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Research Paper

Activation of cellular apoptosis in the caecal epithelium is associated with increased oxidative reactions in lactating goats after feeding a high-concentrate diet

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New Findings

- **What is the central question of this study?**
  What are the ultrastructural changes of the caecal mucosa and the status of epithelial cellular apoptosis and oxidative reactions in lactating goats after prolonged feeding with a high-concentrate diet?
- **What is the main finding and its importance?**
  High-concentrate diet results in ultrastructural damage to the caprine caecal epithelium. Increased oxidative and decreased antioxidative reactions are involved in the process of activating epithelial apoptosis in the caecal epithelium of goats fed a high-concentrate diet. Our results provide new insight into the relationship between abnormal fermentation in the hindgut and damage to the intestinal mucosal barrier.

The effect of feeding a high-concentrate diet (HC) to lactating ruminants on their hindgut epithelial structure remains unknown. In this study, 12 lactating goats were randomly assigned to either HC (65% of dry matter as concentrate; $n = 6$) or a low-concentrate diet (LC; 35% of dry matter as concentrate; $n = 6$). After 10 weeks, the epithelial ultrastructure and cell apoptotic status in the caecal mucosa were determined by transmission electron microscopy and TUNEL, respectively. The results showed that the level of free lipopolysaccharide ($P < 0.05$), total volatile fatty acid concentrations ($P < 0.1$) and starch content ($P < 0.05$) in the caecal digesta were significantly increased in HC- compared with LC-fed goats. The HC-fed goats exhibited obvious epithelial cellular damage, with widened tight junction spaces, nuclear breakdown and mitochondrial swelling. Compared with their LC-fed counterparts, HC-fed goats showed greater apoptosis in the caecal epithelium, as evidenced by more TUNEL-positive apoptotic cells. Western blot analysis showed that there was no significant difference in activated caspase-3, Bax protein expression in caecal epithelial mucosa between HC- and LC-fed goats ($P > 0.05$). However, the level of malondialdehyde content in the caecal epithelium from HC-fed goats was markedly higher than that in LC-fed goats ($P < 0.05$), whereas the level of glutathione peroxidase and the superoxide dismutase activity were significantly decreased. Gene expressions of cytokines, including interleukin-1β, interleukin-6, interleukin-10, tumour necrosis factor-α

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and interferon-γ, as well as myeloperoxidase activity in the caecal mucosa did not show any significant difference between HC- and LC-fed goats. These results indicate that feeding a high-concentrate diet to lactating goats for a prolonged period results in abnormal fermentation and structural disruption in the hindgut, which is accompanied by greater cellular apoptosis and an enhanced oxidative stress response.

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Introduction

To meet the energy or Bcl-2 requirement for supporting maintenance and high milk yields, large amounts of cereal grains or easily degradable dietary byproducts are fed to lactating cows. It is well known that excessive amounts of rapidly fermentable non-structural carbohydrates increase the accumulation of organic acids and, subsequently, result in subacute ruminal acidosis and metabolic disorders in ruminants (Gaebel *et al.* 1989; Plaizier *et al.* 2008; Li *et al.* 2012) that can cause inflammatory responses due to the accumulation of bacterial endotoxins (lipopolysaccharide, LPS) derived from Gram-negative bacteria residing in the rumen and hindgut (Khafipour *et al.* 2009; Zebeli & Ametaj, 2009). Increased LPS in the hindgut is likely to damage the histological structure of the mucosal barrier in the gastrointestinal (GI) tract (Steele *et al.* 2011). The effect of a high-grain diet on rumen fermentation and structural damage has received considerable attention (Klevenhusen *et al.* 2013; Liu *et al.* 2013), but little information is available on hindgut caecal fermentation.

The caecum is one of the main fermentation sites and a major absorptive area in the hindgut of ruminants. It has been reported that caecal fermentation accounts for 12% of the total volatile fatty acid (VFA) production in sheep (Faichney, 1968). Furthermore, it was reported that a high-grain diet increases caecal fermentation, with high concentrations of VFA in the caecal digesta (Gressley *et al.* 2011; Li *et al.* 2012), indicating an important energy supply from the hindgut. However, luminal acidity is one of the most important determining factors of the status of the epithelial barrier; high levels of VFA induced cellular apoptosis (Lan *et al.* 2007). Moreover, higher endotoxin LPS derived from Gram-negative bacteria is an extreme risk factor for damage to the integrity of the epithelial barrier. It has been reported that a high concentration of LPS increased localized epithelial apoptosis and permeability and that these changes were dependent on caspase-3 activation (Chin *et al.* 2006). The rumen epithelium consists of a stratum corneum layer and multicellular layers in the middle, whereas the caecal epithelium is composed of only a single layer of epithelial cells (Plaizier *et al.* 2012), making it more susceptible to damage and much more prone to 'leakiness' of bacterial endotoxins compared with the rumen.

It is well documented that several pathways are involved in the cell apoptotic programme. One pathway is mediated by the formation of the death-inducing signalling complex and activation of caspases (Plaizier *et al.* 2012); another is mediated by pro-apoptotic signals at the mitochondrial level, which include the B-cell lymphoma 2 (Bcl-2) family. Additionally, oxidative stress, defined as the failure of antioxidant defense mechanisms in mitigating the harmful effects of reactive oxygen species (ROS; Sies, 1997), is an important process that can lead to epithelial cell damage in GI mucosal diseases (Bhattacharyya *et al.* 2014). Several essential events, including production of ROS, caspase activation and loss of mitochondrial membrane potential, commit a cell to undergo apoptosis (Anuradha *et al.* 2001). To the best of our knowledge, the effect of feeding ruminants with a diet enriched with a high level of concentrate on caecal epithelial structure at either the tissue or the molecular level has not been reported. The aim of this study, therefore, was to investigate the ultrastructural changes in the caecal mucosa, the status of epithelial cellular apoptosis and the oxidative stress response in lactating goats after prolonged feeding with a high-concentrate diet (HC).

Methods

Ethical approval

All animal procedures were approved by the Institutional Animal Care and Use Committee of Nanjing Agricultural University. The protocol of this study was reviewed and approved specifically, with the project number 2011CB100802. The slaughter and sampling procedures strictly followed the '*Guidelines on Ethical Treatment of Experimental Animals*' (2006) no. 398 set by the Ministry of Science and Technology, China and the '*Regulation regarding the Management and Treatment of Experimental Animals*' (2008) no. 45 set by the Jiangsu Provincial People's Government.

Animals and experimental procedures

The details of animals and the experimental procedures were presented previously (Tao *et al.* 2014). In brief, 12 lactating goats were allowed free access to a control diet containing a forage-to-concentrate ratio of 65:35 for 2 weeks. After dietary adaptation, goats were randomly allocated to two groups. Control group goats were fed a control diet comprising 65% forage and 35% mixed concentrate [low-concentrate (LC) group], while the other group received a high-grain diet containing 65% mixed concentrate and 35% forage [high-concentrate (HC) group]. The animals were fed the respective diets for 10 weeks and had free access to water during the experimental period.

Sample collection

At the end of the experiment, after an overnight fast, all goats were killed by I.V. injections of xylazine [0.5 mg (kg body weight)$^{-1}$; Xylosol; Ogris Pharme, Wels, Austria] and pentobarbital [50 mg (kg body weight)$^{-1}$; Release; WDT, Garbsen, Germany]. Immediately after death, caecal mucosal tissues were carefully removed. Digesta from the proximal caecum was aseptically collected and kept on ice until being stored at −20 °C. Within 20 min of death, a segment of the caecal mucosa from the same position in each animal was collected. The caecal epithelium was separated from the muscular layers by blunt dissection and immediately washed three times in ice-cold PBS. The tissue samples were frozen immediately in liquid nitrogen and then used for extraction of RNA and proteins.

Caecal digesta sampling and assay

Caecal digesta samples were thoroughly mixed with an equal amount of physiological saline (0.90% w/v of NaCl). The mixtures were immediately centrifuged at 3000 *g* for 15 min, and the supernatants were stored at −20 °C until analysed for LPS and VFA detection.

The concentration of LPS in caecal digesta was measured by a Chromogenic End-point Tachypleus Amebocyte Lysate Assay Kit (Chinese Horseshoe Crab Reagent Manufactory Co. Ltd, Xiamen, China). Pretreated caecal digesta samples were diluted until their LPS concentrations were in the range of 0.1–1.0 endotoxin units ml^{-1} relative to the reference endotoxin, and assayed as described by Gozho *et al.* (2005).

Volatile fatty acids were measured using capillary column gas chromatography (GC-14B; Shimadzu, Tokyo, Japan; Capillary Column: 30 m × 0.32 mm × 0.25 mm film thickness; column temperature 110°C; injector temperature 180°C; and detector temperature 180°C).

Caecal digesta samples were dried at 60°C for 48 h. Dried samples were subsequently ground using a Wiley mill through a 1 mm screen (Thomas-Wiley, Philadelphia, PA, USA) and stored at −20°C until analysis for starch using a Total Starch assay kit (Comin Biotechnology Co. Ltd, Suzhou, China).

Transmission electron microscopy and TUNEL

Caecal mucosal tissue samples were separated and fixed immediately with 2% glutaraldehyde (24 h), postfixed with 1% osmium tetroxide (1 h) and embedded in resin. Ultrathin sections were cut and stained with uranyl acetate and lead citrate. Epithelial tissue ultrastructure was determined with a transmission electron microscope (Hitachi H-7650; Hitachi Technologies, Tokyo, Japan).

Apoptotic epithelial cells in caecal tissue were analysed using the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labelling (TUNEL) assay according to the manufacturer's instructions. The apoptosis detection kit was supplied by Boster Bio-engineering Limited Company (Wuhan, China). TUNEL-positive nuclei were clearly identified as brown-stained nuclei, which indicate the presence of DNA fragmentation due to apoptosis. TUNEL-positive cells were determined by observing 1000 cells in randomly selected fields.

RNA isolation, cDNA synthesis and real-time PCR

Caecal mucosal tissue was quickly collected, immediately frozen in liquid nitrogen and stored at −80 °C until RNA isolation. Total RNA was extracted from colonic samples with TRIzol reagent (15596026; Invitrogen, Shanghai, China). The concentration and quality of the RNA were measured with a NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Madison, WI, USA). Then 2 μg of total RNA was treated with RNase-Free DNase (M6101; Promega, Madison, WI, USA) and reverse transcribed according to manufacturer's instructions. Two microlitres of diluted cDNA (1:40, v/v) was used for real-time PCR, which was performed in an Mx3000P (Stratagene, USA). Glyeraldehyde 3-phosphate dehydrogenase (GAPDH), which is not affected by the experimental factors, was chosen as the reference gene. All the primers chosen to study the expression of genes related to cytokine and endoplasmic reticulum stress, as listed in Table 2, were synthesized by Generay Company (Shanghai, China). The method of $2^{-\Delta\Delta C_t}$ was used to analyse the real-time PCR results, and gene mRNA levels were expressed as the fold change relative to the mean value of the control group.

Table 1. The concentrations of free lipopolysaccharide, starch and volatile fatty acids in caecal digesta of low-concentrate (LC)- and high-concentrate (HC)-fed goats

Paramter	LC group	HC group	*P* Value
Free lipopolysaccharide (endotoxin units ml^{-1})	1426.30 ± 753.79	18,611.83 ± 6333.99	0.049
Starch (mg g^{-1})	3.58 ± 0.17	4.11 ± 0.11	0.025
Total volatile fatty acids (mM)	46.52 ± 4.34	56.98 ± 2.89	0.068
Acetate (mM)	28.13 ± 3.35	35.45 ± 2.34	0.099
Propionate (mM)	10.99 ± 0.64	12.83 ± 0.34	0.026
Butyrate (mM)	4.37 ± 0.39	5.74 ± 0.34	0.026
Isobutyrate (mM)	0.91 ± 0.05	0.84 ± 0.01	0.232
Valerate (mM)	1.15 ± 0.04	1.22 ± 0.08	0.424
Isovalerate (mM)	0.97 ± 0.04	0.89 ± 0.01	0.073
Acetate:propionate ratio	2.53 ± 0.16	2.75 ± 0.14	0.334

Values are expressed as the means ± SEM, $n = 6$.

Western blotting analysis

One hundred milligrams of frozen caecal mucosal tissue was minced and homogenized in 1 ml of ice-cold RIPA buffer containing the protease inhibitor cocktail Complete EDTA-free (Roche, Penzberg, Germany). The homogenates were centrifuged at 12235 *g* for 20 min at 4 °C and then the supernatant fraction was collected. The protein concentration was determined using a BCA Protein Assay kit (Pierce, Rockford, IL, USA). Eighty micrograms of protein extract from each sample was then loaded into 15% SDS-PAGE gels, and the separated proteins were transferred onto nitrocellulose membranes (BioTrace; Pall Corp., New York, NY, USA). After transfer, membranes were blocked for 2 h at room temperature in blocking buffer and then incubated with the following primary antibodies: rb-anti-Bax (1:500; BS6420; Bioworld, St. Louis Park, MN, USA), rb-anti-Bcl-2 (1:500; BS1511; Bioworld), rb-anti-actived-caspase-3 (1:500; BS7004; Bioworld) and anti-GAPDH (1:10,000; AP0066; Bioworld) in dilution buffer overnight at 4 °C. After several washes in Tris-buffered-saline with Tween, membranes were incubated with goat anti-rabbit HRP-conjugated secondary antibodies (1:10,000; Bioworld) in dilution buffer for 2 h at room temperature. Finally, the blot was washed and detected by enhanced chemiluminescence using the LumiGlo substrate (Super Signal West Pico Trial Kit; Pierce, Thermo Fisher Scientific, Madison, WI, USA) and the signals were recorded by an imaging System (Bio-Rad, Hercules, CA, USA) and analysed with Quantity One software (Bio-Rad).

Malondialdehyde (MDA) content and glutathione peroxidase (GSH-Px), superoxide dismutase (SOD) and myeloperoxidase (MPO) activity assays

Malondialdehyde content, GSH-Px, SOD and MPO activity of the caecal mucosa tissue were measured by goat MDA, GSH-Px, SOD and MPO ELISA kits (Shanghai Enzyme-linked Biotechnology Co. Ltd, Shanghai, China), res-pectively. The procedures were performed according to the manufacturer's instructions.

Statistical analysis

Data are presented as means ± SEM. The data were tested for normal distribution, and statistical significance was assessed by Student's unpaired *t* test using the software package SPSS version 11.0 for Windows (SPSS Inc., Chicago, IL, USA). Data were considered statistically significant when $P < 0.05$. Numbers of replicates used for statistics are noted in the tables and figures.

Results

Volatile fatty acid and LPS concentrations in caecal digesta

Compared with LC-fed goats, propionate ($P < 0.05$) and butyrate concentrations ($P < 0.05$) in caecal digesta were significantly increased, and the level of acetate ($P < 0.1$) and total VFA ($P < 0.1$) showed a tendency to increase in caecal digesta of HC-fed goats. The HC-fed goats showed a significantly higher level of free LPS ($P < 0.05$) and starch content ($P < 0.05$) in caecal digesta than the LC-fed goats (Table 1).

Ultrastructure and TUNEL of the caecal epithelial tissues

The ultrastructure of caecal epithelium was detected by the transmission electron microscopic method. The results showed that HC-fed goats exhibited damaged tight junctions with wider intercellular spaces, while LC-fed goats displayed integrity and normal tight junctional

Table 2. PCR primer sequences of the target genes

Target genes	Reference/Genbank accession	PCR products(bp)	Primer sequence (5′→3′)
Glyeraldehyde 3-phosphate dehydrogenase	HM043737.1	180	Forward: GGGTCATCATCTCTGCACCT
			Reverse: GGTCATAAGTCCCTCCACGA
Interleukin-1β	D63351.1	173	Forward: CATGTGTGCTGAAGGCTCTC
			Reverse: AGTGTCGGCGTATCACCTTT
Interleukin-6	D86569.1	241	Forward: CCAATCTGGGTTCAATCAGG
			Reverse: ACCCACTCGTTTGAGGACTG
Interleukin-10	DQ837159.1	239	Forward: TTAAGGGTTACCTGGGTTGC
			Reverse: CCCTCTCTTGGAGCATATTGA
Tumour necrosis factor-α	X14828.1	155	Forward: CAAGTAACAAGCCGGTAGCC
			R: AGATGAGGTAAAGCCCGTCA
Interferon-γ	U34232.1	166	Forward: TGATTCAAATTCCGGTGGAT
			Reverse: GCAGGCAGGAGAACCATTAC
GRP78	AJ586431.1	87	Forward: CCCTGACGAAAGACAATC
			Reverse: TGACTTCAATCTGTGGGAC
CHOP	NM_001078163.1	284	Forward: TCTGGCTTGGCTTACTG
			Reverse: TTGCCTCCACTTCCCT

structure (Fig. 1*A* and *D*). Compared with LC-fed goats, with normal cell nuclei and mitochondrial structure, HC-fed goats displayed apparent nuclear breakdown and mitochondrial swelling (Fig. 1*B*–*F*). Figure 2 shows that the proportion of TUNEL-positive apoptotic cells in the caecal epithelium of HC-fed goats was markedly increased compared with LC-fed goats ($P < 0.05$).

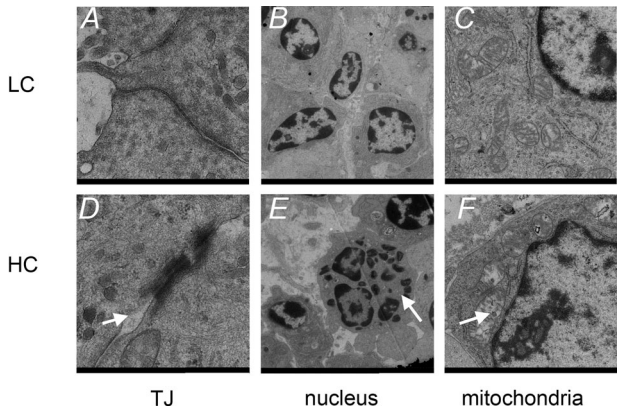

Figure 1. Comparison of ultrastructure of the caecal mucosa between goats fed hig-concentrate diet (HC) and low-concentrate diet (LC)
Caecal mucosal epithelium samples ($n = 6$) from each group were processed for ultrastructural evaluation. Shown are tight junctions (TJ) of the LC group (*A*), a nucleus of the LC group (*B*), mitochondria of the LC group (*C*), tight junctions of the HC group (*D*), a nucleus of the HC group (*E*) and mitochondria of the HC group (*F*). Transmission electron microscopy, ×10,000 magnification (scale bar represents 500 nm). Arrow indicates the location of the tight junctions, nucleus or mitochondria.

Protein expression in the caecal mucosa

As shown in Fig. 3, there was no significant difference in actived caspase-3, Bax and Bcl-2 protein levels in the caecal mucosa between LC- and HC-fed goats ($P > 0.05$).

Malondialdehyde content and GSH-Px, SOD and MPO activities in the caecal mucosa

The level of MDA in the caecal mucosa of HC-fed goats was significantly higher than that in LC-fed goats ($P < 0.05$). The GSH-Px and SOD activities were markedly decreased in HC-fed goats compared with LC-fed goats ($P < 0.05$). There was no significant difference in MPO activity in the caecal mucosa between LC- and HC-fed goats (Fig. 4).

Gene expression in the caecal mucosa

In the caecal mucosa, CHOP mRNA expression was significantly increased in HC-fed goats compared with control ($P < 0.05$). However, the mRNA expression of GRP78, interleukin-1β, interleukin-6, interleukin-10, tumour necrosis factor-α and interferon-γ in the caecal mucosa did not show any significant differences between HC- and LC-fed goats ($P > 0.05$; Fig. 5).

Discussion

Feeding excessive amounts of highly fermentable forages and insufficient dietary coarse fibre leads to subacute ruminal acidosis as well as disordered fermentation in the hindgut, which is likely to result in the accumulation of

bacterial endotoxin derived from Gram-negative bacteria in the GI tract (Zebeli & Ametaj, 2009). As a physical barrier, the intestinal epithelial mucosa separates toxic compounds from the deeper intestinal layers (Turner, 2009; Wardill & Bowen, 2013). Feeding high-grain diets to ruminants causes a high risk of damage to the integrity of the barrier and the functions of the ruminal epithelium in goats (Steele *et al.* 2011; Liu *et al.* 2013). Histological differences between the rumen and the large intestine may imply that the barrier function of the epithelium of the large intestine is more easily compromised by high acidity and high LPS concentrations than that of the rumen. Our previous study demonstrated that feeding a high-concentrate diet to lactating goats led to severe damage of the colonic mucosal tissues (Tao *et al.* 2014). In the present study, we found that feeding a high-concentrate diet to lactating goats for 10 weeks resulted in ultrastructural disruption in the caecal mucosa, as evidenced by wider tight junctions, apparent nuclear breakdown and mitochondrial swelling. The control goats fed a low-concentrate diet displayed integrity and normal structure of the caecal mucosa.

Luminal acidity of the GI tract is one of the most important factors in determining the status of the epithelial barrier. A previous study demonstrated that acetate treatment in the pH range of 6.0–7.0 induced

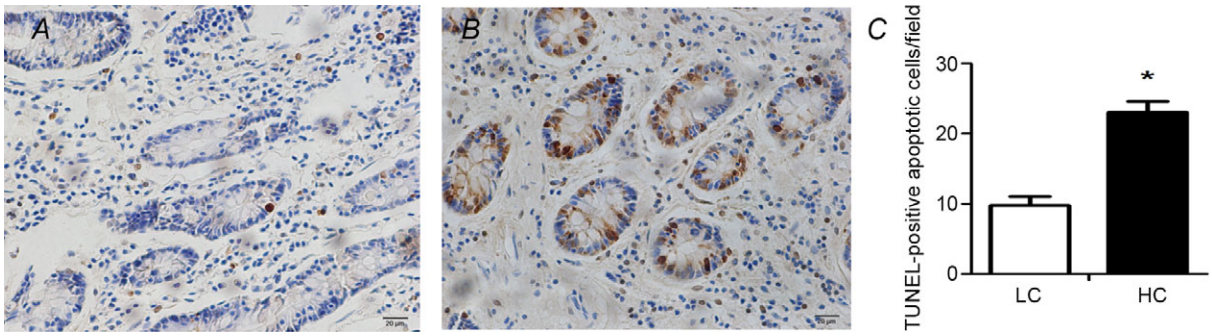

Figure 2. TUNEL comparison of the caecal mucosa between HC- and LC-fed goats
Caecal mucosal epithelium samplese (n = 6) from each group were processed for evaluation of TUNEL-positive apoptotic cells. Shown are sections of caecum from the LC group (*A*) and from the HC group (*B*). Scale bar represents 20 μm. *C*, analysis of the positive apoptotic cells. The results are shown as means + SEM. The data were analysed by Student's unpaired *t* test using the Compare Means function of SPSS 11.0 for Windows. $^{*}P < 0.05$ *versus* LC.

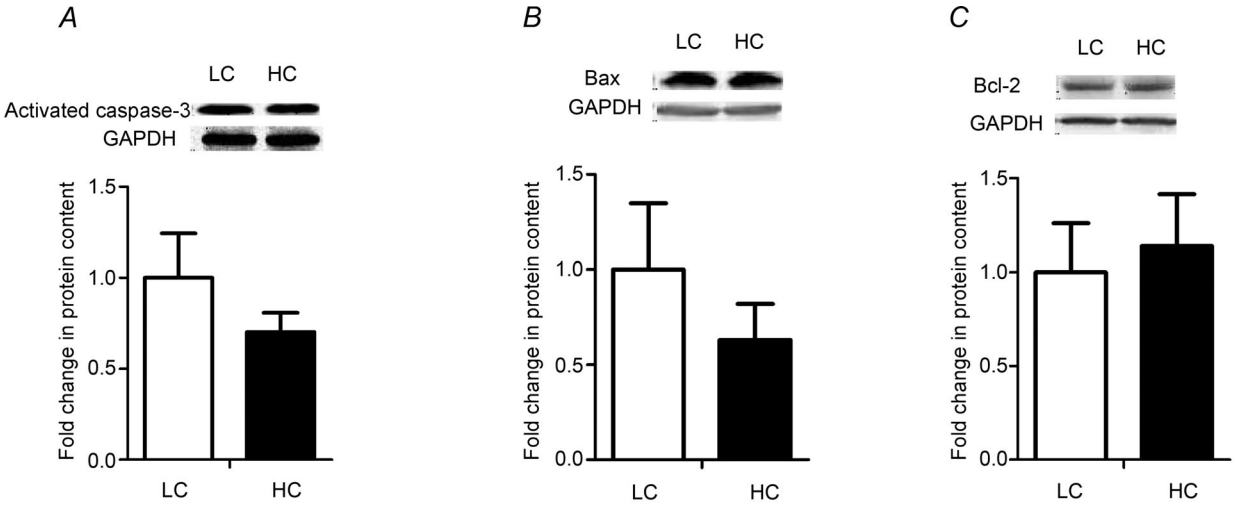

Figure 3. Actived-caspase-3, Bax and Bcl-2 protein expression in the caecal mucosa
Protein level are normalized to glyeraldehyde 3-phosphate dehydrogenase (GAPDH) and shown as the fold change of actived-caspase-3 (*A*), Bax (*B*) and Bcl-2 protein content (*C*). The results are shown as means + SEM. The data were analysed by Student's unpaired *t* test using the Compare Means function of SPSS 11.0 for Windows.

cellular apoptosis rather than necrosis, while acetate treatment at pH 5.5 caused cellular necrosis in a human colon adenocarcinoma cell line (Lan *et al.* 2007). Feeding a high-grain diet to goats increased the caecal digesta concentrations of total VFA (Liu *et al.* 2014). Consistently, we found that HC-fed goats showed a higher level of total VFA in caecal digesta than LC-fed goats. Unfortunately, in this study the pH values were not measured because of insufficient volume of caecal digesta. The effects of luminal pH in the caecum on the epithelial barrier still requires further study.

As one of the most potent inflammatory mediators and a major structural component of Gram-negative bacteria, LPS has been hypothesized to be an important risk factor for intestinal bowel disease (Caradonna *et al.* 2000). In ruminants, the increase in the ruminal LPS concentration due to increased starch feeding is well documented. It has been reported that the increased LPS concentration in the caecum in grain-based subacute ruminal acidosis challenge is due to increased growth of LPS-producing bacteria in the hindgut but not in the rumen (Takizawa *et al.* 2012). In addition, after postruminal infusion of starch, an increase in the Gram-negative bacteria in the caecal digesta was observed (Van Kessel *et al.* 2002). In good agreement with previous studies, in our study we observed a significant increase in LPS in the caecal digesta from HC-fed goats; the starch content in caecal digesta was also markedly increased compared with LC-fed goats. It was reported that a high-grain diet caused an increased concentration of LPS in the caecum and created a health risk for cows (Li *et al.* 2012). Treatment of IEC-6 cells (intestinal epithelial cell line) with LPS can reduce proliferation and promote increased rates of apoptosis and necrosis (Cai *et al.* 2014). In pigs, acetic acid showed a time- and pH-dependent ability to damage the colonic epithelium (Argenzio, 1999). Based on these investigations, it is reasonable to speculate that the increased concentration of VFA and starch content may contribute to the damaged caecal epithelial ultrastructure in HC-fed goats. However, in the present study, the increased concentration of LPS in the caecal epithelium caused by the high-concentrate diet was not accompanied by any change of MPO activity or mRNA expression of the cytokines interleukin-1β, interleukin-6, interleukin-10,

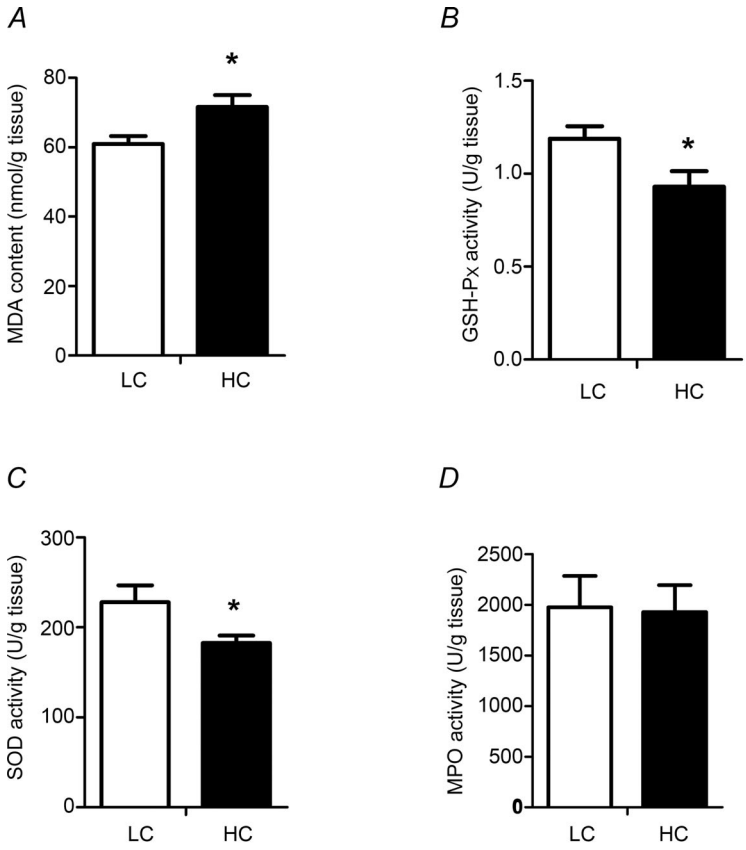

Figure 4. The malondialdehyde (MDA) content (*A*) and the gluathione peroxidase (GSH-Px; *B*), superoxide dismutase (SOD; *C*) and myeloperoxidase (MPO) activities (*D*) in the caecal mucosa
The results are shown as means + SEM. The data were analysed by Student's unpaired *t* test using the Compare Means function of SPSS 11.0 for Windows. $^*P < 0.05$ *versus* LC.

tumour necrosis factor-α and interferon-γ. These results indicate that the LPS signalling pathways may not involved in the damage to caecal mucosa in HC-fed goats.

It has been reported that high concentration of LPS increased the localized epithelial apoptosis and permeability and that these changes were dependent on caspase-3 activation (Chin *et al.* 2006). In addition, short-chain fatty acids increased localized epithelial apoptosis and necrosis, and these changes were dependent on caspase activation (Lan *et al.* 2007). In the present study, epithelial apoptotic status was determined by TUNEL staining, which showed that the number of positive apoptotic cells was significantly higher in HC- than LC-fed goats. Moreover, HC-fed goats demonstrated a marked appearance of dark brown apoptotic cells and intercellular apoptotic fragments compared with LC-fed goats. It is well documented that caspases are key effectors responsible for many morphological and biochemical changes in apoptosis (Thornberry & Lazebnik, 1998); Bcl-2-related proteins are believed to be intracellular mediators of cellular apoptosis. Our previous study showed that the severe structural disruption in the colonic mucosa induced by the high-concentrate diet was associated with the activation of cellular apoptosis and an increase of caspases, as well as a significant decrease in antiapoptosis as indicated by the lower ratio of Bcl-2/Bax mRNA expression (Tao *et al.* 2014). In the present study, however, the activated cellular apoptosis in the caecal epithelium caused by the high-concentrate diet was not accompanied by any change of caspase-3, Bax or Bcl-2 protein content or mRNA expression (data not shown), indicating that other pathways are involved in this activated apoptosis process. It has been reported that the activation of endoplasmic reticulum stress mediated apoptosis and suppressed antioxidant protection (Sun *et al.* 2014). In the present study, as a biomarker of endoplasmic reticulum stress, CHOP mRNA expression was significantly increased in HC-fed goats compared with LC-fed goats. It is reasonable to speculate that the endoplasmic reticulum stress pathway could be involved in the activated apoptosis process in HC-fed goats.

It has been suggested that oxidative stress plays a role as a common mediator of apoptosis (Buttke & Sandstrom, 1994). The ability of oxidative stress to provoke apoptosis as a result of massive cellular damage has been associated with lipid peroxidation and alterations in proteins and nuclei (Halliwell & Gutteridge, 1986; Korsmeyer *et al.* 1995). Reactive oxygen species have many positive functions in living organisms (Ullrich *et al.* 1989; Sohal, 1993), but an uncontrolled increase in their production may have harmful effects (Barclay & Vinqvist, 1994). In the present study, as a biomarker for peroxidation, the MDA content in the caecal mucosa of HC-fed goats was markedly higher than that in LC-fed goats. Living organisms protect themselves against ROS by antioxidant defense systems that are responsible for establishing the balance between the generation and removal of ROS (Sies, 1993). The defense systems against ROS include enzymes such as GSH-Px and SOD, which are able to scavenge free radicals. Our results showed that HC-fed goats showed a significant decrease in GSH-Px and SOD activity in caecal mucosa compared with LC-fed goats. These results suggest that the high-concentrate diet induced increased oxidative reactions and decreased antioxidative effects in the caecal mucosa of lactating goats.

In conclusion, these results suggest that feeding a high-concentrate diet to lactating goats for a long period results in the accumulation of LPS and VFA in the caecal digesta and causes severe damage to the caecal epithelial ultrastructure. Increased oxidative reactions and

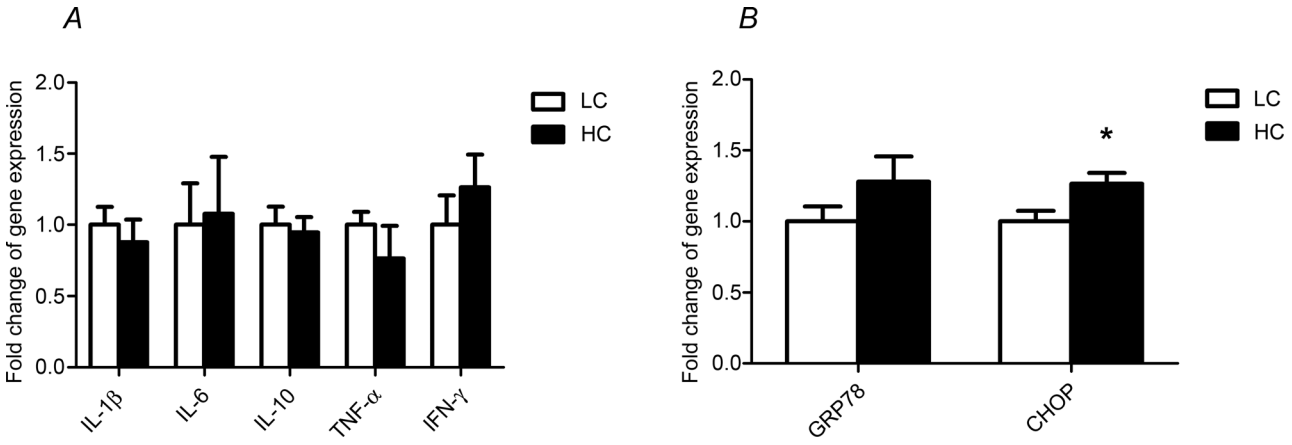

Figure 5. Gene expression in the caecal mucosa
Glyeraldehyde 3-phosphate dehydrogenase was used as the reference gene for gene expression. Abbreviations: IFN-γ, interferon-γ; IL, interleukin; and TNF-α, tumour necrosis factor-α. The results are shown as means + SEM. The data were analysed by Student's unpaired *t* test using the Compare Means function of SPSS 11.0 for Windows. $^*P < 0.05$ *versus* LC.

decreased antioxidative effects might be involved in the process of activating epithelial cellular apoptosis in the caecal epithelium of HC-fed goats.

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Additional information

Competing interests

None declared.

Author contributions

T.S. performed the experiment and drafted the manuscript. R.C., J.T., L.S., Y.D. and H.D. performed the experiment and analysed the data. R.Z. contributed to experimental design and manuscript revision. Y.N. conceived the idea, designed the experiment and finalized the manuscript. All authors read and approved the final manuscript.

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