Effects of Fermented and Unfermented Qizha Oral Liquid Residue on Colonic Microbiota, Metabolites, and Cytokine Expression in Weaned Piglets (Postprint)

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Abstract

To investigate the feasibility of utilizing traditional Chinese medicine residue in weaned piglet production, a comparative study was conducted on the effects of Qizha oral liquid residue before and after fermentation on colonic microecology and health in weaned piglets. A total of 120 21-day-old weaned piglets were randomly assigned to four groups: a blank control group, a Qizha oral liquid residue group (supplemented with 5 kg/t Qizha oral liquid residue), a fermented Qizha oral liquid residue group (supplemented with 5 kg/t fermented Qizha oral liquid residue), and a positive control group (supplemented with 0.04 kg/t virginiamycin + 0.2 kg/t colistin sulfate + 3,000 mg/kg zinc oxide), with 5 replicates per group and 6 piglets per replicate. On day 28 of the trial, colonic contents and tissues were collected to determine microbial counts, metabolite concentrations, and cytokine mRNA expression levels. The results indicated that compared with the blank control group, both the Qizha oral liquid residue group and fermented Qizha oral liquid residue group showed no significant differences in the counts of 7 bacterial species and total bacteria ($P>0.05$), while interleukin (IL)-12 mRNA expression levels were significantly lower than those in both control groups ($P<0.05$), and indole concentration was significantly lower than that in the positive control group ($P<0.05$). The fermented Qizha oral liquid residue group exhibited significantly lower concentrations of acetic acid, butyric acid, valeric acid, isobutyric acid, straight-chain fatty acids, and total short-chain fatty acids, as well as significantly lower IL-4 mRNA expression levels in colonic tissue, compared with the Qizha oral liquid residue group ($P<0.05$). The positive control group displayed significantly higher granulocyte-macrophage colony-stimulating factor mRNA expression levels and significantly lower Clostridium cluster XIVa and Bifidobacterium counts compared with the

blank control group (P<0.05). In conclusion, Qizha oral liquid residue can increase short-chain fatty acid concentrations in the colon of weaned piglets and regulate colonic cytokine expression; both unfermented and fermented Qizha oral liquid residue do not affect intestinal flora homeostasis, whereas antibiotics and zinc oxide affect the stability of piglet intestinal flora; microbial fermentation did not enhance the ameliorative effects of Qizha oral liquid residue on intestinal microbial metabolism.

Full Text

Effects of Qi-Zha Oral Solution Residues Before and After Fermentation on Colonic Microbes, Metabolites, and Cytokine Expression in Weaned Piglets

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Abstract

To investigate the feasibility of using herbal residues in weaned piglet production, this study compared the effects of Qi-Zha oral solution residues (QOR) before and after fermentation on colonic microecology and health in weaned piglets. A total of 120 piglets weaned at 21 days of age were randomly assigned to four groups: blank control group, QOR group (supplemented with 5 kg/t QOR), fermented QOR group (supplemented with 5 kg/t fermented QOR), and positive control group (supplemented with 0.04 kg/t virginiamycin + 0.2 kg/t colistin sulfate + 3,000 mg/kg zinc oxide), with 5 replicates per group and 6 piglets per replicate. On day 28 of the experiment, colonic contents and tissues were collected to determine microbial counts, metabolite contents, and cytokine mRNA expression levels. The results showed that compared with the blank control group, both QOR and fermented QOR groups exhibited no significant differences in the counts of seven tested bacterial species and total bacteria (P>0.05), but significantly decreased interleukin (IL)-12 mRNA expression compared with both control groups (P<0.05), and significantly reduced indole content compared with the positive control group (P<0.05). The fermented QOR group displayed significantly lower contents of acetate, butyrate, valerate, isobutyrate, straight-chain fatty acids, and total short-chain fatty acids, as well as

lower colonic IL-4 mRNA expression, compared with the QOR group (P<0.05). The positive control group showed significantly higher granulocyte-macrophage colony-stimulating factor mRNA expression and significantly lower counts of *Clostridium* cluster XIVa and *Bifidobacterium* compared with the blank control group (P<0.05). In conclusion, QOR can increase short-chain fatty acid content and regulate cytokine expression in the colon of weaned piglets; neither QOR nor fermented QOR affects intestinal microbial homeostasis, whereas antibiotics and zinc oxide disrupt the balance of intestinal microflora in piglets; microbial fermentation did not enhance the beneficial effects of QOR on intestinal microbial metabolism.

Keywords: Qi-Zha oral solution residues; fermentation; weaned piglets; colon micro-ecology; cytokines

Introduction

Weaning stress often disrupts the structure and function of the gastrointestinal tract in piglets, leading to diarrhea and reduced growth performance. Currently, the swine industry commonly adds multiple antibiotics and high-dose zinc oxide to diets to prevent piglet diarrhea. Although effective, this practice has caused a series of problems including immune suppression, intestinal dysbiosis, bacterial resistance, and drug residues [1]. Therefore, reducing the use of antibiotics and zinc oxide in feed has become an urgent issue for the pig industry. Chinese herbal medicine, as a functional feed additive, offers advantages such as abundant resources, green and pollution-free nature, and prominent biological functions. Due to limitations in extraction methods and efficiency, herbal residues generated by processing enterprises still retain various bioactive components and nutrients [2]. However, intracellular active substances in plant-based herbal residues are not easily absorbed and utilized by animals, and the high cellulose content results in large dietary inclusion rates and poor palatability, wasting herbal resources while disrupting the nutritional balance of diets [3]. Modern fermentation technology can process herbal residues by breaking down plant cell walls, degrading cellulose, and enabling microbial enzymes to directionally transform certain compounds into new functional substances [4]. Li et al. [2] reported that crude protein and amino acid contents increased after fermenting residues from Zishen Bunao liquid and *Bupleurum*. Li et al. [5] found that adding fermented herbal residues composed of *Astragalus*, *Angelica*, *Leonurus*, and Honeysuckle to peripartum sow diets improved the number of live-born piglets and litter weight gain at weaning; supplementation with fermented products from *Astragalus*, *Angelica*, *Rehmannia*, and Peony significantly increased litter weight gain at weaning [6]. Our previous research also found that dietary supplementation with 5 kg/t fermented Qi-Zha oral solution residues (QOR) increased average daily feed intake by 4.8% and average daily gain by 8.3% in weaned piglets, while reducing feed-to-gain ratio; moreover, its effect on improving intestinal morphology was superior to that of non-fermented QOR [7].

The colon harbors a large number of microorganisms that metabolize various nutrients, producing metabolites such as ammonia, short-chain fatty acids, biogenic amines, phenols, and indole compounds, which affect host metabolism and health [8]. However, few studies have examined the effects of fermented herbal residues on colonic microbiota and metabolism in piglets. Therefore, this experiment evaluated the regulatory effects of QOR before and after fermentation on colonic microecology and health by measuring their impacts on colonic microbes, metabolites, and cytokine expression, providing a basis for their application in weaned piglet feeding.

Materials and Methods

1.1 Preparation of Fermented QOR

The QOR was provided by Hunan Sheng Yakai Biotechnology Co., Ltd., with main active components including astragaloside, chrysophanol, and rhein. The residues were obtained after water extraction of herbs including *Astragalus*, Hawthorn, *Atractylodes*, Malt, Rhubarb, and Isatis leaf, with total moisture content controlled at 40%–60%. The residues were first sterilized, then inoculated with *Bacillus subtilis* and yeast for aerobic fermentation at 28–32 °C for 22–26 h; subsequently, *Lactobacillus* and *Clostridium butyricum* were inoculated for anaerobic fermentation at 31–34 °C for 24 h. The ratio of *Bacillus subtilis*:yeast:*Lactobacillus*:*Clostridium butyricum* was 5:2:2:1, with viable cell count $ 2×10^{10}$ CFU/g, and the mixture was turned 1–2 times daily. After fermentation, the product was dried under vacuum, pulverized, and stored. The measured contents of dry matter, crude protein, crude fiber, crude fat, crude ash, and gross energy for QOR before and after fermentation were 95.04% and 95.84%, 7.72% and 8.55%, 2.46% and 1.09%, 5.70% and 3.85%, 25.38% and 25.82%, and 13.09 and 13.64 MJ/kg, respectively [7].

1.2 Animals, Groups, and Management

The animal trial was conducted at the Xinfeng Yongan Experimental Base of the Institute of Subtropical Agriculture, Chinese Academy of Sciences from July 16 to August 12, 2015. A total of 120 Duroc × Landrace × Yorkshire crossbred piglets weaned at 21 days of age, with an average body weight of approximately 6 kg and equal sex distribution, were randomly allocated to 4 groups based on litter origin and body weight, with 5 replicates per group and 6 piglets per replicate. Piglets in the blank control group were fed a basal diet, while those in the QOR, fermented QOR, and positive control groups received diets supplemented with 5 kg/t QOR, 5 kg/t fermented QOR, or 0.04 kg/t virginiamycin + 0.2 kg/t colistin sulfate + 3,000 mg/kg zinc oxide, respectively. The inclusion rates for QOR before and after fermentation were determined based on preliminary trials evaluating growth performance and diarrhea incidence; antibiotic and zinc oxide levels followed commercial feed production standards. The basal diet was a commercial nursery feed (free of antibiotics and zinc oxide) with nutrient levels slightly below NRC (2012) standards; its composition and nutrient levels

are shown in Table 1 . The experimental period lasted 28 days. Piglets were housed in elevated nursery pens, fed ad libitum (slight excess remaining after satiation), and provided free access to water. Management and immunization protocols followed commercial pig farm standards.

1.3 Sample Collection

On day 28 of the experiment at 08:00, after a 14-hour fast, all piglets were weighed and one medium-sized piglet per pen was slaughtered. Colonic tissues and contents were collected, snap-frozen in liquid nitrogen, and stored at -80 °C.

1.4 Intestinal Microbial Quantification

Colonic contents were used for microbial DNA extraction using the QIAamp DNA Stool Mini Kit (QIAGEN, Germany) following the manufacturer' s protocol for quantitative PCR analysis. Microbial quantification PCR was performed according to the method of Jiao et al. [9]. Specific primers for absolute quantification PCR are listed in Table 2 and were synthesized by Shanghai Sangon Biotech Co., Ltd.

1.5 Microbial Metabolite Measurement

Straight-chain fatty acids (including acetate, propionate, butyrate, and valerate), branched-chain fatty acids (including isobutyrate and isovalerate), and total short-chain fatty acids (sum of straight-chain and branched-chain fatty acids) were measured by gas chromatography [10], while indole content was determined by high-performance liquid chromatography [11].

1.6 Intestinal Cytokine mRNA Expression

Total RNA was extracted from frozen colonic tissues (-80 °C) using RNA Isolation Solvent, and reverse transcription was performed using the PrimeScript RT kit. Real-time quantitative PCR was conducted using β-actin as the internal reference to determine mRNA expression levels of pro-inflammatory cytokines [interleukin (IL)-4, IL-10, and IL-18] and anti-inflammatory cytokines [granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-6, and IL-12]. Primer sequences are listed in Table 2 and were synthesized by Shanghai Sangon Biotech Co., Ltd. The reaction mixture (10.0 µL) contained 5.0 µL Luminaris Color Higreen high ROX qPCR Master Mix (2×), 0.4 µL forward primer, 0.4 µL reverse primer, 2.2 µL nuclease-free H_2O, and 2.0 µL cDNA template. The thermal cycling program was: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

Table 3 shows the primer sequences for cytokines.

1.7 Statistical Analysis

Experimental data were analyzed using SPSS 17.0 software by one-way ANOVA, with Duncan' s multiple comparison test used to compare group means. Results are expressed as 'mean ± standard error' , and $P<0.05$ was considered statistically significant.

Results

2.1 Effects of QOR and Fermented QOR on Colonic Microbial Counts

As shown in Table 4 , the positive control group exhibited significantly lower counts of *Bifidobacterium* and *Clostridium* cluster XIVa compared with the blank control group ($P<0.05$). No significant differences were observed in the counts of *Bacteroidetes*, *Bifidobacterium*, *Clostridium* cluster IV, *Clostridium* cluster XIVa, *Escherichia coli*, *Firmicutes*, *Lactobacillus*, or total bacteria between the QOR and fermented QOR groups and the blank control group ($P>0.05$).

2.2 Effects of QOR and Fermented QOR on Microbial Metabolite Contents

As shown in Table 5 , both QOR and fermented QOR groups had significantly lower indole content than the positive control group ($P<0.05$). The fermented QOR group exhibited significantly lower acetate and butyrate contents in colonic contents compared with the QOR group ($P<0.05$), but showed no significant differences from either control group ($P>0.05$). The QOR group had significantly higher contents of valerate, isobutyrate, straight-chain fatty acids, and total short-chain fatty acids in colonic contents compared with both the fermented QOR and positive control groups ($P<0.05$).

2.3 Effects of QOR and Fermented QOR on Colonic Cytokine mRNA Expression

As shown in Table 6 , the QOR and positive control groups showed significantly higher IL-4 mRNA expression in colonic tissues compared with the blank control and fermented QOR groups ($P<0.05$). No significant differences in IL-10 and IL-18 mRNA expression were observed among the four groups ($P>0.05$). The blank control group exhibited significantly lower GM-CSF mRNA expression than the positive control group ($P<0.05$). Both QOR and fermented QOR groups had significantly lower IL-12 mRNA expression compared with the two control groups ($P<0.05$).

Discussion

The animal gastrointestinal tract harbors over 100 trillion bacteria belonging to thousands of different microbial species. These microorganisms extensively

participate in host nutrient metabolism, enhance immunity, prevent pathogenic infection, and inhibit production of putrefactive substances in the intestine [12]. Imbalance of intestinal microbiota leads to impaired gut health, resulting in diarrhea and inflammation. *Firmicutes* and *Bacteroidetes* are the dominant phyla in the porcine gut [13], with *Bifidobacterium*, *Lactobacillus*, *Clostridium* cluster IV, and *Clostridium* cluster XIVa being beneficial groups within *Firmicutes*. *Escherichia coli* is a pathogenic bacterium causing piglet diarrhea. In this study, dietary antibiotics and zinc oxide reduced total bacterial counts, *E. coli*, and other beneficial bacteria in the colon, and significantly decreased *Clostridium* cluster XIVa and *Bifidobacterium*, indicating that antibiotics and zinc oxide kill not only harmful but also beneficial bacteria, disrupting intestinal microbial homeostasis. This is consistent with the report by Ma et al. [14] that broad-spectrum antibiotics disrupt intestinal microecological balance and cause dysbiosis in SD rats. Additionally, no significant differences in colonic bacterial counts were observed between the QOR and fermented QOR groups, suggesting that intestinal microbial homeostasis was not affected. Although *Lactobacillus* was added during the fermentation of QOR, the fermented QOR group showed lower *Lactobacillus* counts than the blank control and QOR groups, which may be related to the fermentation process and small sample size; the specific reasons require further investigation.

Tryptophan derived from dietary protein or sloughed intestinal epithelial cells is anaerobically fermented by microorganisms in the posterior ileum of pigs to produce volatile compounds such as indoleacetic acid and indole [15-16]. Indole-containing compounds in feces are reported to be the main odorous substances in pig manure [17]. In this study, the positive control group showed significantly higher indole content than both QOR groups, consistent with Han et al. [18] who reported that antibiotics can induce indole biosynthesis, thereby increasing *E. coli* drug resistance. Additionally, dietary QOR and fermented QOR reduced ammonia nitrogen and indole contents, which could decrease environmental pollution from volatile compounds in pig feces. Short-chain and branched-chain fatty acids in feces are produced by intestinal microbiota metabolizing undigested fiber, resistant starch, and nitrogenous substances in the gut [19]. The fermented QOR group exhibited significantly lower contents of acetate, butyrate, valerate, isobutyrate, and total short-chain fatty acids in the colon compared with the QOR group, which may be related to the reduced crude fiber content in fermented QOR, thereby decreasing the amount of undigested cellulose reaching the colon [20]. Furthermore, the content and composition of microbially produced total short-chain fatty acids depend on substrate availability, intestinal transit time, and microbial community composition [12]. Therefore, measured total short-chain fatty acid content does not necessarily correlate positively with intestinal microbial counts [21].

Pro-inflammatory cytokines (GM-CSF, IL-6, and IL-12) and anti-inflammatory cytokines (IL-4, IL-10, and IL-18) are important immunomodulatory factors. Interleukins are cytokines that mediate interactions between leukocytes and other cells, while GM-CSF stimulates the growth and differentiation of various bone

marrow precursor cells [22]. Thus, balanced expression of multiple immune cytokines is crucial for maintaining normal immune function [23]. In this study, compared with the blank control group, dietary QOR significantly upregulated IL-4 mRNA expression and downregulated IL-12 mRNA expression, suggesting that QOR can enhance immune function in weaned piglets, possibly related to increased total short-chain fatty acid content as microbial metabolites. Total short-chain fatty acids play important physiological roles in host-microbe interactions, such as providing energy for host colonic epithelial cells, inhibiting pathogenic microorganisms, and exerting anti-inflammatory effects by reducing release of immune cytokines and chemokines [24]. Additionally, compared with the blank control group, dietary antibiotics and zinc oxide significantly upregulated pro-inflammatory cytokine GM-CSF mRNA expression in colonic tissue, which is associated with massive release of bacterial cellular components during pathogen killing, thereby triggering inflammatory responses [25].

Conclusions

1. Dietary supplementation with Qi-Zha oral solution residues can increase short-chain fatty acid content and regulate cytokine expression in the colon of weaned piglets.
2. Neither QOR nor fermented QOR affects intestinal microbial homeostasis, whereas dietary antibiotics and zinc oxide disrupt the balance of intestinal microflora in piglets.
3. The microbial fermentation process used in this study did not enhance the beneficial effects of QOR on intestinal microbial metabolism.

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