slaughter and collected fresh rumen epithelial samples for measurement, whereas our study used short-term simulation in Ussing chambers, which may account for the differences.

Guo et al. [29] reported that blood HIS content increased with dietary NFC/NDF ratio. Aschenbach et al. [4] demonstrated that HIS induces apoptosis, increases cell shedding, or interferes with nuclear division and cell maturation, suggesting that the abnormal metabolite HIS can interfere with epithelial cell regeneration during SARA, causing cell damage and triggering inflammatory responses. Our results showed that the pH $\times$ HIS interaction affected rumen epithelial tight junction protein mRNA expression differently. Decreasing pH or adding HIS significantly reduced Claudin-1 and Claudin-7 mRNA expression. When HIS was the main effect, 10.0 ng/mL HIS significantly increased ZO-1 mRNA expression compared with 0.5 ng/mL HIS, which in turn was significantly higher than 0 ng/mL HIS. When pH was the main effect, Claudin-1 mRNA expression was significantly reduced at pH 5.5 and pH 5.2 compared with pH 7.4, reaching its lowest level at pH 5.2. Claudin-7 mRNA expression showed a decreasing trend. The pH $\times$ HIS interaction significantly affected Claudin-1, Claudin-7, and ZO-1 mRNA expression. The pH 5.5 $\times$ 0.5 ng/mL HIS group had the lowest Claudin-1 mRNA expression, not significantly

different from the pH 5.2 × 0.5 ng/mL HIS group. The pH 7.4 × 10.0 ng/mL HIS group had significantly lower Claudin-7 mRNA expression than the pH 7.4 × 0 ng/mL HIS group, but was not significantly different from the pH 5.5 × 10.0 ng/mL HIS group. Compared with the pH 7.4 × 0 ng/mL HIS group, decreasing pH or adding HIS generally increased ZO-1 mRNA expression, with the pH 5.2 × 10.0 ng/mL HIS group being significantly higher than all other groups. These findings demonstrate that the interaction between rumen pH and HIS significantly reduces expression of some tight junction protein genes, destroys rumen epithelial structure and function, and represents one of the main causes of SARA.

4 Conclusion

After SARA onset, the interaction between pH and LPS or HIS acts on rumen epithelium to reduce mRNA expression levels of tight junction proteins, thereby increasing rumen epithelial mucosal permeability.

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