Effects of Clostridium butyricum on Growth Performance, Intestinal Morphology, and Intestinal Permeability in Weaned Piglets (Postprint)

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Abstract

This experiment aimed to investigate the effects of Clostridium butyricum on growth performance, intestinal tissue morphology, and intestinal permeability in weaned piglets. A total of 18 "Duroc × Landrace × Large White" three-way crossbred piglets, weaned at 21 days of age with a body weight of (5.35$±$1.33) kg, were randomly assigned to 3 groups and fed a basal diet (control group), basal diet + 500 mg/kg Clostridium butyricum (Clostridium butyricum group), or basal diet + 3,000 mg/kg zinc oxide (zinc oxide group), with 6 replicates per group and 1 pig per replicate, for a trial period of 14 days. The results showed that: 1) There were no significant differences in average daily gain and feed-to-gain ratio among the groups (P>0.05); the diarrhea rate of piglets in the Clostridium butyricum group and zinc oxide group was significantly reduced compared with the control group (P<0.05). 2) Compared with the control group, the ileal crypt depth of weaned piglets in the Clostridium butyricum group and zinc oxide group was significantly decreased (P<0.05), and the villus height-to-crypt depth ratio was highly significantly increased (P<0.01). 3) The serum endotoxin and D-lactate concentrations on day 14 of weaned piglets in the Clostridium butyricum group and zinc oxide group were both significantly lower than those in the control group (P<0.05), with no significant difference between the Clostridium butyricum group and the zinc oxide group (P>0.05). 4) Compared with the control group, the mRNA expression levels of Occludin in the ileum and colon of weaned piglets in the Clostridium butyricum group and zinc oxide group were significantly increased (P<0.05); compared with the zinc oxide group, there were no significant differences in the mRNA expression levels of zonula occludens-1 (ZO-1) and Occludin in the ileum and colon of weaned piglets in the Clostridium butyricum group (P>0.05). Based on the above results, dietary supplementation of weaned piglets with Clostridium butyricum can significantly reduce the diarrhea rate, improve intestinal mucosal morphology, decrease intestinal mucosal permeability, and upregulate the expression of

tight junction proteins, achieving the same effect as zinc oxide supplementation.

Full Text

Effects of Clostridium butyricum on Growth Performance, Intestinal Morphology and Intestinal Permeability of Weanling Piglets

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Abstract: This study investigated the effects of Clostridium butyricum on growth performance, intestinal morphology, and intestinal permeability of weanling piglets. Eighteen "Duroc×Landrace×Yorkshire" crossbred piglets were weaned at 21 days of age with an average body weight of (5.35$±$1.33) kg and randomly allocated into three groups: a basal diet group (control), a basal diet supplemented with 500 mg/kg Clostridium butyricum (CB group), and a basal diet supplemented with 3,000 mg/kg zinc oxide (ZnO group). Each group comprised six replicates with one piglet per replicate, and the experimental period lasted 14 days. The results showed: (1) No significant differences were observed in average daily gain or feed-to-gain ratio among the three groups ($P>0.05$). The diarrhea rate in both the CB and ZnO groups was significantly lower than in the control group ($P<0.05$). (2) Compared with the control group, the ileal crypt depth in the CB and ZnO groups decreased significantly ($P<0.05$), while the villus height-to-crypt depth ratio increased markedly ($P<0.01$). (3) On day 14, serum endotoxin and D-lactic acid concentrations in the CB and ZnO groups were significantly lower than in the control group ($P<0.05$), with no significant difference between the CB and ZnO groups ($P>0.05$). (4) The mRNA expression of Occludin in the ileum and colon of piglets in the CB and ZnO groups was significantly higher than in the control group ($P<0.05$). Compared with the ZnO group, the CB group showed no significant difference in ZO-1 and Occludin mRNA expression in the ileum and colon ($P>0.05$). These findings indicate that dietary supplementation with Clostridium butyricum significantly reduces diarrhea rate, improves intestinal mucosal morphology, decreases intestinal permeability, and upregulates tight junction protein expression in weanling piglets, producing effects comparable to zinc oxide supplementation.

Keywords: weanling piglets; Clostridium butyricum; zinc oxide; diarrhea; intestinal mucosal barrier

Weaning stress in piglets (including environmental, dietary, and management stress, as well as separation from the sow) adversely affects growth performance and gut health, leading to diarrhea, reduced growth rate, and decreased immunity [1-2]. Diarrhea in piglets remains a major challenge in swine production. High-dose zinc oxide is commonly added to diets to prevent diarrhea and promote growth [3]. Research has demonstrated that adding zinc oxide

at 3,000 mg/kg to weanling piglet diets significantly reduces diarrhea and improves growth performance [4-5]. Studies indicate that the anti-diarrheal effect of zinc oxide is associated with alterations in the intestinal mucosal barrier [6-7]. However, despite its efficacy, zinc oxide has low bioavailability; only 5-10% is utilized by the animal, with the majority excreted in feces and urine, causing severe environmental pollution and threatening the sustainability of livestock production [8-11].

Microecological preparations are increasingly used as novel, environmentally friendly feed additives in livestock production, effectively reducing piglet diarrhea and improving growth performance, though effects vary by probiotic strain. Clostridium butyricum (CB) is a normal intestinal bacterium in animals, named for its butyric acid production. Studies have shown that dietary supplementation with 500 mg/kg CB significantly improves small intestinal morphology and enhances immune and antioxidant capacity in weanling piglets [12]. CB supplementation also effectively promotes growth performance and immune function in broilers [13]. Ling et al. [14] found that CB reduces diarrhea in mice, promotes intestinal tissue repair, and decreases intestinal permeability. While research on CB application in livestock exists, studies on alleviating weaning stress, reducing diarrhea, and decreasing intestinal permeability in piglets remain limited. This experiment investigated the effects of CB supplementation on growth performance, diarrhea, and tight junction protein expression in weanling piglets, using high-dose zinc oxide as a reference, to provide theoretical basis and data support for developing zinc oxide alternatives and understanding CB' s role in mitigating weaning stress and improving diarrhea.

1.1 Experimental Materials

Clostridium butyricum was purchased from a biotechnology company in Qingdao with an activity of 1$×10^{10}$ CFU/g, added at 500 mg/kg. Zinc oxide was purchased from a chemical reagent company under China National Pharmaceutical Group, with a zinc content $ $79%, added at 3,000 mg/kg.

1.2 Basal Diet

The basal diet was a corn-soybean meal type formulated according to NRC (2012) nutrient standards, without antibiotics, meeting the nutritional requirements of weanling piglets (5-10 kg). The composition and nutrient levels of the basal diet are shown in Table 1 .

1.3 Experimental Design

Eighteen healthy "Duroc×Landrace×Yorkshire" crossbred piglets weaned at 21 days of age, with body weight (5.35$±$1.33) kg, were randomly divided into three groups: basal diet (control group), basal diet + 500 mg/kg Clostridium butyricum (CB group), and basal diet + 3,000 mg/kg zinc oxide (ZnO group). Each group had six replicates with one piglet per replicate.

Table 1 Composition and nutrient levels of the basal diet (air-dry basis), %

Items	Content
Ingredient	
Corn	
Full-fat expanded soybean	
Soybean meal	
Fish meal	
Dried whey	
Soybean oil	
$CaHPO_4$	
NaCl	
Limestone	
Choline chloride	
Premix[1])	
Lysine · HCl	
Met	
Thr	
Sugar	
Glucose	
Total	
Nutrient levels[2])	
DE/(MJ/kg)	
CP	
Lys	
Met	
Thr	
Ca	
AP	

[1]) Premix provided the following per kg of diet: VA 1,500 IU, VD_3 200 IU, VE 10 IU, VK_3 1 mg, VB_1 0.9 mg, VB_2 1 mg, VB_{12} 9 g, biotin 0.05 mg, folic acid 0.3 mg, pantothenate 10 mg, nicotinic acid 10 mg, Cu (as copper sulfate) 6 mg, Fe (as ferrous sulfate) 100 mg, Zn (as zinc sulfate) 80 mg, Mn (as manganese sulfate) 4 mg, Se (as sodium selenite) 0.3 mg, I (as potassium iodide) 0.14 mg.
[2]) DE was a calculated value and the others were measured values.

1.4 Feeding Management and Slaughter

The experiment was conducted at the Changping Experimental Base of the State Key Laboratory of Animal Nutrition. Piglets had ad libitum access to feed and water throughout the 14-day trial period. On the final day, all piglets were slaughtered for sample collection. The terminal ileum and mid-colon segments were isolated, and intestinal tissue and mucosal samples were collected. Tissue

samples were fixed in 4% paraformaldehyde, while mucosal samples were snap-frozen in liquid nitrogen and stored at -80°C.

1.5.1 Growth Performance Measurement

All piglets were weighed (after fasting) on the day of weaning (day 1) and on day 14 of the experiment. Daily feed intake was recorded to calculate average daily gain, average daily feed intake, and feed-to-gain ratio.

1.5.2 Diarrhea Rate Measurement

Piglet health and diarrhea were observed and recorded daily. Diarrhea rate was calculated as:
Diarrhea rate (%) = [Number of piglets with diarrhea during experiment / (Experimental days × Number of pigs in group)] × 100.

1.5.3 Intestinal Villus Height and Crypt Depth Measurement

After slaughter, approximately 1 cm ileal rings were excised, rinsed with cold physiological saline, and fixed overnight in 4% paraformaldehyde solution. Samples were paraffin-embedded, sectioned serially, and stained with hematoxylin-eosin (HE). Images were captured using a Nikon eclipse ci optical microscope with a Tucsen CCD ICE5.0 imaging system, and measurements were performed using Image Pro Insight software.

1.5.4 Goblet Cell Number Measurement

Ileal rings (approximately 1 cm) were fixed in 4% paraformaldehyde, paraffin-embedded, and sectioned for Alcian Blue-Periodic Acid-Schiff (AB-PAS) staining. Goblet cells were counted under an optical microscope (5-10 complete intestinal villi per tissue section). Staining criteria: red-stained cells were neutral goblet cells secreting neutral mucin; blue-stained cells were acidic goblet cells secreting acidic mucin; purple-blue stained cells were neutral-acidic goblet cells capable of secreting both neutral and acidic mucin.

1.5.5 Intestinal Permeability Indicators Measurement

Blood samples were collected from the anterior vena cava on days 3, 7, and 14 of the experiment (after fasting), allowed to stand overnight at 4°C, then centrifuged at 4,000 r/min (4°C) for 20 minutes to separate serum. Serum was aliquoted into 0.5 mL tubes and stored at -80°C. Serum D-lactate and endotoxin concentrations were measured using a D-lactate colorimetric assay kit (Sigma) and a chromogenic limulus amebocyte lysate kit (Xiamen Houshiqi Reagent Experimental Factory Co., Ltd.).

1.5.6 Tight Junction Protein mRNA Expression Measurement

Total RNA from ileal and colonic mucosa was extracted using Trizol reagent (Invitrogen) and reverse-transcribed to cDNA using a PrimeScript™ RT reagent kit with gDNA Eraser (TaKaRa). Real-time quantitative PCR was performed using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the reference gene. Reaction conditions were: 95°C pre-denaturation for 2 min; 40 cycles of 95°C denaturation for 10 s and 60°C annealing/extension for 40 s. Fluorescence acquisition and melting curve analysis were performed according to the manufacturer' s instructions for the ABI PRISM 7500 Real-Time PCR System. mRNA expression levels of Occludin and zonula occludens-1 (ZO-1) were calculated using the 2^(-ΔΔCt) method. Primer information is provided in Table 2 and was synthesized by Invitrogen (Shanghai) Trading Co., Ltd.

1.6 Statistical Analysis

Based on a completely randomized single-factor design, basic statistics were analyzed using the MEANS module of SAS 9.2. Data were subjected to ANOVA using the GLM module, and multiple comparisons were performed using Tukey's method. Differences were considered significant at $P<0.05$ and highly significant at $P<0.01$.

Table 2 Primer sequences information

Gene	NCBI No.	Primer sequences (5' $→$3')	Annealing temperature (℃)	Product size (bp)
GAPDH	NM_{001206359.1	F:CGTTCG-GTTGTGGATCT-GAR: TGACGAAGTG-GTCGTTGAGG		
Occludin	NM_{001163647.2	F:CTGCACC-CTCCAGATTGR: TAT-GTCGTTGCTGGGT-GCAT		
ZO-1	XM_{005659811.1	F:CCAGGCC-CTTACCTTTCGR: GGGGTAGGGGTC-CTTCCTAT		

2.1 Effects of Clostridium butyricum on Growth Performance and Diarrhea Rate

As shown in Table 3 , no significant differences were observed in average daily gain or feed-to-gain ratio among groups during the experimental period

(P>0.05). Compared with the control group, the diarrhea rate of weanling piglets in the CB and ZnO groups decreased by 44.45% and 66.67% during days 1-7 (P<0.05), and by 46.88% and 62.49% during days 1-14 (P<0.05), respectively. No significant difference in diarrhea rate was found between the CB and ZnO groups throughout the experiment (P>0.05).

Table 3 Growth performance and diarrhea rate of weanling piglets

Items	Control group	ZnO group	CB group	P-value
IBW (kg)				
FBW (kg)				
ADG (g)				
F/G				
Diarrhea rate of day 1-7	64.29[a]	21.43	35.71	
Diarrhea rate of day 1-14	38.10[a]	14.29	20.24	

In the same row, values with the same or no letter superscripts mean no significant difference (P>0.05), while different small letter superscripts indicate significant difference (P<0.05), and different capital letter superscripts indicate highly significant difference (P<0.01). The same applies below.

2.2 Effects of Clostridium butyricum on Intestinal Morphology and Goblet Cell Number

As shown in Table 4 , compared with the control group, the ileal crypt depth of weanling piglets in the CB and ZnO groups decreased significantly (P<0.05), while villus height increased (P>0.05) and the villus height-to-crypt depth ratio increased markedly (P<0.01). No significant difference in total goblet cell number was observed between the CB and ZnO groups (P>0.05), but the number of neutral-acidic goblet cells increased significantly in the ZnO group (P<0.05). Both the CB and ZnO groups showed a trend toward increased total goblet cell numbers compared with the control group (P>0.05). As shown in Figure 1 [Figure 1: see original paper], no obvious differences in ileal tissue morphology were observed between the CB and ZnO groups.

Table 4 Ileum morphology and goblet cell number of weanling piglets

Items	Control group	ZnO group	CB group	P-value
Villus height (m)				
Crypt depth (m)	140.13[a]	109.06	106.47	
V/C ratio	2.38	3.52	3.65	<0.001
Neutral goblet cells number				
Acidic goblet cells number				
Neutral-acidic goblet cells number				
Total goblet cells number				

Items	Control group	ZnO group	CB group	P-value

Figure 1 Ileum morphological structure graphs of weanling piglets

2.3 Effects of Clostridium butyricum on Intestinal Permeability

As shown in Table 5 , differences in serum endotoxin and D-lactate concentrations among groups began to emerge on day 7. Compared with the control group, the ZnO group showed significantly decreased serum endotoxin concentration on day 7 ($P<0.05$), while both the ZnO and CB groups exhibited significantly reduced serum endotoxin and D-lactate concentrations on day 14 ($P<0.05$). No significant differences in these parameters were observed between the CB and ZnO groups ($P>0.05$).

Table 5 Serum endotoxin and D-lactate concentration of weanling piglets

Item	Time	Control group	ZnO group	CB group	P-value
Endotoxin (EU/mL)	Day 3				
	Day 7	0.194[a]	0.116	0.129	
	Day 14	0.149[a]	0.065	0.081	
D-lactate (ng/ L)	Day 3				
	Day 7	86.32[a]	26.84	26.33	

As shown in Table 6 , compared with the control group, the mRNA expression of Occludin in the ileum and colon of weanling piglets in the ZnO and CB groups increased significantly ($P<0.05$). The CB group showed no significant difference in ZO-1 mRNA expression in the ileum and colon ($P>0.05$), while the ZnO group exhibited significantly increased ZO-1 mRNA expression in the ileum ($P<0.05$). No significant differences in ZO-1 and Occludin mRNA expression in the ileum and colon were observed between the CB and ZnO groups ($P>0.05$).

Table 6 The mRNA expression of ZO-1 and Occludin in intestine of weanling piglets

Item	Indicator	Control group	ZnO group	CB group	P-value
Ileum	ZO-1	0.57	0.97[a]	0.82[a]	
	Occludin	0.51	0.95[a]	1.15[a]	
Colon	ZO-1	0.56	1.01[a]	0.89[a]	
	Occludin				

Weaning is a critical stage in pig production, and weaning stress causes diarrhea and growth retardation. High-dose zinc oxide is commonly used to address

these issues. In this experiment, the ZnO group showed significantly reduced diarrhea rates during the first 7 days and throughout the entire experimental period, consistent with findings by Zhang et al. [6] and Hu et al. [15-16]. The CB group exhibited similar anti-diarrheal effects as the ZnO group, with no significant difference between them, indicating that CB is equally effective against piglet diarrhea. However, no significant differences in growth performance were observed among groups during the experimental period. This contrasts with some studies showing growth-promoting effects of zinc oxide [6,16-17], though other reports indicate no significant growth promotion within 2 weeks when adding 1,000, 2,000, or 3,000 mg/kg zinc oxide to weanling piglet diets [18]. Liu et al. [12] found that dietary CB supplementation alone increased growth performance trends in weanling piglets, but without significant differences compared to the control group. The lack of significant differences in growth performance in this study may be attributed to the relatively short experimental period and individual variation among piglets.

Diarrhea in piglets is closely related to intestinal health. Post-weaning, intestinal morphology changes due to weaning stress, manifested by reduced villus height, increased crypt depth, and decreased proliferative capacity of intestinal mucosal cells, which worsen with earlier weaning [19]. Carlson et al. [20] reported that high-dose zinc supplementation improves intestinal morphology by deepening crypts and lengthening villi. Studies have also shown that 3,000 mg/kg zinc oxide significantly increases small intestinal villus height and related gene expression at both mRNA and protein levels [4,21]. Zinc, as an essential trace element for intestinal epithelial cell development and maturation, benefits intestinal morphology and enhances protein synthesis and cell proliferation [11,17]. In this study, both ZnO and CB groups showed significantly reduced crypt depth and increased villus height-to-crypt depth ratio, indicating that CB, like zinc oxide, protects against intestinal morphological damage. This effect may be related to butyric acid production by CB metabolism, as butyrate is a primary nutrient for intestinal epithelial cell regeneration and repair, playing an important role in intestinal morphology restoration [12].

Goblet cells are specialized intestinal epithelial cells that secrete mucins, which attach to the intestinal mucosal surface and are closely associated with intestinal health [22]. Newly formed goblet cells secrete neutral mucus, while more mature cells primarily secrete acidic mucus, which provides stronger defense against harmful microorganisms [5,23]. In this study, piglets fed zinc oxide showed significantly increased numbers of neutral-acidic goblet cells in the ileum and a trend toward increased total goblet cell numbers, consistent with Liu et al. [24]. Piglets fed CB also showed a similar trend toward increased total goblet cell numbers as the ZnO group. Regarding the distribution of goblet cell types, the intestinal cell proliferation in piglets appears to be in early developmental stages, further supporting zinc' s role in promoting cell growth and proliferation. The effect of CB appears weaker than that of zinc oxide, possibly because probiotics act indirectly on intestinal mucosal cells.

Intestinal barrier integrity is fundamental to normal intestinal function, and barrier damage increases permeability [16]. Numerous studies have shown that weaning stress damages the intestinal mucosal barrier and increases permeability [15,25-27]. Serum D-lactate and endotoxin concentrations serve as indicators of intestinal permeability, reflecting its magnitude [7,16,28]. Under normal conditions, serum concentrations of D-lactate and endotoxin are low, but increase when intestinal permeability rises. In this study, serum endotoxin and D-lactate concentrations increased significantly during the early post-weaning period, indicating that weaning stress substantially impacts intestinal permeability, damages intestinal mucosal structure, and disrupts intestinal microbiota, allowing microbial endotoxin and D-lactate to enter the bloodstream. From day 7 onward, piglets fed CB or zinc oxide showed significantly reduced serum endotoxin and D-lactate concentrations, alleviating weaning stress-induced increases in intestinal permeability and diarrhea rates. This suggests that dietary zinc oxide or CB supplementation assists in intestinal repair, further indicating that the antidiarrheal effects of high-dose zinc and CB are related to changes in intestinal permeability.

The structural basis of intestinal permeability is tight junction proteins, with ZO-1 and Occludin commonly used as markers of tight junction barrier and permeability function [7,29]. Zhang et al. [6] found that dietary supplementation with 2,000 mg/kg zinc oxide significantly increased ileal ZO-1 and Occludin mRNA and protein expression. In this study, both CB and ZnO groups showed significantly increased Occludin mRNA expression in the ileum and colon, while the ZnO group also exhibited significantly increased ZO-1 mRNA expression in the ileum, and the CB group showed a trend toward increased ZO-1 mRNA expression. Both treatments thus appear to have similar effects on intestinal mucosal integrity. Under stress conditions, bacteria and endotoxins can regulate ZO-1 and transmembrane protein Occludin expression by modulating cytokines and protein kinase C, thereby reducing intestinal epithelial barrier function [30]. Studies have shown that gene expression of epithelial tight junction proteins is associated with metabolites of intestinal bacteria (such as butyric acid and teichoic acid) [31]. Therefore, CB may reduce intestinal permeability and diarrhea by producing butyric acid through metabolism or altering intestinal microecology, thereby improving intestinal morphology and upregulating tight junction protein gene expression.

In conclusion, dietary supplementation with 500 mg/kg Clostridium butyricum or 3,000 mg/kg zinc oxide in weanling piglets significantly reduces diarrhea rate, improves intestinal mucosal morphology, decreases intestinal permeability, and upregulates tight junction protein gene expression, thereby protecting intestinal mucosal barrier function and mitigating the adverse effects of early weaning stress.

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