Effects of Clotridium butyricum on Growth Performance, Intestinal Barrier Function, and Serum Cytokine Levels in Weaned Piglets (Postprint)

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Abstract

This study aimed to investigate the effects of dietary Clostridium butyricum supplementation on growth performance, intestinal barrier function, and serum cytokine content in weaned piglets. A single-factor design was adopted in the experiment. Twelve 28-day-old weaned piglets (Duroc × Landrace × Yorkshire) with similar body weight and good health status were selected and divided into 2 groups with 6 replicates per group, and housed individually. The control group was fed a corn-soybean meal-based basal diet, while the experimental group was fed the basal diet supplemented with 5$×10^{5}$ CFU/g Clostridium butyricum. The experiment consisted of a 3-day preliminary period and a 14-day formal trial period. The results showed that: compared with the control group, the average daily gain of piglets in the experimental group was significantly increased by 7.83% ($P<0.05$), and the feed-to-gain ratio was decreased by 5.26% ($P>0.05$); the mRNA relative expression levels of NOD-like receptor protein (NLRP) 3 ($P<0.05$), NLRP6 ($P<0.05$), NLRP12 ($P<0.01$), claudin-1 ($P<0.01$), and zonula occludens-2 (ZO-2) ($P<0.05$) in the jejunum of piglets in the experimental group were significantly or extremely significantly up-regulated, while the mRNA relative expression levels of claudin-1 and ZO-2 in the ileum were extremely significantly up-regulated ($P<0.01$); the number of Lactobacillus in the jejunum and ileum of piglets in the experimental group was significantly increased ($P<0.05$), the number of Escherichia coli in the jejunum was decreased by 2.49% ($P>0.05$), and the number of Escherichia coli in the ileum was significantly decreased ($P<0.05$); the serum interleukin (IL)-1β content of piglets in the experimental group was decreased by 5.47%, and the IL-10 content was increased by 25.43% ($P>0.05$). In conclusion, dietary supplementation of Clostridium butyricum can improve small intestinal barrier function, regulate immune response and intestinal flora balance, and promote growth in piglets.

Full Text

Effects of Clostridium butyricum on Growth Performance, Intestinal Barrier Function, and Serum Cytokine Content in Weaned Piglets

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Abstract: This study investigated the effects of dietary Clostridium butyricum supplementation on growth performance, intestinal barrier function, and serum cytokine content in weaned piglets. A single-factor experimental design was employed, selecting 12 healthy Duroc × Landrace × Yorkshire weaned piglets at 28 days of age with similar body weight, divided into 2 groups with 6 replicates per group and individually housed. The control group received a corn-soybean meal basal diet, while the experimental group received the basal diet supplemented with 5$×10^{5}$ CFU/g Clostridium butyricum. The trial consisted of a 3-day adaptation period followed by a 14-day formal experimental period. Results showed that compared with the control group, the experimental group exhibited a significant 7.83% increase in average daily gain ($P<0.05$) and a 5.26% decrease in feed-to-gain ratio ($P>0.05$). The mRNA relative expression levels of NOD-like receptor protein (NLRP) 3 ($P<0.05$), NLRP6 ($P<0.05$), NLRP12 ($P<0.01$), claudin-1 ($P<0.01$), and zonula occludens protein 2 (ZO-2) ($P<0.05$) were significantly or extremely significantly upregulated in the jejunum, while claudin-1 and ZO-2 mRNA expression levels were extremely significantly upregulated in the ileum ($P<0.01$). Lactobacillus counts in both jejunum and ileum were significantly increased ($P<0.05$), Escherichia coli counts in the jejunum decreased by 2.49% ($P>0.05$), and ileal E. coli counts were significantly reduced ($P<0.05$). Serum interleukin (IL)-1β content decreased by 5.47% ($P>0.05$), while IL-10 content increased by 25.43% ($P>0.05$). In conclusion, dietary Clostridium butyricum supplementation enhances small intestinal barrier function, modulates immune status and intestinal microflora balance, and promotes growth performance in weaned piglets.

Keywords: Clostridium butyricum; weaned piglets; growth performance; tight junction; intestinal barrier

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Weaning imposes various stressors on piglets, impairing intestinal function and reducing nutrient digestion and absorption capacity, which seriously compromises animal health. Intestinal health in piglets has been a hot topic and research challenge in swine nutrition and feed science in recent years. Numerous

studies have demonstrated that Clostridium butyricum is an important probiotic that can maintain or restore dominant intestinal microflora, promote the proliferation of beneficial bacteria such as Lactobacillus and Bifidobacterium, and inhibit the growth of pathogenic bacteria like Salmonella and E. coli, thereby stabilizing the animal' s intestinal microecology. Research indicates that dietary supplementation with 5$×10^{8}$ CFU/g C. butyricum effectively increases body weight gain and feed conversion ratio in weaned piglets, while supplementation with 1$×10^{8}$ CFU/g C. butyricum combined with 1$×10^{9}$ CFU/g Enterococcus faecalis reduces diarrhea incidence. Intestinal microorganisms and their metabolites activate NOD-like receptors in intestinal epithelial cells, promoting epithelial proliferation, enhancing tight junction protein expression, secreting antimicrobial peptides and secretory immunoglobulin A, and regulating cytokine expression to improve intestinal barrier function and immunity. However, the systematic mechanisms by which C. butyricum regulates intestinal health in weaned piglets remain unclear. Therefore, this study investigated the effects of dietary C. butyricum supplementation on growth performance, NLRP and tight junction-related protein mRNA expression in intestinal epithelial cells, and serum cytokine content to elucidate its mechanisms of action and provide a theoretical basis for its application in piglet diets.

1.1 Experimental Design and Animal Management

A single-factor experimental design was used. Twelve healthy Duroc × Landrace × Yorkshire crossbred weaned piglets at 28 days of age with an initial body weight of $(6.97\$\pm0.68)kgwereselectedandrandomlyallocatedto2groupswith6replicatespergroup,withindividu$ CFU/g. The diet formulation referenced Li et al., with basal diet composition and nutrient levels shown in Table 1 .

The experiment was conducted at the Animal Research Center of Tianjin Institute of Animal Husbandry and Veterinary Medicine. All pigs had ad libitum access to feed and water, with room temperature maintained at 25–28°C. The trial consisted of a 3-day adaptation period and a 14-day formal experimental period. Piglets showing no adverse reactions during the adaptation period entered the formal experimental period. Health status was monitored daily, with mortality, diarrhea, and medication use recorded. Other management and immunization procedures followed commercial piglet-rearing recommendations.

1.2 Experimental Materials and Treatment

The C. butyricum freeze-dried powder used in this experiment was a commercial product containing 1$×10^{10}$ CFU/g viable bacteria, which was uniformly mixed with the basal diet prior to the trial.

1.3 Growth Performance

Body weights were recorded on days 31 and 45 of the experimental period to calculate average daily gain (ADG). Daily feed intake was recorded to calculate

average daily feed intake (ADFI). The feed-to-gain ratio (F/G) was calculated based on ADG and ADFI.

1.4 Sample Collection of Blood, Jejunal and Ileal Segments, and Contents

At the end of the experiment, 3 piglets were randomly selected from each group. After 12 hours of fasting, blood was collected via the anterior vena cava using vacuum coagulation-promoting tubes before euthanasia. The abdominal cavity was immediately opened under sterile conditions, and the cardiac, pyloric, and distal rectal sphincters were ligated. Jejunal and ileal contents were aseptically collected, snap-frozen in liquid nitrogen, and stored at -80°C for analysis. Approximately 5 cm mid-segments of jejunum and ileum were dissected, rinsed with phosphate-buffered saline (PBS), aliquoted into cryovials, and stored at -80°C for determination of mRNA relative expression levels of NLRP3, NLRP6, NLRP12, ZO-2, and claudin-1. Identical anatomical regions were sampled for both jejunum and ileum.

1.5 Measurement Indices and Methods

Blood samples were centrifuged at 4°C and 4,000×g for 10 minutes after clotting, and serum was collected and stored at -20°C. Serum pro-inflammatory cytokine interleukin (IL)-1β and anti-inflammatory cytokine IL-10 contents were measured using enzyme-linked immunosorbent assay (ELISA) kits. Primer pairs for six genes (NLRP3, NLRP6, NLRP12, claudin-1, ZO-2, and glyceraldehyde-3-phosphate dehydrogenase [GAPDH]) were synthesized by Sangon Biotech (Shanghai) Co., Ltd., with primer sequences listed in Table 2 .

Quantitative jejunal and ileal samples were homogenized using a T165-48 multi-sample tissue grinder (Shanghai Jingxin Industrial Development Co., Ltd.). Total RNA was extracted from jejunal and ileal epithelial cells using the TacoTM RNA kit and reverse-transcribed into cDNA using the All-in-OneTM First-cDNA Synthesis kit according to the manufacturer' s instructions. Using the SYBR® Premix Ex TaqTM kit, 1 µL of cDNA template was amplified in a final reaction volume of 15 µL with four replicates per gene to detect mRNA relative expression levels of NLRP3, NLRP6, NLRP12, ZO-2, and claudin-1. Quantitative reverse transcription PCR (qRT-PCR) conditions were: 1) 95°C for 10 min; 2) 40 cycles of 95°C for 10 s, 61°C for 20 s, and 72°C for 10 s with fluorescence signal acquisition; 3) 72°C for 10 s, 95°C for 10 s with automatic fluorescence signal acquisition. Negative controls without reverse transcription and internal reference controls were included for each sample to obtain threshold cycle (Ct) values. Relative quantification was performed using the $2^{(-\Delta\Delta Ct)}$ method. E. coli counts were determined using MacConkey agar, and Lactobacillus counts were determined using Rogosa agar, following the methods described by Qiao et al.

1.6 Statistical Analysis

Data were analyzed on a per-pig basis. Raw data were processed using Excel 2007, and intestinal microflora data were log-transformed prior to statistical analysis. One-way ANOVA and least significant difference (LSD) tests were performed using SAS 9.1.3 software, with t-tests used for inter-group comparisons. Results are expressed as "mean ± standard error." $P<0.05$ was considered statistically significant, and $P<0.01$ was considered extremely significant.

2.1 Effects of Clostridium butyricum on Growth Performance of Weaned Piglets

As shown in Table 3 , compared with the control group, dietary C. butyricum supplementation significantly increased average daily gain by 7.83% ($P<0.05$), increased average daily feed intake by 2.49% ($P>0.05$), and decreased feed-to-gain ratio by 5.26% ($P>0.05$) in weaned piglets.

2.2 Effects of Clostridium butyricum on mRNA Relative Expression Levels of NLRP and Tight Junction-Related Proteins in Jejunal and Ileal Epithelial Cells of Weaned Piglets

Tables 4 and 5 show that dietary C. butyricum supplementation extremely significantly increased NLRP12 and claudin-1 mRNA expression levels ($P<0.01$) and significantly increased NLRP3, NLRP6, and ZO-2 mRNA expression levels ($P<0.05$) in jejunal epithelial cells. In ileal epithelial cells, claudin-1 and ZO-2 mRNA expression levels were extremely significantly increased ($P<0.01$), while NLRP3, NLRP6, and ZO-2 expression levels showed no significant differences compared with the control group ($P>0.05$).

2.3 Effects of Clostridium butyricum on Intestinal Microflora of Weaned Piglets

As shown in Table 6 , dietary C. butyricum supplementation significantly increased Lactobacillus counts in both jejunum and ileum ($P<0.05$), decreased jejunal E. coli counts by 2.49% ($P>0.05$), and significantly reduced ileal E. coli counts ($P<0.05$).

2.4 Effects of Clostridium butyricum on Serum Cytokine Content of Weaned Piglets

Table 7 demonstrates that dietary C. butyricum supplementation decreased serum IL-1β content and increased serum IL-10 content ($P>0.05$), with IL-1β decreasing by 5.47% and IL-10 increasing by 25.43% compared with the control group.

3.1 Effects of Clostridium butyricum on Growth Performance of Weaned Piglets

Clostridium butyricum produces various beneficial substances in the animal intestine, including amino acids, B vitamins, and vitamin K, which promote fat and protein digestion and absorption, stimulate proliferation of beneficial bacteria, and inhibit pathogenic bacteria. It can also serve as an amino acid carrier for amino acid transport, making it an effective feed additive for improving feed efficiency and animal growth performance. As a spore-forming bacterium, C. butyricum resists high temperatures during feed pelleting and tolerates gastric acid, bile acids, and digestive enzymes. It is sensitive to only a few antibiotics such as novobiocin, vancomycin, and tetracycline, while showing strong resistance to many others, making it a novel probiotic for livestock production. Liao et al. reported that feeding broilers with different concentrations of C. butyricum significantly improved growth performance, increased daily gain, and decreased feed-to-gain ratio. The piglet digestive tract is sterile at birth and gradually colonized by various bacteria during the pre-weaning period, but microbial balance is not yet established. Dietary probiotic supplementation can improve piglet growth performance and intestinal microbial balance. Wang et al. demonstrated that 0.2% C. butyricum supplementation significantly increased average weight gain from weaning to relocation and decreased feed-to-gain ratio by 0.08%. Consistently, this study showed that dietary C. butyricum improved growth performance in weaned piglets, with increased ADG and ADFI and a 5.26% reduction in feed-to-gain ratio. These results indicate that C. butyricum supplementation enhances nutrient supply and absorption while maintaining original nutritional levels, thereby improving piglet growth performance.

3.2 Effects of Clostridium butyricum on Intestinal Barrier Function of Weaned Piglets

Intestinal barrier function is formed by a monolayer of intestinal epithelial cells connected via tight junction proteins, preventing harmful microorganisms, antigens, and toxins from entering the bloodstream. Intercellular tight junctions regulate intestinal barrier permeability and maintain epithelial structural integrity, with normal tight junction protein expression being essential for survival. Studies have shown that C. butyricum promotes proliferation of beneficial intestinal microbiota and healthy intestinal development, inhibits expression of pathogenic virulence proteins through direct cell contact, and reduces pathogen invasion and colonization. It provides both direct nutritional benefits and stimulates intestinal mucosal immune responses.

The effects of probiotics on intestinal barrier function have attracted widespread attention. Lactobacillus salivarius can alleviate or prevent barrier damage caused by pathogens or harmful substances. Lactobacillus rhamnosus GG accelerates intestinal barrier maturation in mice by upregulating claudin-3 expression. Piglets administered Lactobacillus reuteri show increased expression of tight junction proteins including claudin-1, zonula occludens protein 1 (ZO-1), and

occludin in jejunum and ileum. Claudin-1 and ZO-2 are crucial tight junction proteins for maintaining intestinal mucosal mechanical barrier integrity. Consistent with previous studies, this study found that mRNA expression levels of claudin-1 and ZO-2 were significantly higher in both jejunum and ileum of the experimental group. These results demonstrate that dietary C. butyricum stimulates intestinal epithelial cell proliferation and enhances tight junction-related protein expression, thereby improving intestinal barrier function.

3.3 Effects of Clostridium butyricum on NLRP Expression in Intestinal Epithelial Cells of Weaned Piglets

NOD-like receptors are a major class of pattern recognition receptors in innate immunity. NLRP3, NLRP6, and NLRP12 are highly expressed in the small intestine as negative feedback regulators of intestinal inflammation and play important roles in maintaining mucosal barrier function and microbial homeostasis. Upon ligand recognition, NLRPs form inflammasomes that activate caspase-1, ultimately regulating maturation and secretion of IL-1 family cytokines including IL-1β, IL-18, and IL-33. This study found that C. butyricum increased mRNA expression levels of NLRP3, NLRP6, and NLRP12 in jejunum but had no significant effect on their expression in ileum, indicating differential tissue-specific regulation of gene expression by C. butyricum. Additionally, serum IL-1β content was slightly lower in the experimental group, though not significantly. This may be explained by: (1) elevated NLRP expression enhancing claudin-1 and ZO-2 expression and intestinal barrier function, reducing intestinal permeability and stimulation of epithelial cells by harmful microorganisms and danger signals, resulting in lower pro-inflammatory IL-1β levels; and (2) high NLRP expression potentially regulating other cytokines such as IL-18, which may negatively feedback on IL-1β expression and reduce intestinal inflammation.

3.4 Effects of Clostridium butyricum on Intestinal Microflora of Weaned Piglets

Beneficial microorganisms play crucial roles in the animal intestine, maintaining dynamic microbial balance. Weaning stress often causes changes in gastrointestinal physiology, immunity, and microbiota, leading to intestinal dysfunction. Lactobacillus, as an anaerobe, becomes the dominant microflora and consumes most oxygen in the gastrointestinal tract while regulating pH, preventing pathogen proliferation. Liang et al. reported that dietary C. butyricum supplementation in weaned piglets decreased intestinal pH, increased C. butyricum and Lactobacillus counts, and reduced E. coli counts, consistent with our findings. This study demonstrated that C. butyricum supplementation significantly increased Lactobacillus counts in jejunum and ileum and significantly reduced ileal E. coli counts, promoting beneficial bacteria as dominant microflora and alleviating weaning stress.

3.5 Effects of Clostridium butyricum on Serum Cytokines of Weaned Piglets

Clostridium butyricum activates the animal immune system, enhances immunity, and maintains animal health. In vitro studies show that C. butyricum recognizes and activates Toll-like receptor 2, modulating secretion of appropriate levels of pro-inflammatory cytokines IL-8, IL-6, and tumor necrosis factor-α (TNF-α) to resist pathogen infection while maintaining immune homeostasis. Moderate inflammatory responses help clear pathogens, whereas excessive inflammation causes tissue damage and can be life-threatening, requiring a balanced inflammatory and anti-inflammatory state. IL-1β is an early pro-inflammatory cytokine that promotes inflammatory cell infiltration and plays important roles throughout inflammatory activation and regulation. IL-10 is an anti-inflammatory cytokine that effectively inhibits pro-inflammatory cytokine secretion, reduces inflammation, and provides immune stimulation and modulation, facilitating probiotic colonization and enabling probiotic functions.

Hua et al. reported that C. butyricum stimulation of human peripheral blood mononuclear cells and dendritic cells decreased IL-4 expression and upregulated IL-10 expression. Chen et al. demonstrated that combined C. butyricum and Enterococcus faecalis increased serum IL-10 content. Consistently, this study showed that C. butyricum supplementation decreased serum IL-1β and increased IL-10 content. Therefore, dietary C. butyricum modulates inflammatory responses and provides probiotic benefits. Liang et al. found elevated NLRP3 expression and downstream IL-1β secretion in lung tissue of asthmatic mice, whereas this study showed increased jejunal NLRP3 expression but slightly decreased serum IL-1β in piglets. This discrepancy suggests that IL-1β secretion may be regulated by proteins other than NLRP3, requiring further investigation.

In conclusion, dietary Clostridium butyricum supplementation improved growth performance, modulated intestinal microflora balance, enhanced mRNA expression of tight junction-related proteins in intestinal epithelial cells, increased serum anti-inflammatory cytokine IL-10 content, and decreased pro-inflammatory cytokine IL-1β content, thereby improving intestinal mucosal barrier function and inflammatory responses in weaned piglets.

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