Effect of Clostridium butyricum on Lipopolysaccharide-Induced Intestinal Injury in Acutely Stressed Rats: A Postprint

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

Abstract

This experiment aimed to investigate the effects of Clostridium butyricum on growth performance in healthy rats and on intestinal structure, intestinal disaccharidase activity, and intestinal inflammation in rats with LPS-induced acute stress. Thirty-six male SD rats were selected and divided into control, LPS, and LPS+Clostridium butyricum groups based on body weight, with 4 rats per cage and 3 cages per group. The control and LPS groups were fed a basal diet, while the LPS+Clostridium butyricum group was fed the basal diet supplemented with 0.05% Clostridium butyricum. On day 40 of the experiment, rats in the LPS and LPS+Clostridium butyricum groups were weighed and intraperitoneally injected with LPS at a dose of 4 mg/kg BW (LPS concentration 1.5 mg/mL, injection volume approximately 1.0 mL), while control rats were intraperitoneally injected with 1 mL of normal saline, and were euthanized for sampling 3 hours later. The results showed: 1) Clostridium butyricum had no significant effect on final body weight, average daily gain, average daily feed intake, or feed-to-gain ratio in healthy rats ($P<0.05$). 2) Compared with the control group, LPS injection significantly increased duodenal crypt depth ($P<0.05$), and significantly decreased duodenal villus-to-crypt ratio, jejunal mucosal thickness, and the number of intraepithelial lymphocytes in the jejunum and ileum ($P<0.05$). Compared with the LPS group, prophylactic feeding of Clostridium butyricum significantly decreased duodenal crypt depth ($P<0.05$), and significantly increased jejunal mucosal thickness and the number of intraepithelial lymphocytes ($P<0.05$). 3) Compared with the control group, LPS injection significantly decreased duodenal sucrase and jejunal maltase activities ($P<0.05$). Compared with the LPS group, prophylactic feeding of Clostridium butyricum inhibited the LPS-induced decrease in duodenal sucrase activity, and the duodenal sucrase activity in the LPS+Clostridium butyricum group was not significantly different from that in the control group ($P>0.05$). 4) Compared with the control group, LPS injection significantly increased myeloperoxidase (MPO)

activity and interleukin-6 (IL-6) and tumor necrosis factor- (TNF-) contents in the jejunum and ileum (P<0.05). Compared with the LPS group, prophylactic feeding of Clostridium butyricum significantly decreased MPO activity in the jejunum and ileum and IL-6 and TNF- contents in the jejunum (P<0.05); although IL-6 and TNF- contents in the ileum of the LPS+Clostridium butyricum group showed no significant decrease (P>0.05), they also did not show significant increase compared with the control group (P>0.05). It was concluded that Clostridium butyricum had no negative effects on growth in healthy rats, and that prophylactic feeding of Clostridium butyricum to rats could inhibit the LPS-induced decrease in intestinal sucrase activity under LPS acute stress, alleviate damage to intestinal mucosal structure, enhance intestinal immune function, and reduce intestinal inflammation.

Full Text

Inhibitory Effect of Clostridium butyricum on Intestinal Damage in Lipopolysaccharide-Induced Acute Stress Rats

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Abstract: This experiment was conducted to investigate the effects of Clostridium butyricum on the growth performance of healthy rats and on intestinal structure, intestinal disaccharidase activities, and intestinal inflammation in rats with lipopolysaccharide (LPS)-induced acute stress. Thirty-six male SD rats were randomly allocated to three groups: a control group, an LPS group, and an LPS+Clostridium butyricum group. Rats were housed four per cage, with three cages per group. The control and LPS groups were fed a basal diet, while the LPS+Clostridium butyricum group received the basal diet supplemented with 0.05% Clostridium butyricum.

On day 40 of the experiment, rats in the LPS and LPS+Clostridium butyricum groups were administered an intraperitoneal injection of LPS at 4 mg/kg body weight (LPS concentration: 1.5 mg/mL, injection volume: approximately 1 mL). Control rats received an intraperitoneal injection of 1 mL saline solution. All rats were sacrificed three hours later for sample collection.

The results showed: (1) Clostridium butyricum had no significant effects on final body weight, average daily gain, average daily feed intake, or feed-to-gain ratio in healthy rats (P>0.05). (2) Compared with the control group, LPS injection significantly increased duodenal crypt depth (P<0.05) and significantly decreased duodenal villus height-to-crypt depth ratio, jejunal mucosal thickness, and intraepithelial lymphocyte counts in both jejunum and ileum (P<0.05). Compared with the LPS group, preventive administration of Clostrid-

ium butyricum significantly reduced duodenal crypt depth (P<0.05) and significantly increased jejunal mucosal thickness and intraepithelial lymphocyte count (P<0.05). (3) LPS injection significantly decreased duodenal sucrase activity and jejunal maltase activity compared with the control group (P<0.05). Preventive administration of Clostridium butyricum inhibited the LPS-induced reduction in duodenal sucrase activity, with no significant difference observed between the LPS+Clostridium butyricum group and the control group (P>0.05). (4) LPS injection significantly increased myeloperoxidase (MPO) activity and interleukin-6 (IL-6) and tumor necrosis factor- (TNF-) contents in jejunum and ileum compared with the control group (P<0.05). Preventive administration of Clostridium butyricum significantly decreased MPO activity in jejunum and ileum and reduced IL-6 and TNF- contents in jejunum compared with the LPS group (P<0.05). Although IL-6 and TNF- contents in ileum of the LPS+Clostridium butyricum group showed no significant decrease (P>0.05), they also did not differ significantly from the control group (P>0.05).

In conclusion, Clostridium butyricum shows no negative effects on the growth performance of healthy rats. Preventive dietary supplementation with Clostridium butyricum can inhibit LPS-induced reduction of intestinal sucrase activity, alleviate damage to intestinal mucosal structure, enhance intestinal immune function, and reduce intestinal inflammation in rats under acute stress.

Keywords: Clostridium butyricum; rat; growth performance; intestine; inflammation; lipopolysaccharide

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In livestock production, stress factors such as weaning can cause intestinal inflammation and damage to intestinal barrier function, leading to diarrhea and digestive disorders. Probiotics are beneficial active microorganisms that colonize the host and improve intestinal microecological balance. In animal production, probiotics are commonly used as growth promoters to help establish and improve intestinal microbial flora, increase feed efficiency, enhance immunity, and improve product quality, representing a promising alternative to antibiotics [1-2]. Various probiotic species exist with different efficacies. Clostridium butyricum is an anaerobic, Gram-positive spore-forming bacterium. Unlike other probiotics, it primarily exists as endospores, making it easy to store and highly resistant to adverse conditions. Research has shown that Clostridium butyricum produces various digestive enzymes, vitamins, butyric acid, and other nutrients, demonstrating excellent intestinal nutritional functions [3]. Additionally, its metabolic products—including butyric acid, acetic acid, and hydrogen—can reduce intestinal inflammation and accelerate intestinal damage repair [4-5], showing promising application potential for weaning-induced diarrhea, intestinal damage, and microbial flora disorders. In July 2009, it was approved by the Ministry of Agriculture as a new generation of microecological feed additive. Currently, Clostridium butyricum is used preventively in feed, but research on its application effects in healthy animals under acute stress-induced damage remains limited. Whether preventive supplementation can help healthy hosts alleviate

acute stress damage requires further investigation. Therefore, this study used rats as a model to investigate the effects of preventive Clostridium butyricum supplementation on growth performance of healthy rats and its protective effects on intestinal structure, disaccharidase activities, and intestinal inflammation in LPS-induced acute stress, aiming to provide theoretical basis for using preventive Clostridium butyricum supplementation to mitigate acute stress damage in animals.

1.1 Experimental Animals and Materials

The Clostridium butyricum preparation was provided by the Institute of Plant Protection and Microbiology, Zhejiang Academy of Agricultural Sciences, with a viable count of 4×10 CFU/g. Thirty-six male SD rats weighing approximately 120 g were purchased from Zhejiang Academy of Medical Sciences. The basal diet was maintenance rodent chow purchased from Zhejiang Academy of Medical Sciences, with composition and nutrient levels shown in Table 1 .

1.2 Experimental Design and Animal Management

SD rats were randomly divided into three groups according to body weight: control group, LPS group, and LPS+Clostridium butyricum group. Rats were housed four per cage, with three cages per group. Room temperature was maintained at 25°C, and bedding was changed every three days to keep cages clean and dry. All rats had free access to water and feed. After a 3-day adaptation period, the experiment began. The control and LPS groups were fed the basal diet, while the LPS+Clostridium butyricum group received the basal diet supplemented with 0.05% Clostridium butyricum. Body weight and feed intake were recorded every five days. On day 40, all rats were weighed, and those in the LPS and LPS+Clostridium butyricum groups received an intraperitoneal injection of LPS at 4 mg/kg body weight (approximately 1 mL of 1.5 mg/mL solution). The control group received an intraperitoneal injection of 1 mL saline solution. All rats were sacrificed three hours later, and small intestine samples were collected.

1.3 Sample Collection and Detection Indicators

Rats were euthanized by cervical dislocation. The abdominal cavity was opened, and intestinal tissues were isolated. Duodenum, jejunum, and ileum segments were collected in duplicate. One portion of intestinal tissue was fixed overnight in 4% paraformaldehyde, then paraffin-embedded, sectioned serially, and stained with hematoxylin-eosin (HE). Images were captured using an Olympus DP-71 imaging system. The Image-Pro Plus 5.0 system was used to acquire, measure, and analyze villus height, crypt depth, mucosal thickness, and intraepithelial lymphocyte (IEL) count, and to calculate the villus height-to-crypt depth ratio (V/C = villus height/crypt depth). IEL counting method: For each specimen, 3-6 villi were observed, and the number of lymphocytes per 100 columnar epithelial cells in each villus epithelium was counted and converted to a percentage (%).

The average value represented the intraepithelial lymphocyte count for that specimen. The other portion of intestinal tissue was placed in centrifuge tubes, snap-frozen in liquid nitrogen, and stored at -80°C for subsequent analysis.

Intestinal total protein content, myeloperoxidase (MPO) activity, and sucrase, lactase, and maltase activities were measured using colorimetric methods. Interleukin-6 (IL-6) and tumor necrosis factor- (TNF-) contents were determined by enzyme-linked immunosorbent assay (ELISA). All assay kits were purchased from Nanjing Jiancheng Bioengineering Institute.

1.4 Data Processing

All experimental data are expressed as mean ± standard deviation. One-way ANOVA was performed using SPSS 17.0 software, and LSD multiple comparison tests were used to detect significant differences among the three groups. $P<0.05$ was considered statistically significant.

2.1 Effects of Clostridium butyricum on Growth Performance of Healthy Rats

As shown in Table 2 , body weight changes were consistent across all groups, and Clostridium butyricum had no significant effects on average daily feed intake, average daily gain, or feed-to-gain ratio ($P>0.05$).

2.2 Effects of Clostridium butyricum on Intestinal Structure and Intraepithelial Lymphocyte Count in LPS-Treated Rats

Table 3 shows that compared with the control group, LPS injection significantly increased duodenal crypt depth ($P<0.05$) and significantly decreased the villus height-to-crypt depth ratio in duodenum ($P<0.05$), but did not significantly damage villus structure in jejunum or ileum ($P>0.05$). Compared with the LPS group, preventive administration of Clostridium butyricum significantly reduced duodenal crypt depth ($P<0.05$) but did not prevent the LPS-induced decrease in duodenal villus height-to-crypt depth ratio, which remained significantly lower than the control group ($P<0.05$). LPS injection significantly decreased jejunal mucosal thickness and intraepithelial lymphocyte counts in jejunum and ileum compared with the control group ($P<0.05$). Preventive administration of Clostridium butyricum effectively inhibited these LPS-induced effects on mucosal thickness and intraepithelial lymphocyte count, with the most pronounced effects observed in jejunum. The LPS+Clostridium butyricum group showed significantly increased jejunal mucosal thickness and intraepithelial lymphocyte count compared with the LPS group ($P<0.05$), reaching levels that did not differ significantly from the control group ($P>0.05$).

2.3 Effects of Clostridium butyricum on Intestinal Sucrase, Lactase, and Maltase Activities in LPS-Treated Rats

Table 4 demonstrates that compared with the control group, LPS injection significantly decreased duodenal sucrase activity and jejunal maltase activity ($P<0.05$). Activities of other disaccharidases in different intestinal segments showed decreasing trends but were not significantly different ($P>0.05$). Preventive administration of Clostridium butyricum inhibited the LPS-induced reduction in duodenal sucrase activity, with no significant difference observed between the LPS+Clostridium butyricum group and the control group ($P>0.05$).

2.4 Effects of Clostridium butyricum on Intestinal Inflammation in LPS-Treated Rats

As shown in Table 5 , LPS injection significantly increased MPO activity and IL-6 and TNF- contents in jejunum and ileum compared with the control group ($P<0.05$). Preventive administration of Clostridium butyricum effectively suppressed inflammation in jejunum and ileum. Compared with the LPS group, the LPS+Clostridium butyricum group showed significantly decreased MPO activity in jejunum and ileum and reduced IL-6 and TNF- contents in jejunum ($P<0.05$). Although IL-6 and TNF- contents in ileum of the LPS+Clostridium butyricum group were not significantly reduced compared with the LPS group ($P>0.05$), they also did not differ significantly from the control group ($P>0.05$).

3.1 Effects of Clostridium butyricum on Growth Performance of Healthy Rats

Probiotics can regulate intestinal flora and produce beneficial substances in the host intestine, potentially exerting positive effects on body weight. In rats fed high-fat diets, Lactobacillus supplementation reduced body weight and inhibited obesity by regulating intestinal flora structure and increasing blood leptin levels [6]. In animal production, probiotics are commonly used as growth promoters to mitigate effects of environmental or feed stress. Meng et al. [7] found that supplementation with Bacillus subtilis and Clostridium butyricum effectively improved average daily gain and feed-to-gain ratio in growing-finishing pigs throughout the entire period, while also significantly increasing apparent digestibility of gross energy and nitrogen. Cao et al. [8] reported that adding Clostridium butyricum at 2.5×10 or 5×10 CFU/kg to broiler diets significantly improved average daily gain and feed-to-gain ratio at 21 and 42 days of age, with effects comparable to antibiotic supplementation. However, probiotic effects on body weight are not always significant. Kuo et al. [9] found that probiotics had limited effects on body weight and feed intake in healthy mice during 3-4 week trials, consistent with our findings. This study found that Clostridium butyricum supplementation had no significant effects on growth parameters including average daily gain and feed-to-gain ratio in healthy rats. This may be related to animal health status and living environment. The rats used in this experiment were in a rapid growth phase, and the clean, hygienic cage conditions

may have masked the growth-promoting effects of probiotics.

3.2 Effects of Clostridium butyricum on Intestinal Structure and Intraepithelial Lymphocyte Count in LPS-Treated Rats

Intestinal villus height, crypt depth, villus height-to-crypt depth ratio, and mucosal thickness are important indicators reflecting intestinal development and nutrient absorption. Wang [10] demonstrated that Clostridium butyricum ZJU-F1 could inhibit weaning stress-induced reductions in villus height and villus height-to-crypt depth ratio in duodenum, jejunum, and ileum, with piglets fed Clostridium butyricum showing more orderly and dense microvilli structures under light microscopy. Liu [11] obtained similar results, finding that dietary supplementation with 1,000 mg/kg Clostridium butyricum significantly increased villus height and villus height-to-crypt depth ratio in duodenum, jejunum, and ileum, and increased jejunal mucosal thickness in broilers. This study found that Clostridium butyricum supplementation effectively reduced duodenal crypt depth and increased jejunal mucosal thickness, consistent with previous research. This intestinal nutritional function of Clostridium butyricum may be related to its metabolic products. During fermentation, Clostridium butyricum produces glycosidases, cellulases, vitamins, butyric acid, and other nutrients, particularly butyric acid, which can provide energy for intestinal mucosal cells and accelerate their proliferation and maturation [3,12].

Intraepithelial lymphocytes distributed among intestinal mucosal epithelial cells constitute the first line of immune defense in the intestine. They directly participate in mucosal immune responses, resist invasion by intestinal pathogens, and can accelerate epithelial cell regeneration through pseudopodia contact [13]. Increased intraepithelial lymphocyte count helps improve immune capacity [14]. This study found that after 40 days of Clostridium butyricum supplementation, the LPS+Clostridium butyricum group showed significantly increased jejunal intraepithelial lymphocyte count compared with the LPS group, reaching levels comparable to the control group, consistent with previous studies. Rieger et al. [15] found that Enterococcus faecium feeding significantly increased intraepithelial lymphocyte counts at apical and nuclear positions in weaned piglets. Bai et al. [16] also observed that dietary supplementation with 0.1% or 0.2% probiotics significantly increased intestinal intraepithelial lymphocyte counts, particularly T lymphocytes, in broilers at 21 and 42 days of age. These results indicate that Clostridium butyricum and other probiotics can enhance intestinal mucosal immunity, particularly cellular immunity.

3.3 Effects of Clostridium butyricum on Intestinal Disaccharidase Activities in LPS-Treated Rats

The primary energy source for animals is carbohydrates, whose digestion and absorption mainly depend on small intestinal disaccharidases. Disaccharidase activity can be used to measure the developmental status of intestinal mucosal epithelial cells [17]. Dou [18] reported that dietary supplementation with 6×10

CFU/g Lactobacillus effectively alleviated LPS-induced reductions in lactase activity in jejunum and ileum. In Giardia-infected mice, administration of Lactobacillus before or during infection significantly increased intestinal sucrase and lactase activities [19]. This study found that Clostridium butyricum supplementation showed a trend toward increasing sucrase and lactase activities in rat intestine.

3.4 Effects of Clostridium butyricum on Intestinal Inflammation in LPS-Treated Rats

TNF- and IL-6 are important inflammatory mediators involved in immune responses. Their increased content can trigger a cascade release of other inflammatory factors, causing cellular damage [20-21]. MPO is a heme protease secreted by neutrophils and macrophages, and its activity can reflect the degree of intestinal inflammatory infiltration. This study found that preventive Clostridium butyricum supplementation effectively reduced MPO activity in jejunum and ileum and decreased IL-6 and TNF- contents in jejunum. Although IL-6 and TNF- contents in ileum were not significantly reduced compared with the LPS group, they also did not differ significantly from the control group, indicating that Clostridium butyricum can effectively inhibit LPS-induced intestinal inflammation. These results are consistent with previous studies. Zhang et al. [22] found that daily gavage with 2 mL Clostridium butyricum (2.3×10^{11} CFU/L) for 21 days effectively reduced blood IL-23 and TNF- contents and inhibited oxazolone-induced colonic inflammation in a rat colitis model. Pang et al. [23] also observed that oral administration of Clostridium butyricum for 8 days effectively reduced serum endotoxin and D-lactate contents and decreased ileal IL-10 mRNA expression in diarrheal rats. The anti-inflammatory effects of Clostridium butyricum are primarily mediated through its metabolic products butyric acid and hydrogen. Research has shown that butyric acid can promote epithelial cell proliferation, accelerate damage repair, and reduce inflammation [4], while hydrogen has recently been identified as an anti-inflammatory gas that can alleviate oxidative stress and treat various inflammatory diseases [5,24]. Additionally, Clostridium butyricum can stimulate macrophage aggregation near inflammatory sites and activate IL-10 release through the Toll-like receptor 2 (TLR2)/myeloid differentiation factor 88 (MyD88) signaling pathway, thereby inhibiting inflammatory factor production [25].

4 Conclusion

1. Under the conditions of this experiment, dietary supplementation with 0.05% Clostridium butyricum had no significant effect on the growth performance of healthy rats.
2. Preventive dietary supplementation with Clostridium butyricum in rats can inhibit LPS-induced reduction of intestinal sucrase activity, alleviate LPS-induced effects on intestinal villus height and crypt depth, improve intestinal immune function, and reduce intestinal inflammation.

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Note: Figure translations are in progress. See original paper for figures.