Dietary supplementation of thiamine enhances colonic integrity and modulates mucosal inflammation injury in goats challenged by lipopolysaccharide and low pH

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Abstract

The current study aimed to investigate the protective effects of dietary thiamine supplementation on the regulation of colonic integrity and mucosal inflammation in goats fed a high-concentrate (HC) diet. Twenty-four Boer goats (live weight of 35·62 (sem 2·4) kg) were allocated to three groups (CON: concentrate/forage = 30:70; HC; concentrate/forage = 70:30 and HCT: concentrate/forage = 70:30 with 200 mg thiamine/kg DMI) for 12 weeks. Results showed that compared with the HC treatment, the HCT group had a significantly higher ruminal pH value from 0 to 12 h after the feeding. The haematoxylin–eosin staining showed that desquamation and severe cellular damage were observed in the colon epithelium of the HC group, whereas the HCT group exhibited more structural integrity of the epithelial cell morphology. Compared with the HC treatment, the HCT group showed a markedly increase in pyruvate dehydrogenase and α-ketoglutarate dehydrogenase enzymes activity. The mRNA expressions in the colonic epithelium of SLC19A2, SLC19A3, SLC25A19, Bcl-2, occludin, claudin-1, claudin-4 and ZO-1 in the HCT group were significantly increased in comparison with the HC diet treatment. Compared with the HC treatment, the HCT diet significantly increased the protein expression of claudin-1 and significantly decreased the protein expression of NF-κB-related proteins p65. The results show that dietary thiamine supplementation could improve the colon epithelial barrier function and alleviate mucosal inflammation injury in goats after lipopolysaccharide and low pH challenge.

Key words: Thiamine: Colonic integrity: Inflammation: Apoptosis: High-concentrate diet: Goat

In the current ruminant production systems, large amounts of high-concentrate (HC) diets are fed to goats or dairy cows to maximise the yield of meats and milk[1]. Although these short-term feeding regimens might be effective to support energy requirements, the excessive amounts of non-structural fermentable carbohydrates lead to a lot of negative influences as the accumulation of SCFA and microbial translocation in rumen and hindgut[2,3]. Moreover, the decline of pH in the rumen and gastrointestinal tract likely results in the lysis of gram-negative bacteria and the release of lipopolysaccharide (LPS) that translocate into the blood circulation system, which enhances mucosal damage of the hindgut[4]. Under normal physiological conditions, only a small amount of LPS penetrates the gastrointestinal epithelial barrier by a special immune mechanism as a consequence of an endocytotic pathway[5,6]. In contrast, under physiological stresses caused by endotoxin and cytokines, the barrier properties of tight junctions (TJ) can be provoked, causing increased epithelial permeability and shift of LPS[7].

Accumulation evidence shows that the large intestine is invaded easily by gut LPS translocation in ruminants. Furthermore, not only multiple approaches of transcellular transport but the monolayer intestinal mucosa is also more prone to damage by low pH resulting in an increase in the paracellular permeability compared with the rumen epithelial structure[8]. Ruminants can experience hindgut acidosis if the rumen cannot maintain physiological degradability, increasing the flow rate of

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substrates for gastrointestinal (caecum and colon) microbial fermentation[9]. Bertok (2004) testified that bile acids cause degradation of LPS in the small intestine[10]. LPS is more easily degraded in the small intestine due to the detoxification by intestinal alkaline phosphatase and antimicrobial peptide[11–13]. Moreover, the animal's colon or caecum has a higher permeability to macromolecules than the small intestine, probably connected with a peculiar paracellular pathway[14]. Thus, the large intestine is more LPS susceptible than the small intestine. After translocation from the rumen to the hindgut, LPS interacts with LPS-binding protein (LBP), which markedly enhances LPS activity and augments the production of pro-inflammatory cytokines[15]. Subsequently, the LPS–LBP conjugates were transferred to cell surfaces and interact with toll-like receptors 4 (TLR4), and then NF-κB were activated, which resulting in a range of cascade immune responses[16].

As a water-soluble vitamin, thiamine plays an important role not only in energy metabolism but also in the regulation of barrier function[17,18]. Studies have shown the fact that an increase of dietary non-fibre carbohydrate levels (HC diet) can decrease the content of thiamine in the rumen and may cause thiamine deficiency[19,20]. Feeding on long-term HC diets caused a low ruminal pH and microbial activity[21] and may, therefore, affect thiamine production[22]. Pan *et al.* (2017) revealed that excessive feeding of HC diets caused disturbance of bacterial community associated with thiamine metabolism, resulting in thiamine deficiency[23]. Our prior studies have confirmed that exogenous thiamine supplementation not only relieved inflammation in ruminal epithelium via regulating the NF-KB pathway but promoted epithelial development in goats[18,24]. However, the literature revealed no data regarding thiamine regulation of inflammation and intestinal integrity in the colon during a long period of feeding HC diet. Thus, we hypothesised that thiamine supplementation could protect colonic integrity via modulating mucosal inflammation injury. The aim of this study focuses on the evaluation of the anti-inflammatory properties of thiamine and enzyme activities related to thiamine function in colonic mucosa during long-term HC diet feeding.

Methods

Ethics statement

Animal care and procedures were under the Chinese guidelines for animal welfare and approved by the Guidelines for the Ethics Committee of Yangzhou University (SXXY 2015-0054).

Animals and experimental design

The experimental sample size was determined according to the prior studies on the function of thiamine during a long period of HC feeding[18,24,25]. We expanded the sample size in the study to obtain more valid information. Twenty-four female Boer goats (body weight = 35·62 (SEM 2·4) kg, body condition score = 3·15 (SEM 0·14), where 0 = emaciated and 5 = obese)[26] bought from Lingtang, a village in Jiangsu province, were used in this study. During the 2-week adaptation period, all trial animals were housed in separate pens (1·5 × 1·5 m) and receiving *ad libitum*

the same diet (concentrate:forage = 30:70). At the end of the dietary adaptation period, goats (dry period) in parity 1 or 2 were randomly allocated to three groups (complete randomised design). The first group were fed a low-concentrate diet comprising 70 % forage and 30 % mixed concentrate (CON, *n* 8), the second group were offered a HC diet containing 70 % mixed concentrate and 30 % forage (HC, *n* 8), while the last group received a a HC diet supplemented with 200 mg of thiamine/ kg DMI (HCT, *n* 8). Based on NRC standard (2007)[27], the diets components and nutrient compositions were formulated to meet the nutrient and energy requirements of 35-kg Boer goats (online supplementary Table S1). The feedstuff thiamine content was determined using thiamine measurement kits (TSZ Biological Trade Co. Ltd), according to the manufacturer protocols. The dietary supplementation dose of thiamine was confirmed according to our previous research[28]. For daily thiamine supplementation method, 100 g of concentrate diet mixed with thiamine (half of daily thiamine total intake) (T283819, thiamine hydrochloride, purity≥99 %, Aladdin) was firstly fed to each animal. Then, the remaining ration was fed until the goats had consumed the entire 100 g of concentrate to ensure full intake of thiamine. The goats were fed the respective diets twice daily at 07.00 h and 18.00 h for 12 weeks and had free access to freshwater during the experimental period. All goats were dewormed with 0·2 mg of ivermectin/kg (I8411, Solarbio) of BW for controlling gastrointestinal parasites once a week during the entire experiment. Moreover, diets and orts of goats were tracked and gathered daily. At the end of the experimental period, all goats were euthanised with an intravenous injection of sodium pentobarbital (200 mg/kg BM, provided by Experiment Management Center of Yangzhou University).

Sample collection and analysis

On the last day of weeks 10, 11 and 12, approximately 30 ml of representative rumen fluid sample was collected via the oral cannula at 0, 1, 2, 4, 6, 8, 10 and 12 h after feeding to measure the pH value by a high-accuracy portable pH metre (Testo 205, Testo AG) as described by Gozho *et al.* (2005)[29]. The pH value of each sample was measured three times to avoid anthropogenic errors. After death, approximately 50 ml of colonic digesta from the proximal colon was collected, following by the immediate determination of pH values using a portable pH metre (Testo 205, Testo AG). Colonic digesta samples were divided into three portions. One portion was mixed with an equal amount of physiological saline (0·90 % wt/vol of NaCl), immediately centrifuged at 10 000 × g for 15 min at 4 °C, and the supernatants were taken and stored at −20°C until subsequent analyses for VFA according to previous studies[18,23,25,28,30]. The second portion of each of the colon samples was transferred into pyrogen-free tubes and processed for LPS concentration analyses using Rogers *et al.* (1985) method, and a lower measurement limit of LPS as 0·1 endotoxin units/ml[31]. The last portion of the colon samples was used to determine the concentration of thiamine and lactate using a similar method described by Ma *et al.* (2021b)[18]. Within 5 min after slaughtering, representative colonic epithelium fragments were collected from the same position from each animal and the muscular layer was stripped using blunt

dissection. Then immediately, they were washed three times in precooled PBS buffer, followed by snap-freezing in liquid N_2 and preservation under −80 °C until further analysis. In addition, 2 cm of the colonic fragments were fixed in 4 % paraformaldehyde (Sigma) for histomorphometric microscopic analysis.

Histological analysis

Samples of the intestinal wall of the colonic mucosa were collected for histological observation, fixing in 4 % formaldehyde buffered solution (Beyotime Institute of Biotechnology), embedded in paraffin and then sectioned. Specimens were measured for injury after haematoxylin–eosin staining as described by Yue *et al.* (2012)[32]. A scoring criterion was adopted for determining histological damage as described previously[33].

Immunohistochemistry of colonictight junction analysis

The expression and distribution of the intestinal wall of the colonic mucosa TJ proteins were conducted using immunohistochemistry. The following antibodies were used in the immunohistochemistry assay: claudin-1 (diluted at 1:100, ab15098; Abcam), claudin-4 (diluted at 1:100, ab210796; Abcam), occludin (diluted at 1:100, ab167161; Abcam) and ZO-1 (diluted at 1:100, ab214228; Abcam). Histological sections were prepared by embedding in paraffin, based on the method of histological analysis. Then sections were incubated with antibodies and dyed with hematein for light microscope observation. Image-Pro Plus v.6.0 software (Media Cybernetics) was used to choose the same brown colour as the consistent criterion for estimating all photos. Each photo was measured to acquire the cumulative optical density of each image.

Enzyme activities assay of pyruvate dehydrogenase, α-ketoglutarate dehydrogenase and lactate dehydrogenase in colon tissue

The pyruvate dehydrogenase (PDH), *α*-ketoglutarate dehydrogenase (*α*-KGDH) and lactate dehydrogenase (LDH) enzyme activities in the colonic tissue were measured using commercial high-precision kits (Jiehuigao biological technology Co., Ltd), according to the supplier protocols. The absorbance of all samples was read at 605 nm and 340 nm, using a multi-function microplate reader (FLx800, Bio-Tek Instruments, Inc.). All trial tissue samples were normalised to total protein concentration by the BCA kits (Beyotime Biotechnology Institute).

Assay of matrix metalloproteinase activity in colon tissue

The matrix metalloproteinase (MMP)-2 and MMP-9 activities were determined using gelatin zymography. Total protein was extracted from 100 mg of a ground colon tissue sample based on a previous method[34]. Briefly, 60 μg of total protein was utilised to electrophoresis with a 12 % polyacrylamide gel containing 1 % gelatin for 90 min. Subsequently, the gel was incubated with a renaturation buffer and a developing buffer at 37 °C, respectively. Then, the gel was stained with 0·5 % Coomassie blue R-250 for the following analysis. The Quantity One software (Bio-Rad Laboratories Inc.) was used to analyse the relative zymographic intensity of each sample[28]. All

experiments were repetitively conducted thrice, and all values were expressed as fold changes compared with the CON group.

Caspase-3 and caspase-8 activities assay in colon tissue

Caspase-3 and caspase-8 enzyme activities in the colonic mucosa tissue were determined using a caspase activity Assay Kit (Nanjing Jiancheng Bioengineering Institute) according to the manufacturer instructions. In brief, 7-amino-4-trifluoromethylcoumarin (AFC) was used to label the caspase molecule in each sample, then fluorescence value were detected by an automatic fluorescence microplate reader (FLx800, Bio-Tek Instruments, Inc.) at 405 nm[35]. All values were expressed as fold changes relative to the CON group.

Total RNA extraction and real-time quantitative PCR

Total RNA for all samples were extracted from approximately 100 mg of colonic tissues by using total RNAiso Plus Kit V2 (Vazyme Biotech Co., Ltd) according to the manufacturer's protocols. Total RNA was quantified using a Nanodrop (NC2000, Thermo Fisher), and RNA integrity number (RIN) was measured on a Bioanalyzer (Bioanalyzer2100, Agilent). The experiment of electrophoresis with a 1·2 % (wt/vol) denaturing agarose gel was conducted to measure the integrity of RNA, and clear 28S and 18S RNA bands were confirmed in each sample before being used in the additional trials. Subsequently, 2 μg of total RNA was reverse-transcribed into cDNA using a PrimeScript RT reagent Kit (Vazyme Biotech Co., Ltd) based on the manufacturer's guidance. The primers information of reference and target genes were displayed in Table 1 (Sangon Biotech). Primers were designed to span exon-exon junctions, where possible and were assessed for amplification efficiency (calculated as $-1 + 10^{(-1/slope)} \times 100$) using a serial 10-fold dilution of pooled cDNA using a serial dilution of pooled cDNA. Melt curves for primers were checked to verify the presence of a single product and avoid dimer formation. Based on Gao *et al.* (2013)'s method in the screening of reference genes, RefFinder (http://www.leonxie.com/referencegene.php), including Normfinder, geNorm and the comparative ΔCT method, was used to select the first-rank reference gene (ACTB, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and hypoxanthine phosphoribosyltransferase (HPRT)) by determining the candidate genes' ranking[36]. A lower gene geomean of ranking value means a higher expression stability. The order of gene expression stability, from most to least stable, was HPRT1, ACTB and GAPDH. Therefore, GAPDH was chosen as the reference gene to normalise mRNA expression. The quantitative reverse transcription PCR experiments were performed as described by Ma *et al.* (2021b) using SYBR green plus reagent kit (Vazyme Biotech Co., Ltd)[24]. Final reference genes were used to standardise target gene abundance using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001)[37]. All reactions were performed in triplicate.

Western blotting analysis

The ground colonic tissue samples (about 100 mg) were pyrolysed with RIPA lysis and extraction buffer (Solabio Biotech Co., Ltd) for 10 min. Colonic lysates were centrifuged at

Table 1. Primers for quantitative real-time PCR

Gene name	Sequences (5′-3′)	GenBank accession	Product size (bp)	Efficiency* (%)
NF-κB	F: TGGCGAGAGGAGCACAGACAC R: TGACCAGCGAGATGCGGACTG	XM_018043384·1	92	99·26
IL-1β	F: CATGTGTGCTGAAGGCTCTC R: AGTGTCGGCGTATCACCTTT	XM_013967700·2	172	94·35
IL-6	F: ACACTGACATGCTGGAGAAGATGC R: CCGAATAGCTCTCAGGCTGAACTG	NM_001285640·1	116	95·08
IL-10	F: AAACAAGAGCAAGGCGGTGGAG R: ACTCACTCATGGCTTTGTAGACACC	XM_005690416·3	83	97·42
CXCL-10	F: CCACGTGTCGAGATTATTGCCA R: TGCCTCTTTCCGTGTTCGAG	NM_001285721·1	141	102·90
CXCL-13	F: CTCTCCTGTCCACGGTGTTC R: CCCACGGGGGATGATTTGAA	XM_005681804·3	121	99·35
MMP-9	F: TTGAGGGCGAACTCAAGTGG R: CCCATCTCCGTGCTCTCTAAC	NM_001314269·1	206	99·82
MMP-13	F: TGTTGCTGCCCATGAGTTTG R: TCATCTCCTGGACCGTAGAG	XM_005689359·3	159	92·05
Bcl-2	F: AGGCTCACAGCACACTCTTC R: GGCCTGTGGGCTTCACTTAT	XM_018039337·1	193	101·54
Bax	F: TGAAGCGCATTGGAGATG R: GGCCTTGAGCACCAGTTT	XM_013971446·2	185	97·90
Occludin	F: GCCTGTGTTGCCTCCACTCTTG R: CATAGCCATAGCCACTTCCGTAGC	XM_018065677·1	118	95·62
Claudin-1	F: GCTGTGGATGTCGTGCGTGTC R: TGCCTCCTCGTCGTAACTGTCC	XM_005675123·3	161	95·54
Claudin-4	F: TCATCGGCAGCAACATCGTCAC R: CAGCAGCGAGTCGTACACCTTG	XM_005697785·2	109	91·76
ZO-1	F: TGGCAATGGTTAATGGCGTCTCC R: TGCCTCCTCGTCGTAACTGTCC	XM_018066118·1	185	96·55
SLC19A2	F: GAGGAACAGGAATCCAAGCCAGAC R: GAGCAGAGGGCGAGAGGAGTAG	XM_018060285·1	90	97·48
SLC19A3	F: TGGACCTACTCTTACCTGGCACTAC R: CTGGAGGATGATGACTGGCTTGTAG	XM_005676643·3	84	97·31
SLC25A19	F: CAGCTCCTTGAAGCGTGCCTAC R: TCCAGTGGGTATGTGAGGGTCTTG	XM_013972239·2	126	93·12
GAPDH	F: GGGTCATCATCTCTGCACCT R: GGTCATAAGTCCCTCCACGA	XM_005680968·3	176	103·68
β-actin	F: CTGGCACCACACCTTCTACA R: GGGTCATCTTCTCACGGTTG	NM_001314342·1	189	96·79
HPRT1	F: CACCAGCTGGCTCCGTTATG R: AGTCGTTCGGTCCTGTCCAT	XM_018044253·1	163	94·22

CXCL, C-X-C motif chemokine ligand; MMP-9, matrix metalloproteinase 9; Bcl-2, B-cell lymphoma/leukaemia 2; Bax, Bcl-2-associated X protein; ZO-1, zonula occludens-1; SLC25A2, solute carrier family 19, member 2; SLC19A3, solute carrier family 19; SLC25A19, mitochondrial thiamine pyrophosphate transporter; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HPRT1, hypoxanthine phosphoribosyltransferase 1; F, forward; R, reverse.
* Efficiency $= -1 + 10^{(-1/\text{slope})} \times 100$.

12 000 × g at 4 °C for 30 min and then the supernatant was collected. Total protein concentrations were measured in triplicate using an Enhanced BCA Protein Detection Kit (Vazyme Biotech Co., Ltd). The protein samples were denatured at 100 °C for 5 min, and then 60 mg of protein was run on a 12 % SDS-PAGE gel electrophoresis at 60 V for 30 min, then 100 V for 2 h and then transferred to a nitrocellulose membrane (Millipore). These membranes were blocked with 5 % BSA (Beyotime Biotechnology) for 1 h at 25 °C, and then incubated overnight at 4 °C with primary antibodies, including anti-ZO-1 (ab214228, diluted at 1:1000, Abcam), anti-occludin (ab167161, diluted at 1:1000, Abcam), anti-claudin-1 (ab15098, diluted at 1:1000, Abcam) and anti-β-actin (diluted at 1:1500, Santa Cruz). β-Actin was used as a loading control for standardisation of target protein by Western blotting. After washing step with Tris-Buffered Saline Tween (TBST), membranes were incubated with the secondary antibody horseradish peroxidase (HRP-conjugated goat anti-rabbit IgG, diluted at 1:1000, Beyotime)

for 45 min at room temperature. Then, enhanced chemiluminescence Plus kit (Vazyme Biotech Co., Ltd) was used to visualise the protein bands. The signals were captured using a LAS4000 imaging system (GE Healthcare Bio-Sciences AB) and converted to grey values by Image-Pro Plus 6.0 (Media Cybernetics Inc.) software. The protein expression was showed as fold change relative to the average value of the CON[38].

Statistical analysis

Shapiro–Wilk's and Levene's tests were used to test for normality and homogeneity of variances, respectively. The general linear model repeated measures was repetitively performed to analyse ruminal pH by IBM SPSS 21.0 statistics (SPSS Inc.). For each goat, pH value resluts for the last day of consecutive weeks 10, 11 and 12 were averaged before analysis[39]. Other data were analysed using one-way ANOVA with Dunnett's post-test[39,40]. The fixed effect of parity, which was included in the original models and

was not significant ($P > 0.05$), was excluded from the final model, in which only treatment was the fixed effect. The data were considered statistically significant at $P < 0.05$.

Results

Changes in the ruminal pH

Compared with the CON group, the HC group had ($P < 0.05$) lower ruminal pH value ($P < 0.05$; Fig. 1), and pH was below 6.0 within 12 h after feeding. The HCT group had a significantly higher pH value than the HC groups from 0 to 12 h ($P < 0.05$) after the feeding, and ruminal pH was above 6.0 after 4 h of feeding.

SCFA, lipopolysaccharide, thiamine and lactate concentrations and pH in colonic digesta

Compared with the CON group, the HC group showed a markedly decrease (Table 2; $P < 0.05$) in pH value and thiamine concentration of colonic digesta, but a markedly increase ($P < 0.05$) in free LPS, lactate, acetate, propionate, butyrate and total SCFA concentrations. On the contrary, the pH value and thiamine concentration of colonic digesta were significantly increased ($P < 0.05$) in the HCT group compared with the HC goats, but free LPS, lactate, acetate, propionate, butyrate and total SCFA concentrations were significantly decreased ($P < 0.05$).

Morphological analysis in colon tissue

Haematoxylin–eosin staining showed desquamation and severe cellular damage that was observed in the colon epithelium of the HC group, whereas the CON and HCT goats exhibited structural integrity of the epithelial cell morphology (Fig. 2(a)–(c)). Meanwhile, the colonic epithelial damage score was significantly higher in the HC group compared with the CON and HCT group ($P < 0.05$).

Matrix metalloproteinase-, caspase- and thiamine-dependent enzyme activities in the colonic mucosa tissues

As shown in Table 3, the HC goats showed significantly higher levels of MMP-2, MMP-9, caspase-3, caspase-8 and LDH enzyme activities ($P < 0.05$) and lower levels of PDH and α-KGDH enzyme activities ($P < 0.05$) in the colonic mucosal tissue than that in the CON goats. Moreover, compared with the HC goats, the HCT group showed a markedly increase ($P < 0.05$) in PDH and α-KGDH enzyme activities and a markedly decrease ($P < 0.05$) in MMP-2, MMP-9, caspase-3, caspase-8 and LDH enzyme activities.

Immunohistochemistry of colonic epithelium tight junction

To investigate the differences in colonic epithelium barrier with different diet treatments, we examined the expression and distribution of TJ (zonula occludens-1, occludin, claudin-1 and claudin-4). The HC diet treatment showed significantly downregulated protein expression levels ($P < 0.05$; Fig. 3) in occludens-1 (ZO-1), occludin, claudin-1 and claudin-4 that in the CON goats, whereas the HCT had a significant increase ($P < 0.05$)
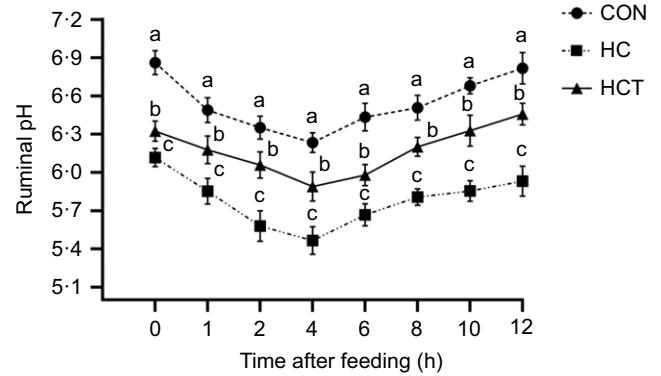

Fig. 1. Ruminal pH values for goats fed the low-concentrate diet (CON), high-concentrate diet (HC) and HC diet supplemented with 200 mg of thiamine/kg of DMI (HCT). Data are shown as means values with their standard errors. Means with different letters (a–c) are significantly different ($P < 0.05$) at 0–12 h.

Table 2. Effects of dietary thiamine supplementation in colonic digesta parameters, contents of thiamine and lipopolysaccharide of goats fed with a high-concentrate diet at the time of slaughter
(means values with their standard errors)*

Item	Dietary treatment			SEM	P
	CON	HC	HCT		
Ph	7.24*	5.86	6.31	0.18	0.001
Thiamine (µg/l)	5.47*	1.13	2.56	0.32	0.003
Free LPS (×10^3 EU/ml)	18.29	36.75*	22.53	4.21	0.024
Lactate (mM)	0.47	0.82*	0.51	0.06	0.022
Acetate (mM)	26.28	48.36*	35.92	4.67	0.013
Propionate (mM)	12.32	19.45*	16.18	1.81	0.018
Butyrate (mM)	7.92	11.54*	8.78	0.67	0.013
Acetate: Propionate	2.13	2.48*	2.22	0.06	0.012
TSCFA (mM)	48.67	83.21*	64.58	6.36	0.007

CON, control; HC, high-concentrate diet; HC, high-concentrate diet supplemented with 200 mg of thiamine/kg of DMI; LPS, lipopolysaccharide; EU, endotoxin unit; TSCFA, total SCFA.
* Data are expressed as means values with their standard errors. Mean values with different superscripts are significantly as determined by Tukey's test ($P < 0.05$).
n 8 goats/treatment.

in protein expression levels of ZO-1, occludin, claudin-1 and claudin-4.

Gene expression in colon tissue

The relative mRNA expression of SLC19A2, SLC19A3, SLC25A19, Bcl-2, occludin, claudin-1, claudin-4 and ZO-1 were lower ($P < 0.05$; Table 4) and NF-κB, IL-1β, IL-6, IL-10, CXCL-10, CXCL-13, MMP-9, MMP-13 and Bax were higher ($P < 0.05$) in the HC group than that in the CON group. Compared with the HC treatment, dietary thiamine supplementation (HCT group) reversed this change for the expression of the above genes ($P < 0.05$).

Protein expression in colon tissue

Western blotting analysis showed significantly higher p65 protein levels ($P < 0.05$; Fig. 4) and markedly lower claudin-1 protein levels ($P < 0.05$) than those in the CON group, whereas the expression levels of p65 were lower ($P < 0.05$) and claudin-1
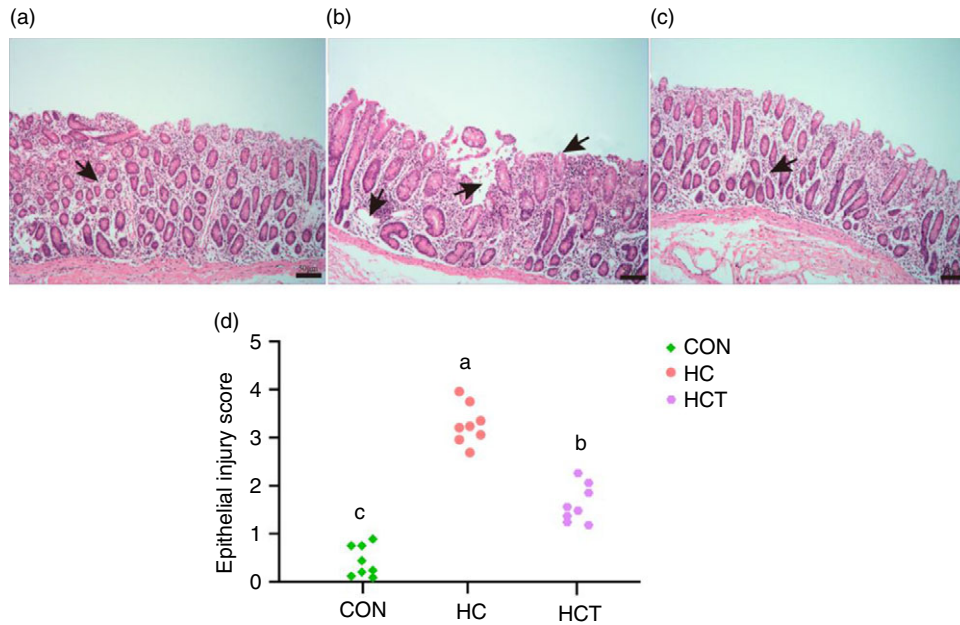
Fig. 2. Representative histology sections with haematoxylin–eosin staining of the colon in goats from the low-concentrate diet (CON), high-concentrate diet (HC) and high-concentrate diet with thiamine (HCT) group. (a) The colonic mucosa epithelium in the CON group was intact and showed no disruption (n 8; scale bar = 50 μm). (b) In the HC treatment, desquamation and severe cellular damage were observed in the colonic mucosa epithelium (n 8; scale bar = 50 μm). (c) The colonic mucosa epithelium showed slight damage in the HCT treatment (n 8; scale bar = 50 μm). (d) Colonic epithelial injury score. Arrow indicates the degree of injury in the colonic mucosa epithelium. The mean values in columns without a common superscript letter differ ($P < 0.05$).

Table 3. Effects of dietary thiamine supplementation on enzyme activities in the colonic mucosa of goats fed with a high-concentrate diet (means values with their standard errors)*

Item	Dietary treatment			SEM	P
	CON	HC	HCT		
MMP-2 (fold change)	1·00	3·64*	2·73	0·25	0·008
MMP-9 (fold change)	1·00	2·88*	2·19	0·28	0·014
Caspase-3 (fold change)	1·00	1·65*	1·42	0·12	0·007
Caspase-8 (fold change)	1·00	1·49*	1·22	0·12	0·013
PDH (nmol/mg protein)	2·23*	1·36	2·14*	0·13	0·016
α-KGDH (nmol/mg protein)	1·48*	0·62	1·56*	0·07	0·021
LDH (U/g of protein)	254·28	371·64*	239·73	18·43	0·019

CON, control; HC, high-concentrate diet; HCT, high-concentrate diet supplemented with 200 mg of thiamine/kg of DMI; MMP-2, matrix metalloproteinase 2; PDH, pyruvate dehydrogenase; α-KGDH, α-ketoglutarate dehydrogenase; LDH, lactate dehydrogenase.
* Data are expressed as means values with their standard errors. Mean values with different superscripts are significantly as determined by Tukey's test ($P < 0.05$).
n 8 goats/treatment.

were higher ($P < 0.05$) in the HCT group compared with the HC group.

Discussion

This study was designed to investigate the potential regulatory effects of thiamine in simulated prolonged feeding of excess HC diets (extreme conditions); thus, we chose a diet with a higher concentrate to forage ratio (concentrate:forage = 30:70). The study can provide some guidances for intensive production in ruminants through the overfeeding of HC diets. Feeding the HC diets to ruminants causes a high risk to damage the hindgut epithelial structure[41,42]. Prior studies demonstrated that dietary thiamine supplementation alleviates the HC diets-induced oxidative stress, apoptosis and promotes rumen epithelial development in goats[18,24]. Therefore, we focused on the regulation role of thiamine on colonic integrity and mucosal inflammation injury. Our current results showed that long-term feeding of the HC diets can cause a decrease in the pH values, the SCFA and thiamine concentration and an increase in LPS concentration in the colon, while thiamine supplementation reversed these changes. These data revealed that thiamine addition improved microbial fermentation and the hindgut environment. Plaizier *et al.* (2012) uncovered that the fluid flows out of the rumen into the omasum and subsequently into the hindgut contains LPS[43]. Therefore, it is expected that part of the LPS found in the large intestine is derived from gram-negative bacteria of the rumen. This decline in ruminal pH (pH value has been below 5·6 for a long time) can cause the lysis of gram-negative bacteria and release LPS that translocate into the bloodstream and hindgut[4]. In the current study, dietary thiamine supplementation elevated pH value compared with the HC groups from 0 to 12 h after feeding, and ruminal pH was above 6·0 after 4 h of feeding. Thiamine is essential for the growth of most bacteria strains of Ruminococcus, such as *Ruminococcus albus* (cellulolytic bacteria) and *Ruminococcus flavefasciens* (cellulolytic bacteria)[23,44]. Moreover, Wetzels *et al.* (2016) demonstrated that exogenous thiamine supplementation facilitates higher pH, which helped in the proliferation of *Succinivibrio*[45]. Although the exact mechanism of inhibition of HC diet-induced LPS production via thiamine supplementation is unclear, these data may show more valid evidence for us to study the protective effect of thiamine on colonic integrity.
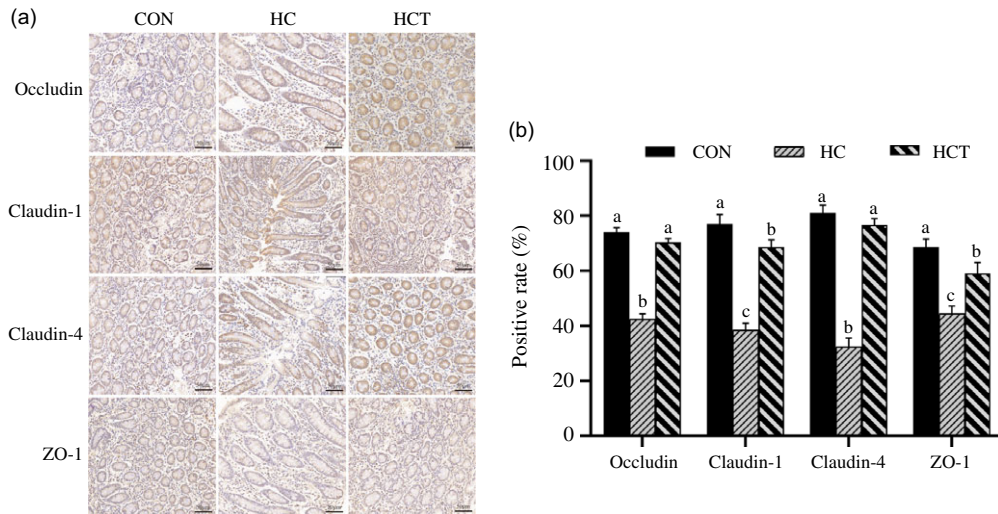
Fig. 3. Effects of dietary thiamine supplementation on the expression and distribution of tight junction proteins in colonic mucosa epithelium. (a) Immunohistochemistry results of occludin, claudin-1, claudin-4 and ZO-1 in colonic mucosa epithelium. (b) Positive rate analysis. All scale bars show 50 μm (n 8 goats/treatment). CON, low-concentrate diet; HC, high-concentrate diet; HCT, high-concentrate diet supplemented with 200 mg of thiamine/kg of DM intake. The mean values in columns without a common superscript letter differ (P < 0·05).

Table 4. Effect of dietary thiamine on the expression of genes in the colonic mucosal tissue of goats
(means values with their standard errors)*

	Dietary treatment				
Item	CON	HC	HCT	SEM	P
Thiamine transport					
SLC19A2	1·0*	0·71	1·13*	0·05	0·034
SLC19A3	1·0	0·42	2·06*	0·12	0·008
SLC25A19	1·0	0·37	4·12*	0·24	0·002
Inflammation					
NF-κB	1·00	1·91*	1·13	0·17	0·023
IL-1β	1·00	2·46*	1·58	0·22	0·016
IL-6	1·00	3·73*	2·54	0·11	0·027
IL-10	1·00	2·15*	1·72	0·13	0·032
CXCL-10	1·00	3·22*	2·94*	0·37	0·041
CXCL-13	1·00	4·27*	2·55	0·42	0·038
MMP-9	1·00	1·75*	1·38	0·09	0·013
MMP-13	1·00	2·08*	1·10	0·11	0·022
Apoptosis					
Bcl-2	1·00*	0·32	0·69	0·08	0·007
Bax	1·00	1·75*	0·91	0·15	0·014
Bcl-2/Bax	1·00*	0·18	0·76	0·11	0·008
Barrier function					
Occludin	1·00*	0·54	0·82	0·06	0·012
Claudin-1	1·00*	0·35	0·93	0·10	0·015
Claudin-4	1·00*	0·56	0·90*	0·13	0·032
ZO-1	1·00*	0·49	0·72	0·15	0·026

CON, control; HC, high-concentrate diet; HCT, high-concentrate diet supplemented with 200 mg of thiamine/kg of DMI; SLC25A2, solute carrier family 19, member 2; SLC19A3, solute carrier family 19; SLC25A19, mitochondrial thiamine pyrophosphate transporter; CXCL, C-X-C motif chemokine ligand; MMP, matrix metalloproteinase; Bcl-2, B-cell lymphoma/leukaemia 2; Bax, Bcl-2 associated X protein; ZO-1, zonula occludens-1.
* Data are expressed as means values with their standard errors. Mean values with different superscripts are significantly as determined by Tukey's test (P < 0 05).
n 8 goats/treatment.

The thiamine concentration in colonic digesta under different dietary concentrate diet levels has rarely been reported. In the present study, the thiamine concentrations in colonic digesta of three groups were 5·47, 1·13 and 2·56 μg/l, respectively.

Dabak and Gul et al. (2004) demonstrated that long-term HC diet feeding decreased thiamine concentration in the rumen due to the reduction in the microbial thiamine synthesis and the degradation by thiaminase under lower pH[22]. Thus, we supposed that the decrease of thiamine concentration in the colon was associated with thiaminase and microbial community. The absorption of thiamine is mediated by carriers[46–47]. In this study, the HC diet reduced the gene expression of SLC19A2, SLC19A3 and SLC25A19 in the colonic epithelium. The transport carriers of thiamine were pH-sensitive[48], and the LPS and pro-inflammatory cytokines could also downregulate the expressions of SLC19A2 and SLC19A3[47]. The current research observed that dietary thiamine supplementation increased the gene expression of transport carriers of thiamine. Our previous results have demonstrated that excessive feeding of the HC diet leading to a decrease in thiamine-related enzyme activities such as PDH, α-KGDH and transketolase in rumen epithelium tissues[24]. In order to investigate whether a similar effect would occur in the colonic mucosa under the stimulation of LPS, we measured the PDH, α-KGDH and LDH enzyme activities in the colonic mucosal epithelium and obtained the same results as the previous study in rumen epithelium, while the supplementation of exogenous thiamine reverse the change. The one possible reason was that exogenous supplementation of thiamine maintains the colon homoeostasis by higher pH and lower LPS, which stimulated the expression of thiamine transport carriers in colonic epithelium. In addition, supplementary thiamine promoted carbohydrate metabolism and production of energy[49], while energy is also a crucial factor for the active transport of thiamine[50].

In intensive production, injury of epithelial barrier function in the hindgut is repeatedly diagnosed in ruminants which are continually fed a HC diet. In contrast to the multilayered structure cellular structure of the ruminal epithelium, the colon only is composed of a monolayer structure[51]. Thus, the integrity of the colon is more easily attacked by a high concentration of
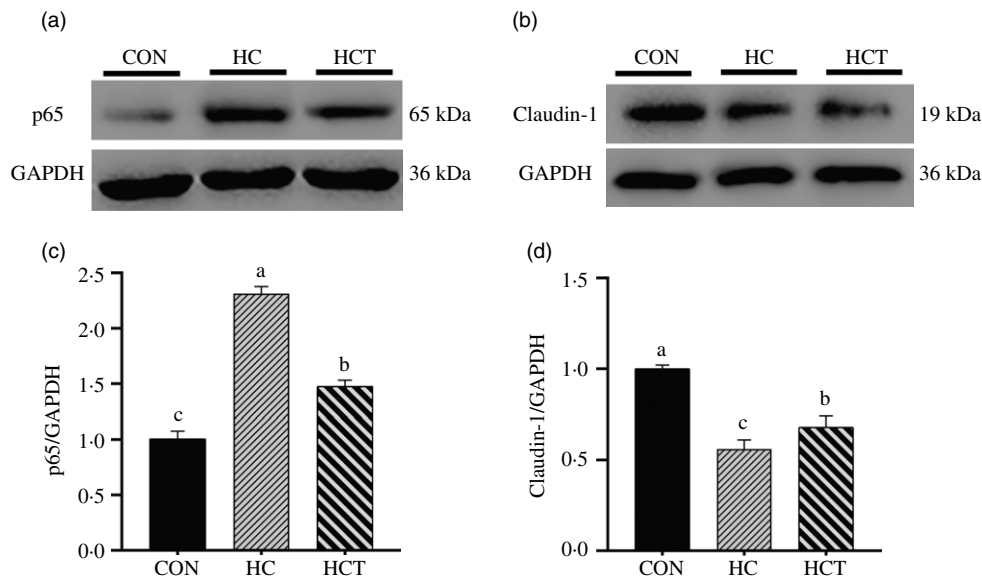
Fig. 4. Effects of dietary thiamine supplementation on barrier function and inflammatory key protein expression. (a) Western blot analysis of p65. (b) Western blot analysis of claudin-1. Results of protein levels expressed as arbitrary units relative to GAPDH protein, fold change of (c) p65 and (d) claudin-1 protein content in the colonic mucosa. CON, low-concentrate diet; HC, high-concentrate diet; HCT, high-concentrate diet supplemented with 200 mg of thiamine/kg of DM intake; GAPDH, glyceraldehyde-3-phosphate dehydrogenase. Values are mean ± SEM. The mean values in columns without a common superscript letter differ ($P < 0.05$).

LPS[52]. Besides, the acidity in the gastrointestinal tract also plays a vital role in maintaining the integrity of the epithelial barrier[53]. The histological analysis showed that dietary thiamine supplementation during long-term the HC diets feeding protects the colonic barrier function, which may be explained that the thiamine supplementation decreases the accumulation of SCFA, lactate and LPS in the colon and weakens the intense stimulation for the colonic epithelial barrier.

As highly dynamic structures, TJ proteins dedicate to the maintenance of physical barrier function[18]. Moreover, TJ also play a crucial role in promoting cell–cell interactions and stabilising the transcellular pathways[54]. Our immunohistochemistry data showed that dietary thiamine supplementation promoted the colon epithelial barrier integrity in comparison with the HC group as confirmed by the markedly changed expression and distribution of TJ in colon epithelium (occludin, claudin-1, claudin-4 and ZO-1). The expression of the TJ gene and protein were also in accord with the data results of immunohistochemistry. Several stimulating factors including lower pH and cumulative toxic compounds from bacterial metabolism have been reported to involve in the process of TJ inhibition caused by feeding the HC diet[55]. Occludin protein is responsible for the transport of the macromolecule[56], with ZO-1 as an organising component of the TJ link occludin to the cortical actin cytoskeleton[57]. Thus, the coordination of occludin and ZO-1 is of profound significance for the integrity of the epithelium and the transport of nutrients. Claudins consist of several gene families, which have the role to enhance cell proliferation[58]. Dietary thiamine supplementation promotes an increase in pH and a decrease in LPS levels in colon, which may be one of the reasons for the protection of the colon integrity. Although our previous studies have confirmed that thiamine can promote the development of rumen epithelium[24], the exact action

mechanism of thiamine for the promotion of TJ expression is still unclear.

The HC diet-induced LPS triggers the injury and death of intestinal mucosal epithelial cells involved in apoptotic signal transduction pathway, which impaired mucosal barrier function, leading to epithelial leakage[59]. The caspases family play a vital role during the initiation and effector stages of cell apoptotic among the multiple molecules involved in the apoptotic programme[60]. By regulation of apoptotic components, cytochrome C forms complex, then the apoptosome which via caspase 9 triggers executioner caspase 3 and eventually induces cell death[61]. The caspase 8 regulates and controls ligand binding triggers apoptosis in the extrinsic pathway[42]. The Bcl-2 families of proteins, including Bax (pro-apoptotic) and Bcl-2 (anti-apoptotic), are vital regulators in the early phase of the apoptotic pathway. The change in the expression of the Bcl2/Bax ratio would affect dramatically the apoptotic rate and would eventually alter the phenotypic status of the cell[59]. Samo *et al.* (2020) revealed that long-term HC diet feeding can induce epithelial injury and apoptosis in the colon. In the present study[42], Bax mRNA levels were significantly decreased, whereas the Bcl-2 mRNA level and the Bcl2/bax ratio were significantly increased in HCT goats compared with that in the HC goats. Moreover, the results of enzyme activity analysis in colonic mucosa showed that the HCT group had a markedly decrease in caspase-3 and caspase-8 enzyme activity compared with the only HC diet feeding, which suggests that thiamine contributes to the attenuation of the HC diet-induced apoptosis through the regulation of the Bax/Bcl-2 ratio and the caspase pathway.

Alleviation of inflammation in the colonic mucosa can improve the integrity of the colon. Zhang *et al.* (2020) confirmed that the effects of thiamine supplementation on local inflammation in the ruminal epithelium could be associated with a

reduced ruminal LPS content[28]. In addition, SCFA have also been demonstrated to involve leucocyte migration and modulate the production of certain cytokines such as TNF-α and IL-6[62]. Therefore, we analysed the mRNA expression levels of NF-κB and other related inflammatory genes, and the results suggested that the gene expression levels of NF-κB, IL-1β, IL-6 and IL-10 were lower in the HCT treatment than in the HC goats. Western blot data showed the decline of NF-κB-associated proteins p65 in HCT goats. Long-term exposure of rumen epithelial cells to LPS significantly upregulates the expression of chemokines and induces acute inflammatory responses[63]. These chemokines are crucial chemo-attractants for the recruitment of leucocytes during inflammatory responses. Thus, the mRNA expression levels of CXCL-10 and CXCL-13 were also lower in the HCT goats than in HC group, though the decrease of CXCL-10 mRNA expression was not significant. These results revealed that thiamine regulates the expression of inflammatory cytokines and chemokines in the colonic mucosa of goats, which may be attributed to the inhibitory effect of thiamine-supplemented HC diet compared with long-term HC diet to induce inflammation. Prior research has also suggested that NF-κB signal pathway can damage barrier function by motivating the release of inflammatory factors[64], while the fact has been confirmed that thiamine alleviates inflammation and protects barrier function by regulating the NF-κB/TLR4 pathway[18,28].

In this study, we also measured the expression of MMP2, MMP9 and MMP13 related to defence functions in the gastrointestinal tract. Thiamine caused a significant down-regulation in mRNA expression of MMP-9 and MMP-13 in colonic mucosa epithelium during long-term HC diet feeding. Furthermore, the enzyme activities of MMP2 and MMP9 were assessed using gelatin zymography, and our results also demonstrated that dietary thiamine supplementation decreased the activation of MMP2 and MMP9 enzymes. The TJ along with the extracellular matrix are the dominating substrates for MMP[65]. Furthermore, Wu and Schmid-Schonbein (2011) found that NF-κB involves in the activation of MMP[66]. As a result, thiamine supplementation can downregulate the expression of MMP that may be associated with alleviation of inflammation.

In general, the results from this study demonstrate that colonic inflammation will be induced in goats fed the HC diet for 12 weeks that is reflected in desquamation and severe cellular damage in colon epithelium. However, thiamine, as a water-soluble vitamin, reversed the adverse effects and decreased the cells apoptosis by suppressing the NF-κB activation and the production of inflammatory cytokines. Thus, these findings provide a new strategy that thiamine could potentially serve as a dietary supplementation to contribute to alleviating the negative effects of the trigger of the HC diet.

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Supplementary material

For supplementary material accompanying this paper visit https://doi.org/10.1017/S0007114522000174

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