Effects of a Composite Additive of Benzoic Acid, Bacillus coagulans, and Oregano Oil on Growth Performance, Antioxidant Capacity, and Jejunal Digestion and Absorption Function in E. coli-Challenged Piglets: Postprint

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Abstract

This experiment aimed to investigate the effects of a compound additive consisting of benzoic acid, Bacillus coagulans, and oregano oil on growth performance, antioxidant capacity, jejunal mucosal disaccharidase activity, and mRNA expression of nutrient transporters in piglets challenged with Escherichia coli. Twenty healthy weaned piglets (Duroc × Landrace × Large White) with an average body weight of (7.64±0.46) kg at (24±1) days of age were randomly allocated to 4 groups with 5 replicates per group and 1 pig per replicate. The control group (CON) and E. coli group (ETEC) were fed the basal diet, while the antibiotic group (AT) and compound additive group (ABO) were fed experimental diets supplemented with antibiotics (20 g/t colistin sulfate + 40 g/t zinc bacitracin) and compound additive (3000 g/t benzoic acid + 400 g/t Bacillus coagulans + 400 g/t oregano oil), respectively. On day 22 of the experiment, piglets in the ETEC, AT, and ABO groups were orally administered a culture containing 3×10^{11} CFU of E. coli, while piglets in the CON group received the same dose of sterile culture. The experimental period lasted 26 days. The results showed that compared with the CON group, the ETEC group exhibited significantly increased diarrhea rate and diarrhea index ($P<0.05$), significantly elevated serum and jejunal mucosal malondialdehyde (MDA) content ($P<0.05$), significantly reduced serum total antioxidant capacity (T-AOC) and total superoxide dismutase (T-SOD) activity, and decreased jejunal mucosal sodium-glucose cotransporter 1 (SGLT1) mRNA expression level ($P<0.05$), along with a tendency to decrease jejunal mucosal T-AOC and T-SOD activity

(P<0.10). Compared with the ETEC group, the ABO group showed significantly increased average daily gain (ADG) (P<0.05), significantly decreased feed to gain ratio (F/G) (P<0.05), significantly reduced diarrhea rate and diarrhea index (P<0.05), significantly decreased serum and jejunal mucosal MDA content (P<0.05), significantly enhanced serum and jejunal mucosal T-AOC and T-SOD activity (P<0.05), and significantly upregulated jejunal mucosal SGLT1 and peptide transporter 1 (PepT1) mRNA expression levels (P<0.05). Furthermore, compared with the AT group, the ABO group demonstrated significantly lower diarrhea index (P<0.05) and significantly higher serum T-AOC (P<0.05). In summary, dietary supplementation with the compound additive of benzoic acid, Bacillus coagulans, and oregano oil can significantly alleviate E. coli-induced diarrhea, enhance antioxidant capacity, and improve growth performance and intestinal digestion and absorption function in piglets.

Full Text

Effects of Compound Additive with Benzoic Acid, Bacillus coagulans and Oregano Oil on Growth Performance, Antioxidant Capacity and Jejunal Digestion-Absorption Function of Piglets Challenged with Enterotoxigenic Escherichia coli

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Abstract: This experiment investigated the effects of a compound additive containing benzoic acid, *Bacillus coagulans* and oregano oil on growth performance, antioxidant capacity, jejunal mucosal disaccharidase activities, and nutrient transporter mRNA expression in piglets challenged with enterotoxigenic *Escherichia coli* (ETEC). Twenty healthy castrated Duroc×Landrace×Yorkshire weaned piglets, aged (24±1) days with an average body weight of (7.64±0.46) kg, were randomly allocated to four groups with five replicates per group and one pig per replicate. The control group (CON) and ETEC-challenged group (ETEC) were fed a basal diet, while the antibiotic group (AT) and compound additive group (ABO) received the basal diet supplemented with antibiotics (20 g/t colistin sulfate + 40 g/t bacitracin zinc) or the compound additive (3,000 g/t benzoic acid + 400 g/t *Bacillus coagulans* + 400 g/t oregano oil), respectively. On day 22 of the experiment, piglets in the ETEC, AT and ABO groups were orally administered a culture containing 3×10^{11} CFU of *E. coli*, while CON

piglets received the same volume of sterile culture medium. The experimental period lasted 26 days. The results showed that compared with the CON group, the ETEC group exhibited significantly increased diarrhea rate and diarrhea index ($P<0.05$), significantly elevated malondialdehyde (MDA) content in serum and jejunal mucosa ($P<0.05$), and significantly reduced total antioxidant capacity (T-AOC) and total superoxide dismutase (T-SOD) activity in serum, as well as decreased sodium-glucose cotransporter 1 (SGLT1) mRNA expression in jejunal mucosa ($P<0.05$), with a tendency toward decreased T-AOC and T-SOD activities in jejunal mucosa ($P<0.10$). Compared with the ETEC group, the ABO group showed significantly improved average daily gain (ADG) ($P<0.05$), reduced feed-to-gain ratio (F/G) ($P<0.05$), decreased diarrhea rate and diarrhea index ($P<0.05$), lower MDA content in serum and jejunal mucosa ($P<0.05$), higher T-AOC and T-SOD activities in serum and jejunal mucosa ($P<0.05$), and increased SGLT1 and oligopeptide transporter 1 (PepT1) mRNA expression levels in jejunal mucosa ($P<0.05$). Furthermore, compared with the AT group, the ABO group demonstrated a significantly lower diarrhea index ($P<0.05$) and higher serum T-AOC ($P<0.05$). In conclusion, dietary supplementation with the compound additive containing benzoic acid, *Bacillus coagulans* and oregano oil can effectively alleviate ETEC-induced diarrhea, enhance antioxidant capacity, and improve growth performance and jejunal nutrient digestion-absorption function in piglets.

Keywords: benzoic acid; *Bacillus coagulans*; oregano oil; growth performance; antioxidant capacity; weaned piglets

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Early weaning has become an essential technique in modern swine production to improve economic efficiency. However, early-weaned piglets have underdeveloped intestinal tracts and are vulnerable to dietary, environmental and psychological stressors, making them susceptible to pathogenic microorganisms that damage intestinal structure and function, leading to diarrhea and mortality. Enterotoxigenic *Escherichia coli* (ETEC) is one of the primary pathogenic factors causing diarrhea and death in piglets. Previous studies have shown that ETEC infection reduces antioxidant capacity in young animals, particularly decreasing antioxidant enzyme activities in intestinal cells and promoting lipid peroxidation, which disrupts intestinal mucosal integrity and function. This process may represent an important mechanism underlying ETEC-induced diarrhea in piglets. Dietary antibiotics are commonly used to prevent post-weaning diarrhea, but long-term and extensive use can compromise animal immunity, enhance bacterial resistance, and cause antibiotic residues, posing serious threats to human health. Therefore, identifying safe and effective antibiotic alternatives is critically important.

Research has demonstrated that benzoic acid, *Bacillus coagulans* and oregano oil possess strong antioxidant capacities and can significantly alleviate oxidative damage induced by various stressors, suggesting their potential as antibiotic substitutes. Chang et al. found that benzoic acid contains carboxyl groups that scav-

enge free radicals, interrupt radical chain reactions, inhibit lipid peroxidation, and mitigate oxidative damage. Kodali et al. reported that *Bacillus coagulans* can scavenge reactive oxygen species (ROS) and inhibit ROS-producing microorganisms, thereby enhancing antioxidant capacity. Oregano oil, extracted from the plant *Origanum vulgare*, contains carvacrol and thymol as its main active components. Thymol, with its phenolic hydroxyl group, can donate hydrogen to hydroperoxyl groups produced in the initial step of fat oxidation, delaying hydroperoxide formation. Additionally, oregano oil can activate antioxidant enzyme systems after entering the animal body, increasing antioxidant enzyme activities and enhancing the ability to scavenge oxygen free radicals. However, field applications show that individual supplementation of these additives has limited effects with high variability. Recently, the "synergistic" and "additive" effects of combining organic acids, probiotics and essential oils in animal diets have attracted widespread attention, though reports on the combined use of benzoic acid, *Bacillus coagulans* and oregano oil to alleviate ETEC-induced oxidative stress in piglets are lacking. Therefore, this study combined these three additives based on their antioxidant functions to investigate their effects on growth performance, antioxidant capacity, jejunal mucosal disaccharidase activities and nutrient transporter mRNA expression in ETEC-challenged piglets, aiming to provide data for rational use of these additives and reference information for antibiotic alternative research.

1.1 Experimental Materials

Benzoic acid was produced by DSM (China) Limited with 99.5% purity and a recommended dosage of 3,000–5,000 g/t. *Bacillus coagulans* was provided by Kunming Sanzheng Group with a content of 5×10 CFU/g and a recommended dosage of 200–400 g/t. Oregano oil was provided by Zhuhai Kemin Industries, Inc., with main active components carvacrol and thymol at concentrations greater than 2.2% and 1.1%, respectively, and a recommended dosage of 300–500 g/t.

1.2 Experimental Design

Twenty healthy (24±1)-day-old weaned Duroc×Landrace×Yorkshire piglets with an average body weight of (7.64±0.46) kg were randomly assigned to four groups according to similar body weight: control group (CON), ETEC-challenged group (ETEC), antibiotic group (AT) and compound additive group (ABO), with five replicates per group and one pig per replicate. The CON and ETEC groups were fed the basal diet, while the AT and ABO groups received the basal diet supplemented with antibiotics (20 g/t colistin sulfate + 40 g/t bacitracin zinc) or the compound additive (3,000 g/t benzoic acid + 400 g/t *Bacillus coagulans* + 400 g/t oregano oil), respectively. On the morning of day 22, piglets in the ETEC, AT and ABO groups were orally infused with 3×10^{11} CFU *E. coli* culture (concentration of 1×10 CFU/mL) via gastric catheter, while CON piglets received the same volume of sterile culture medium. The

experimental period lasted 26 days.

1.3 Experimental Diets

The basal diet was a corn-soybean meal-based diet formulated according to NRC (2012) nutrient requirements for 7–25 kg weaned piglets. Its composition and nutrient levels are shown in Table 1 . Experimental diets were prepared by replacing corn in the basal diet with the respective additive products.

1.4 Animal Management

The experiment was conducted at the experimental base of the Institute of Animal Nutrition, Sichuan Agricultural University. Piglets had free access to water and were fed four times daily (08:00, 12:00, 16:00, 20:00) with small amounts added frequently. Feed allowance was adjusted to ensure slight remaining feed in the trough after satiation. Room temperature was maintained at 25–28 °C with relative humidity of 60–70%. To prevent cross-infection, experimental piglets were housed separately in two metabolic rooms according to ETEC challenge status.

1.5 E. coli Culture and Challenge

The ETEC strain used in this study was provided by the Key Laboratory of Animal Biotechnology Center, College of Veterinary Medicine, Sichuan Agricultural University, with serotypes O149, K88 and K91. On the morning of day 22, after overnight fasting and weighing, challenged piglets received 3×10^{11} CFU *E. coli* culture (concentration of 1×10 CFU/mL) via gastric catheter, while unchallenged piglets received the same volume of sterile culture medium.

1.6 Sample Collection and Processing

On the morning of day 27, after overnight fasting and weighing, 20 mL of blood was collected from the anterior vena cava into regular centrifuge tubes, allowed to stand at room temperature for 30 min, then centrifuged at 3,500 r/min for 10 min to separate serum, which was stored at -20 °C for antioxidant index analysis. After blood collection, all piglets were euthanized under anesthesia, and the abdominal cavity was immediately opened to isolate the intestines. The jejunum was removed from the mesentery and sampled on ice. A 20-cm segment from the middle of the jejunum was longitudinally opened, gently rinsed with precooled physiological saline to remove intestinal contents, blotted dry on filter paper, and the mucosa was scraped with a glass slide in one direction, placed in EP tubes, wrapped in aluminum foil, snap-frozen in liquid nitrogen, and stored at -80 °C for analysis.

1.7 Assay Methods

1.7.1 Growth Performance

Piglets were weighed on the morning of days 1 and 27 after overnight fasting, and daily feed intake was recorded to calculate average daily feed intake (ADFI), average daily gain (ADG) and feed-to-gain ratio (F/G) on a per-replicate basis.

1.7.2 Diarrhea Indices

After ETEC challenge, diarrhea status was observed and recorded daily for each group to calculate diarrhea index and diarrhea rate. Fecal scoring criteria are shown in Table 2 . Diarrhea was defined as fecal score 2. Diarrhea rate and average diarrhea index were calculated according to Yuan et al. and Liao Bo:

Diarrhea rate (%) = [Number of diarrhea episodes/(Number of piglets × Experimental days)] × 100
Diarrhea index = Total diarrhea score/(Number of piglets × Experimental days)

1.7.3 Antioxidant Capacity in Serum and Intestine

Total antioxidant capacity (T-AOC), glutathione peroxidase (GSH-Px) and total superoxide dismutase (T-SOD) activities, and malondialdehyde (MDA) content in serum and intestine were determined using assay kits from Nanjing Jiancheng Bioengineering Institute according to the manufacturer's instructions. For jejunal mucosal homogenate preparation, approximately 1 g of mucosa was weighed using a precision electronic balance, mixed with precooled physiological saline at a 1:9 weight/volume ratio, homogenized on ice, and centrifuged in a refrigerated centrifuge at appropriate speeds for different indices. The supernatant was collected for T-AOC, GSH-Px, T-SOD activity and MDA content analysis.

1.7.4 Jejunal Mucosal Disaccharidase Activities

Jejunal mucosal sucrase and maltase activities were determined using assay kits from Nanjing Jiancheng Bioengineering Institute. Samples were pretreated by homogenizing 0.5 g of jejunal mucosa with precooled physiological saline at a 1:9 weight/volume ratio on ice, followed by centrifugation at 3,500 r/min for 10 min at 4 °C. The supernatant was used for sucrase and maltase activity determination.

1.7.5 Nutrient Transporter mRNA Expression in Jejunal Mucosa

Real-time quantitative PCR (RT-PCR) was used to determine mRNA expression levels of sodium-glucose cotransporter 1 (SGLT1), glucose transporter type 2 (GLUT2) and oligopeptide transporter 1 (PepT1) in jejunal mucosa.

Total RNA was extracted from jejunal mucosa using Trizol Reagent (TaKaRa, Japan) according to the manufacturer' s instructions. RNA concentration was measured using a nucleic acid-protein detector (Beckman Du-800, CA, USA). The A260/A280 ratio (1.8–2.0) indicated good RNA purity. cDNA synthesis was performed using a PrimeScript™ reagent kit (TaKaRa, Japan) following the manufacturer' s protocol, and the products were stored at -20 °C. Target gene fragments were searched in NCBI, and primers were designed using Primer 5 and Oligo 6.0 software, then synthesized by Dalian Baosheng Biological Company.

Primer sequences and annealing temperatures are shown in Table 3 . RT-PCR was performed using an ABI7900HT Real-Time PCR System (ABI, USA) with a 10 L reaction mixture containing 5 L SYBR Premix Ex Taq™ II (2×) (TaKaRa, Japan), 0.4 L forward primer, 0.4 L reverse primer, 3.2 L double-distilled water, and 1 L cDNA template. PCR amplification conditions were: 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and appropriate annealing temperature for 30 s, then 95 °C for 10 s. Melting curve analysis was performed from 55 °C to 95 °C with a temperature increase rate of 0.5 °C/s. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the reference gene, and relative expression was calculated using the 2^(-ΔΔCt) method.

1.8 Data Processing and Statistical Analysis

Experimental data were initially processed using Excel 2013. Differences between CON and ETEC groups were analyzed using independent t-tests in SPSS 17.0 software. Data from ETEC-challenged groups (ETEC, AT and ABO) were analyzed by one-way ANOVA followed by Duncan' s multiple comparison test. All data are expressed as "mean and pooled standard error." Differences were considered significant at $P<0.05$ and trends at 0.05 P 0.10.

2.1 Effects of Compound Additive on Growth Performance of ETEC-Challenged Piglets

As shown in Table 4 , ADFI did not differ significantly among groups during the entire experimental period ($P>0.05$). Compared with the CON group, ADG in the ETEC group decreased by 9.91% ($P>0.05$) and F/G increased by 5.11% ($P>0.05$). Compared with the ETEC group, ADG in the ABO group was significantly increased ($P<0.05$) and F/G was significantly decreased ($P<0.05$). Compared with the AT group, ADG in the ABO group increased by 17.21% ($P>0.05$) and F/G decreased by 5.29% ($P>0.05$).

2.2 Effects of Compound Additive on Diarrhea of ETEC-Challenged Piglets

As shown in Table 5 , during days 22–26 (post-challenge), the ETEC group exhibited significantly higher diarrhea rate and diarrhea index compared with the CON group ($P<0.05$). Compared with the ETEC group, the ABO group showed significantly reduced diarrhea rate and diarrhea index ($P<0.05$), while the AT group showed numerical reductions ($P>0.05$). Compared with the AT group, the ABO group had a significantly lower diarrhea index ($P<0.05$).

2.3 Effects of Compound Additive on Antioxidant Capacity in Serum and Jejunal Mucosa of ETEC-Challenged Piglets

As shown in Table 6 , compared with the CON group, the ETEC group showed significantly increased MDA content in serum and jejunal mucosa ($P<0.05$), significantly decreased T-AOC and T-SOD activities in serum ($P<0.05$), and a trend toward decreased T-AOC and T-SOD activities in jejunal mucosa

(P<0.10). Compared with the ETEC group, the ABO group exhibited significantly increased T-AOC and T-SOD activities in serum and jejunal mucosa (P<0.05) and significantly decreased MDA content in serum and jejunal mucosa (P<0.05), while the AT group showed significantly increased serum T-AOC activity (P<0.05). Compared with the AT group, the ABO group had significantly higher serum T-AOC activity (P<0.05). No significant differences were observed in GSH-Px activity in serum or jejunal mucosa among groups (P>0.05).

2.4 Effects of Compound Additive on Disaccharidase Activities in Jejunal Mucosa of ETEC-Challenged Piglets

As shown in Table 7 , no significant differences in jejunal mucosal maltase or sucrase activities were observed among groups (P>0.05). Compared with the CON group, maltase and sucrase activities in the ETEC group decreased by 21.18% and 9.46%, respectively. Compared with the ETEC group, the ABO group showed increases of 26.17% and 8.59% in maltase and sucrase activities, respectively.

2.5 Effects of Compound Additive on Nutrient Transporter mRNA Expression in Jejunal Mucosa of ETEC-Challenged Piglets

As shown in Table 8 , compared with the CON group, the ETEC group exhibited significantly decreased SGLT1 mRNA expression in jejunal mucosa (P<0.05). Compared with the ETEC group, the ABO group showed significantly increased SGLT1 and PepT1 mRNA expression levels (P<0.05), while the AT group demonstrated significantly increased PepT1 mRNA expression (P<0.05). No significant differences were observed in GLUT2 mRNA expression among groups (P>0.05).

3.1 Establishment of the Animal Challenge Model

In this study, the ETEC challenge model successfully induced diarrhea in piglets, reducing ADG by 9.91% and increasing F/G by 5.11%, consistent with previous reports. This may be attributed to the shift of nutrients from growth support to immune response, as well as impaired intestinal function and reduced nutrient digestion-absorption capacity caused by diarrhea. Previous studies have suggested that ETEC-induced reduction in intestinal antioxidant capacity may be an important mechanism underlying diarrhea. Our findings also demonstrated that ETEC challenge significantly increased MDA content and decreased T-AOC and T-SOD activities in serum and jejunal mucosa, with a trend toward reduced T-AOC and T-SOD activities in jejunal mucosa, consistent with literature reports. Therefore, we hypothesize that enhancing antioxidant capacity through dietary intervention may be an effective strategy to alleviate ETEC-induced diarrhea in piglets.

3.2 Effects of Compound Additive on Growth Performance and Diarrhea of ETEC-Challenged Piglets

Previous studies have shown that benzoic acid, *Bacillus coagulans* and oregano oil as feed additives can improve growth performance and disease resistance in livestock. Papatsiros et al. reported that dietary supplementation with a mixture of benzoic acid and *Bacillus cereus* significantly increased ADG and reduced F/G and diarrhea rate in weaned piglets. Hui et al. found that adding 2,000 mg/kg benzoic acid and 100 mg/kg thymol to weaned piglet diets significantly improved growth performance and reduced diarrhea index. Li et al. observed that combined supplementation of acidifiers, probiotics, essential oils and enzymes significantly increased ADG and reduced F/G and diarrhea rate compared with the AT group. Our study also found that the combination of benzoic acid, *Bacillus coagulans* and oregano oil significantly improved ADG, and reduced F/G, diarrhea rate and diarrhea index in ETEC-challenged piglets, with superior effects compared to the AT group, possibly related to the antioxidant properties of the three additives.

3.3 Effects of Compound Additive on Antioxidant Capacity of ETEC-Challenged Piglets

The enzymatic and non-enzymatic antioxidant systems interact to maintain free radical balance and resist oxidative damage, with T-AOC, T-SOD and GSH-Px being important components of the enzymatic system. MDA, a stable end product of lipid peroxidation, reflects the degree of lipid peroxidation and cellular damage. Studies have demonstrated that benzoic acid, *Bacillus coagulans* and oregano oil possess strong antioxidant capacities that can alleviate oxidative damage under pathological conditions. Diao found that 0.5% benzoic acid supplementation in weaned piglet diets significantly reduced serum MDA content. Tang reported that dietary plant essential oils increased liver GSH-Px activity and heart catalase (CAT) and SOD activities while decreasing MDA content in liver and lung tissues. Our study showed that dietary supplementation with the compound additive significantly ameliorated ETEC-induced reductions in T-AOC and T-SOD activities and increases in MDA content in serum and jejunal mucosa, with superior effects compared to the AT group. These results indicate that the combination of benzoic acid, *Bacillus coagulans* and oregano oil can enhance systemic and intestinal antioxidant capacity and alleviate ETEC-induced oxidative damage.

3.4 Effects of Compound Additive on Jejunal Digestion-Absorption Function of ETEC-Challenged Piglets

The small intestine is the primary site for nutrient digestion and absorption, where mucosal disaccharidases play crucial roles in carbohydrate absorption. Jang et al. reported that 50 mg/kg plant essential oil supplementation in broiler diets significantly increased trypsin, -amylase and maltase activities. Guo found that *Bacillus licheniformis* significantly increased small intestinal mucosal disac-

charidase activities. Our study showed that ETEC challenge decreased maltase and sucrase activities by 21.18% and 9.46%, respectively, while dietary supplementation with the compound additive prevented these reductions. Additionally, intestinal mucosa contains numerous nutrient transporters whose expression levels reflect absorption capacity. GLUT2 and SGLT1 are major glucose transporters, while PepT1 primarily transports di- and tripeptides from protein digestion. We found that ETEC challenge significantly decreased SGLT1 mRNA expression, whereas the compound additive prevented this reduction and increased PepT1 mRNA expression. These results indicate that the compound additive can significantly improve intestinal nutrient digestion-absorption capacity in ETEC-challenged piglets, possibly by enhancing antioxidant capacity, alleviating oxidative damage, and thereby maintaining intestinal barrier structure and function integrity.

4 Conclusion

Dietary supplementation with the compound additive containing benzoic acid, *Bacillus coagulans* and oregano oil can significantly alleviate ETEC-induced diarrhea and oxidative damage, enhance antioxidant capacity, and improve growth performance and jejunal nutrient digestion-absorption capacity in piglets.

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