JAST (Journal of Animal Science and Technology) TITLE PAGE

Upload this completed form to website with submission

ARTICLE INFORMATION	Fill in information in each box below
Article Type	Research article
Article Title (within 20 words without abbreviations)	Dietary supplementation of *Enterococcus faecalis* based parabiotics combined with *Bacillus subtilis* ameliorate intestinal dysfunction in weaned piglets challenged with ETEC
Running Title (within 10 words)	Paraprobiotics protect against ETEC in weaned piglets
Author	Dongcheol Song[1†], Seungwon Jung[1†], Jihwan Lee[2†], Kyeongho Jeon[1], Hyuck Kim[1], Jinmo Yang[1] and Jinho Cho[1*]
Affiliation	[1]Department of Animal Science, Chungbuk National University, Cheongju, South Korea 28644 [2]Department of Animal Science, Jeonbuk National University, Jeonju 54896, Korea
ORCID (for more information, please visit https://orcid.org)	Dongcheol Song / (https://orcid.org/0000-0002-5704-603X) Seungwon Jung/ (https://orcid.org/0009-0004-5592-7097) Jihwan Lee/ (https://orcid.org/0000-0001-8161-4853) Kyeongho Jeon / (https://orcid.org/0000-0003-2321-3319) Hyuck Kim / (https://orcid.org/0000-0002-5280-0734) Jinmo Yang / (https://orcid.org/0009-0007-4272-3441) Jinho Cho / (http://orcid.org/0000-0001-7151-0778)
Competing interests	No potential conflict of interest relevant to this article was reported.
Funding sources State funding sources (grants, funding sources, equipment, and supplies). Include name and number of grant if available.	This research was funded by Winsbio, Korea
Acknowledgements	Not applicable.
Availability of data and material	Upon reasonable request, the datasets of this study can be available from the corresponding author.
Authors' contributions Please specify the authors' role using this form.	Conceptualization: Cho J, Song D, J Lee. Data curation: Jeon K, Kim H. Formal analysis: Song D. Methodology: Jung S. Software: Jeon K, Kim H, Yang J. Validation: Song D, Jung S. Investigation: Song D. Writing - original draft: Song D, Jung S, J Lee. Writing - review & editing: Cho J, Song D, Jung S, Jeon K, Kim H, Yang J.
Ethics approval and consent to participate	The experimental protocol was approved (CBNUA-25-0087-01) by the Institutional Animal Care and Use Committee of Chungbuk National University, Cheongju, Korea.

CORRESPONDING AUTHOR CONTACT INFORMATION

For the corresponding author (responsible for correspondence, proofreading, and reprints)	Fill in information in each box below
First name, middle initial, last name	Jinho Cho
Email address – this is where your proofs will be sent	jinhcho@chungbuk.ac.kr
Secondary Email address	Paul741@daum.net
Address	Department of Animal Science, Chungbuk National University, Cheongju, South Korea 28644

Cell phone number	+82-10-5488-8580
Office phone number	+82-43-261-2544
Fax number	+82-43-273-2240

Abstract

Post-weaning diarrhea caused by enterotoxigenic *Escherichia coli* (ETEC) is a major challenge in pig production, necessitating development of effective alternatives to antibiotic growth promoters. This study aimed to evaluate the effects of dietary *E. faecalis* EF-2001 based parabiotics, supplemented either alone (WB) or alongside BS, β-glucan, and mannan-oligosaccharides (FB) on intestinal health in weaned pigs challenged with ETEC. A total of 24 crossbred weaning pigs were randomly assigned to six treatment groups with four replicates per treatment: positive control (PC, basal diet without challenge), negative control (NC, basal diet with ETEC challenge), WB1 (NC with 0.10% WB alone), FB50 (NC with 0.05% FB), FB75 (NC with 0.075% FB), and FB100 (NC with 0.10% FB). All pigs in challenged groups received oral ETEC inoculation for three consecutive days. The combined supplementation at the highest inclusion level (FB100) completely restored average daily gain and feed efficiency to levels comparable with PC, while markedly reducing diarrhea scores compared to NC. Apparent total tract digestibility of dry matter, crude protein, and gross energy improved progressively with increasing combined supplementation levels. The FB100 group maintained white blood cell counts and pro-inflammatory cytokine levels comparable to PC while increasing serum IgA and IgG concentrations compared to NC. Intestinal morphology was preserved in combined supplementation groups, with maintained villus height and reduced crypt depth resulting in improved villus height-to-crypt depth ratios. Gene expression analysis revealed upregulated claudin-1 in PC and FB100 groups, indicating enhanced intestinal barrier function. Microbiota analysis showed maintained alpha diversity with increased *Blautia* abundance in the FB100 group and reduced *Bacteroidota* in the FB100 group compared to NC. These findings demonstrate that dietary supplementation with *E. faecalis*-based parabiotics combined with *B. subtilis*, β-glucan, and mannan-oligosaccharides effectively protects against ETEC-induced intestinal dysfunction through integrated mechanisms including barrier enhancement, immune modulation, and microbiota optimization, offering a scientifically validated alternative to antibiotic growth promoters in weaned pig production.

Keywords: parabiotics, probiotics, weaned pigs, ETEC, intestinal health

Introduction

Weaning is a critical transition period in swine production, marked by exposure to several stressors such as dietary changes, maternal separation, and environmental adaptations [1]. These stressors often lead to post-weaning diarrhea (PWD), a complex syndrome that hinders intestinal development, triggers inflammatory responses, and reduces productivity [2, 3]. A primary cause of PWD is enterotoxigenic *Escherichia coli* (ETEC), which adheres to intestinal epithelial cells and secretes enterotoxins that disrupt mucosal integrity and immune balance [4, 5].

Probiotics have been widely used as interventions to support intestinal health and modulate immune function in weaned piglets. However, concerns about their consistent efficacy across different environmental conditions, processing methods, and storage durations have led researchers to explore alternative solutions [6]. Parabiotics, which consist of inactivated microbial cells or cell components produced through heat treatment or other inactivation techniques, have emerged as promising alternatives. They retain immunomodulatory and anti-inflammatory properties while providing greater stability compared to live probiotics [7, 8].

Heat-killed *Enterococcus faecalis* has shown bioactive effects, including the reduction of pro-inflammatory cytokines, regulation of immunoglobulins, and protection of the gastrointestinal mucosa, making it a valuable ingredient for stabilizing the intestinal environment in weaned piglets [9]. Modern strategies for enhancing intestinal health go beyond single functional components to include synergistic combinations of ingredients with complementary mechanisms, allowing for more effective modulation of intestinal microbiota and host immune responses [10].

Bacillus subtilis (BS) has been noted for its ability to improve intestinal microbial balance and mucosal health [11], while immunogenic polysaccharides such as β-glucan and mannan-oligosaccharides (MOS) stimulate innate immunity and prevent pathogen adhesion to the intestinal epithelium [12, 13]. Despite the established roles of these components in promoting intestinal health and immune function through distinct physiological pathways, there is limited research comparing the effects of *E. faecalis*-based parabiotics administered alone versus in combination with probiotics and immunogenic polysaccharides.

Heat-killed *E. faecalis* retains structural components such as peptidoglycan, lipoteichoic acid, and surface proteins that engage host pattern recognition receptors, modulating immune responses without requiring viable colonization [14, 15]. In contrast, viable B. subtilis exerts probiotic functions through active metabolism, including secretion of digestive enzymes, production of antimicrobial lipopeptides, generation of short-chain fatty acid precursors, and competitive exclusion of enteric pathogens [16, 17]. β-glucan and MOS further

reinforce these effects by stimulating innate immunity and competitively blocking ETEC fimbrial attachment to enterocytes [12, 13, 18]. The integration of these mechanistically distinct components is therefore expected to provide additive or synergistic protection against ETEC-induced intestinal dysfunction.

We hypothesized that combined supplementation would produce synergistic effects on gut health and immune responses, providing superior protection against ETEC infection compared to administering parabiotics alone. This study aimed to evaluate the effects of dietary *E. faecalis* EF-2001 (WB) based parabiotics, supplemented either alone or alongside BS, β-glucan, and MOS, on growth performance, nutrient digestibility, blood profiles, intestinal morphology, intestinal immunity, and fecal microbiota composition in ETEC-challenged weaned piglets.

Materials and Methods

Ethics approval and consent to participate

The experimental protocols describing the management and care of animals were reviewed and approved by the Animal Care and Use Committee of Chungbuk National University (CBNUA-25-0087-01).

Animals and experimental design

A total of 24 crossbred weaning pigs ([Landrace × Yorkshire] × Duroc) with an initial BW of 11.70 ± 1.08 kg were individually housed in 45cm × 55cm × 45cm stainless steel metabolism cages. There was one pig in each cage and four replicate cages per treatment and housed in individual pen for 17 days, including 3 days before and 14 days after the first ETEC challenge (d 1). All diets were formulated to meet or exceed the NRC [14] requirements for 11-25 kg pigs (Table 1). The diets were mixed with water in a 1:1 ratio before feeding and were fed at 08:30 and 17:30 each day. The pigs had ad libitum access to water. The experimental environment was maintained at relative humidity of 60 ± 2.3%, temperature of 27 ± 1.5 ℃. In the ETEC challenge treatments, all pigs were orally inoculated by dividing a total of 10 mL of ETEC (F18; 1×10^{10} CFU/mL) for three consecutive days from 1 day post-inoculation (DPI) after 3 d of adaptation. Successful establishment of ETEC infection was assessed based on the following indicator; fecal consistency score, evaluated daily from d 0 to d 7 post-challenge. The WB and Immune FB-110 (FB) used in the current experiment is manufactured by Winsbio (Yongin, Korea). FB, a combined parabiotic supplement, was formulated to contain WB (1.0×10^{10} cells/g), BS MORI (2.0×10^{8} CFU/g), β-glucan (5%), and MOS (3%). The dietary treatments evaluated in this study included: 1) PC (basal diet), 2) NC (basal diet + ETEC challenge), 3) WB1 (NC + 0.10% WB containing 1.0×10^{10} cells/g) 4) FB50 (NC + 0.05% FB), 5) FB75 (NC + 0.075% FB), and 6) FB100 (NC + 0.1% FB).

Growth performance

Body weight was recorded at initiation, day 7, and day 14 of the experiment. Daily feed intake was calculated by subtracting residual feed from the amount offered. Average daily gain (ADG) was calculated by dividing body weight gain by the number of days in each period. Feed conversion ratio (FCR) was calculated by dividing average daily feed intake (ADFI) by ADG.

Diarrhea scores

The diarrhea scores were individually recorded at 08:00 and 17:00 h by the same person during the entire experimental period. The diarrhea score was scored using a method used by Zhao et al. [15]. The diarrhea scores were assigned as follows: 0, normal feces; 1, soft feces; 2, mild diarrhea; and 3, severe diarrhea.

Nutrient digestibility

To estimate digestibility, 0.2% chromium oxide (Cr_2O_3) was supplemented with diets as an indigestible marker. Pigs were fed diets mixed with chromium oxide for 4 consecutive days from days post-inoculation (DPI) 4 and 11, and fresh excreta samples were collected in that period. At the end of the experiment, fecal samples were stored at −20℃ and dried at 70℃ for 72 h and then ground to pass through a 1 mm screen. All analysis items (feed and fecal) were analyzed for DM and CP. The procedures utilized for the determination of dry matter (DM) and crude protein (CP) digestibility were conducted with the AOAC methods [16]. Chromium was analyzed with an ultraviolet absorption spectrophotometer (UV-1201, Shimadzu, Kyoto, Japan). The digestibility was calculated using the following formula: digestibility (%) = [1–(Nf × Cd)/(Nd × Cf)] × 100, where Nf is the nutrient concentration in feces (% DM), Nd is the nutrient concentration in diet (% DM), Cd is the chromium concentration in diet (% DM), and Cf is the chromium concentration in feces (% DM).

All chemical analyses of diet and fecal samples were conducted duplicate, and the mean value was used for digestibility calculation. Analyses were repeated when the coefficient variation between duplicate measurements exceeded 5%.

Blood profile

Blood samples were obtained from the jugular vein of 4 pigs, each treatment at DPI 1 and DPI 14. At the time of collection, blood samples were collected into vacuum tubes containing K_3EDTA for CBC analysis and non-heparinized tubes for serum analysis, respectively. After collecting, blood samples were centrifuged (3,000 × g for 15 min at 4◦C). The white blood cells (WBC), basophils, neutrophils, and lymphocyte levels in the whole blood were measured using an automatic blood analyzer (ADVIA 120, Bayer, NY, USA)

Morphological analysis of small intestine

At the end of the experiment (DPI 14), pigs were anesthetized with carbon dioxide gas after blood sampling and euthanized by exsanguination. Intestinal tissues of approximately 10 cm from the ileum (close to the ileocecal junction) were collected and fixed in 10% neutral buffered formalin (NBF; Sigma–Aldrich, St. Louis, MO, United States). After cutting the intestine sample, it was dehydrated and dealcoholized. The samples were, then, installed on slides, treated with paraffin, and stained with hematoxylin and eosin. Villus height (VH) and crypt

depth (CD) were measured under the light microscope (OLYMPUS DP71, BX50F-3, Olympus Optical Co. Ltd., Tokyo, Japan). VH was determined by measuring the distance between the tip of the villi to the villus crypt junction, and CD was determined by measuring the distance between adjacent villi.

Measurements of pro-inflammatory cytokine and immunoglobulin

The inflammatory biomarkers such as interleukin-6 (IL-6) and tumor necrosis factor α (TNF-α) were measured using commercially available ELISA kits, according to the manufacturer's instructions (R&D Systems, Minneapolis, MN). Immunoglobulin G (IgG) and immunoglobulin A (IgA) levels were gauged using an automatic biochemistry blood analyzer (Hitachi 747; Hitachi, Tokyo, Japan).

Expression of tight junction proteins

The Total RNA extraction kit (iNtRON Biotechnology, Seongnam, Korea) was used to extract the RNA from the intestinal mucosa. The mRNA was converted to cDNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA, USA). For cDNA synthesis, the mixed solution was heat treated at 25°C for 10 min, at 37°C for 2 h, and at 85°C for 5 min. Gene amplification was performed using Fast qPCR 2×SYBR Green Master Mix (Applied Biosystems). RT-qPCR was performed in two steps. The first step was an enzyme activation step, which was performed at 95°C for 2 min for 1 cycle. The second step was a denaturation step at 95°C for 15 seconds and an annealing/extend step at 56°C for 1 min, repeating a total of 40 cycles to perform gene amplification. The target genes were zonula occludens-1 (ZO1), claudin-1 (CLDN1), mucin 1 (MUC1) and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Primers used in the amplification are shown in Table 6. Normalization was performed using the reference gene GAPDH. Relative gene expression was analyzed using the $2^{-\Delta\Delta Ct}$ method [17].

16S metagenomic data analysis

Bacterial 16S rRNA amplicon sequencing data were analyzed using QIIME2 next-generation microbiome bioinformatics pipeline for microbial community analysis. The samples were sent to Sanigen (Anyang, South Korea) for microbial sequencing using the 16s rRNA technique. All raw input data were transformed in the form of QIIME2 artifacts, which contain information about the data types and sources for the downstream processing. From raw sequence data, the amplicon sequence variants (ASVs) were obtained using the Divisive Amplicon

Denoising Algorithm 2 (DADA2) within QIIME 2 plugin, which detected and corrected amplicon errors and filtered out the potential base error and chimeric sequences [18, 19]. Per-sample sequencing depth and ASV counts after quality filtering. To control differences in sequencing depth across samples, the ASV table was rarefied to a uniform depth corresponding to the minimum sample read count after quality filtering, prior to alpha and beta diversity analyses. Rarefaction curves were generated to confirm that this sampling depth captured the majority of microbial diversity in each sample. The relative classification frequency table represented differential abundance tests at specific taxonomic levels was created using collapse and feature-table within the QIIME2 plugins. The "diversity" QIIME2 plugin was used to estimate alpha diversity measurements and plots using the R bioinformatics packages. This microbial diversity analysis pipeline was designed to use the ASV table (a higher resolution analog than the traditional OTU table) of the ASV picking step as necessary input data. Analyzing the differences in species richness and evenness scores considering the sampling depth was measured using the observed OTUs and Chao1, Shannon, and Simpson indices. Each index estimates the V3-V4 hypervariable region of the bacterial 16S rRNA gene. In addition, a difference in the relative abundance was analyzed by comparing the average bacterial proportion and composition investigated in each taxonomic ranking. Additionally, according to the different amplicon regions, the bacterial classification accuracy was cross-checked by comparing the taxonomy matching rate of each ASV taxonomy and NCBI bacterial reference genome database at the phylum and genus levels. P-values for differential abundance testing were corrected for multiple comparisons using the Benjamini–Hochberg false discovery rate (FDR) procedure, and adjusted P-values ($q < 0.05$) were considered significant.

Statistical analysis

JMP Pro 16 (SAS Institute Inc., Cary, NC, United States) and GraphPad Prism (Version 9.1.0; GraphPad Software, San Diego, CA) were used for statistical analyses and graph visualization, respectively. All data, except diarrhea frequency, were analyzed by one-way ANOVA using the Standard Least Squares model of JMP, with treatment as the fixed effect. For variables measured at multiple time points (e.g., body weight, ADG, ADFI, G:F, fecal score), each time point or growth phase was analyzed independently as a separate outcome. Diarrhea frequency was compared among treatments using the chi-square test (FREQ procedure of JMP); Fisher's exact test was applied when expected cell counts were less than 5. For microbiota analysis, each treatment group served as the control group for quantitative beta diversity and PROC MIXED with Dunnett's

post-hoc test was used to compare the treatment groups. Statistical significance was defined as a probability level of $P < 0.05$.

Results

Growth performance

The effects of dietary WB based parabiotics, supplemented either alone or alongside BS, β-glucan, and MOS on growth performance are presented in Table 2. No significant differences were observed in BW among treatment groups throughout the experimental period ($p > 0.05$). ADG during DPI 1 to 7 was higher ($p < 0.05$) in PC than in NC. During DPI 8 to 14, ADG was lower ($p < 0.05$) in NC, WB1, FB50, and FB75 than in PC. For the overall period (DPI 1 to 14), ADG was lower in NC, WB1, FB50, and FB75 than in PC ($p < 0.05$), and FB100 did not differ from PC. ADFI did not differ among treatment groups during any period ($p > 0.05$). FCR during d 1 to 7 was higher ($p < 0.05$) in NC than PC, and FB100. For the overall period (DPI 1 to 14), FCR was higher ($p < 0.05$) in NC than PC, and FB100.

Fecal score

The effects of dietary WB based parabiotics, supplemented either alone or alongside BS, β-glucan, and MOS on fecal score are presented in Figure 1. The distribution of fecal scores differed significantly among treatment groups ($\chi^2 = 89.622$, $p < 0.001$). The proportion of severe diarrhea (score 3) was higher in NC than PC, FB75, and FB100, and the proportion of normal feces (score 0) was lower in NC than in PC and FB100. Among the FB-supplemented groups, the proportion of severe diarrhea progressively decreased from FB50 to FB100, with FB100 showing the lowest incidence and a distribution comparable to PC.

Nutrient digestibility

The effects of dietary WB based parabiotics, supplemented either alone or alongside BS, β-glucan, and MOS on ATTD of nutrients are presented in Table 3. On DPI 7, DM digestibility was lower ($p < 0.05$) in NC and WB1 than PC, and FB100. GE digestibility was lower ($p < 0.05$) in NC, WB1, and FB50 than PC, and FB100, whereas CP digestibility did not differ among treatment groups ($p > 0.05$). On DPI 14, DM digestibility was lower ($p < 0.05$) in NC than PC, FB75, and FB100. CP digestibility was lower ($p < 0.05$) in NC than PC, and FB100. GE digestibility was lower ($p < 0.05$) in NC than PC, FB50, FB75, and FB100.

Blood profile

The effects of dietary WB based parabiotics, supplemented either alone or alongside BS, β-glucan, and MOS on blood profile are presented in Table 4. On DPI 1, no significant differences were observed among treatment groups for red blood cells (RBC), WBC, IgA, IgG, and IL-6 ($p > 0.05$). On DPI 14, RBC concentration was lower ($p < 0.05$) in NC than in all other groups. WBC concentration was higher ($p < 0.05$) in NC, and WB1 than PC, and was lower ($p < 0.05$) in FB50, FB75, and FB100 than NC. Serum IgA concentration was higher ($p < 0.05$) in PC, FB75, and FB100 than NC, WB1, and FB50. Serum IgG concentration was lower ($p < 0.05$) in NC, and WB1 than PC. IL-6 concentration was higher ($p < 0.05$) in NC, and WB1 than PC, and was lower ($p < 0.05$) in FB50, FB75, and FB100 than NC.

Intestinal morphology

The effects of dietary WB based parabiotics, supplemented either alone or alongside BS, β-glucan, and MOS on intestinal morphology are presented in Table 5. VH and villus width did not differ among treatment groups ($p > 0.05$). CD was greater ($p < 0.05$) in NC than PC, FB50, FB75, and FB100, and was greater ($p < 0.05$) in WB1 than PC, FB75, and FB100. The VH to CD ratio was lower ($p < 0.05$) in NC and WB1 than PC, FB75, and FB100.

Tight junction gene expression

The effects of dietary WB based parabiotics, supplemented either alone or alongside BS, β-glucan, and MOS on tight junction gene expression are presented in Table 7. Relative mRNA expression of CLDN1 was lower ($p < 0.05$) in NC, WB1, FB50, and FB75 than PC. No significant differences were observed among treatment groups for ZO-1 and MUC 1 expression ($p > 0.05$).

Fecal microbiota

The effects of dietary WB based parabiotics, supplemented either alone or alongside BS, β-glucan, and MOS on fecal microbiota are presented in Table 8 and Figures 2–4. Alpha diversity indices (Chao1, Shannon, and Simpson) did not differ among treatment groups on either DPI 1 or DPI 14 ($p > 0.05$; Table 8). Beta diversity analysis on DPI 1 showed no clear separation among treatment groups (Figure 2), whereas on DPI 14, FB100 was separated from NC and FB50 along the principal coordinate axes ($p < 0.05$; Figure 3). At the phylum level

on DPI 14, the relative abundance of *Bacteroidota* was lower ($p < 0.05$) in FB100 than NC, WB1, and FB50 and *Actinobacteriota* was higher ($p < 0.05$) in FB100 than NC (Figure 4). At the genus level on DPI 14, the relative abundance of *Blautia* was higher ($p < 0.05$) in FB100 than PC, NC, FB50 (Figure 4).

Discussion

ETEC-induced PWD poses a significant challenge in pig production, resulting in economic losses and increased antibiotic usage [20]. This study demonstrated that dietary supplementation with WB combined with BS, β-glucan, and MOS effectively protects weaned pigs from ETEC-induced intestinal dysfunction. The restoration of growth performance in pigs challenged by ETEC may be partly consistent with the complementary mechanisms of paraprobiotics and probiotics. Heat-inactivated probiotics have been reported to maintain their immunomodulatory functions through intact peptidoglycan and lipoteichoic acid, which can activate pattern recognition receptors independently of bacterial viability [21, 22]. Meanwhile, BS contributes active metabolic functions, including the production of antimicrobial compounds, secretion of digestive enzymes, and competitive exclusion of enteric pathogens [23]. In this study, the highest inclusion level completely restored ADG and G:F to levels comparable to the PC, while lower doses showed gradual improvements. The combination of immunomodulation and metabolic functionality likely explains the enhanced protection observed compared to previously reported single-component strategies [10]. These results align with previous research indicating that BS supplementation improved FCR in ETEC challenged weaned pigs [24, 25]. The BS MORI strain, which produces 1-deoxynojirimycin (DNJ), an iminosugar that potently inhibits α-glucosidases [26]. Beyond the probiotic actions of BS, DNJ inhibits the endoplasmic reticulum glucosidases I and II that trim N-linked glycans during glycoprotein folding, thereby blocking maturation of viral envelope glycoproteins such as hemagglutinin and neuraminidase and lowering virion infectivity [27]. This antiviral capacity, together with antimicrobial and anti-inflammatory activity, distinguishes the MORI strain from generic BS isolates and adds a complementary mode of action to the FB formulation.

ETEC-induced diarrhea primarily results from enterotoxin-mediated disruption of electrolyte transport and fluid homeostasis in intestinal epithelial cells [28]. MOS function as decoy receptors for bacterial fimbriae, competitively inhibiting ETEC attachment to intestinal epithelial cells and preventing subsequent toxin delivery [29]. Simultaneously, β-glucan enhances intestinal barrier integrity by stimulating epithelial cell proliferation and upregulating TJ expression, which reduces paracellular permeability and mitigates fluid loss [30, 31]. The antimicrobial lipopeptides produced by BS exhibit direct bactericidal activity against gram-negative pathogens, further reducing ETEC colonization [32]. DNJ from the MORI strain also inhibits α-glucosidase, which may reduce the carbohydrate substrates available to enteric pathogens and complement the anti-adhesive action of MOS and the bactericidal lipopeptides of BS [26]. In this study, diarrhea scores were significantly lower in

supplemented groups compared to the NC, with the highest inclusion level providing the most effective clinical protection. These findings are consistent with previous reports showing that combined probiotic and prebiotic supplementation decreased the incidence and severity of diarrhea in ETEC-challenged pigs [33, 34].

Enteric pathogens hinder nutrient absorption by causing villus atrophy, degrading brush border enzymes, inducing inflammatory cytokine-mediated metabolic changes, and disrupting nutrient transporter expression [33, 35]. The preservation of intestinal morphology, marked by maintained VH and reduced CD in supplemented groups, correlates directly with increased absorptive surface area and a greater population of functional enterocytes [36]. In addition to structural maintenance, probiotic supplementation has been shown to upregulate the expression of nutrient transporter genes, thereby enhancing active nutrient absorption independently of morphological changes [37]. In this study, supplementation improved the digestibility of DM, CP, and GE with the highest inclusion level nearing complete restoration compared to the PC. These findings align with previous studies indicating that combinations of probiotics and prebiotics enhance nutrient digestibility in weaned pigs under pathogen challenge conditions [23].

ETEC infection typically triggers acute inflammatory responses, characterized by elevated levels of pro-inflammatory cytokines and leukocyte infiltration. While these responses are crucial for initial pathogen control, dysregulation can lead to increased intestinal damage and impaired barrier function [20, 33]. Heat-killed *E. faecalis* has been shown to steer immune responses toward regulatory phenotypes, increasing the production of anti-inflammatory IL-10 while modulating the secretion of pro-inflammatory mediators such as IL-6 and TNF-α [21, 38]. This immunoregulatory ability prevents hyperinflammatory responses that could otherwise compromise intestinal integrity and nutrient absorption. In our study, supplemented groups maintained WBC counts and pro-inflammatory cytokine levels comparable to the PC, suggesting effective prevention of excessive systemic inflammation. DNJ has been reported to suppress NF-κB-driven transcription of pro-inflammatory cytokines, consistent with the lower IL-6 and TNF-α concentrations in supplemented groups and likely reinforcing the immunoregulatory effect of the parabiotic component [39]. The enhancement of adaptive immunity, especially mucosal IgA responses, offers sustained protection by neutralizing pathogens before they invade the epithelium [40]. IgA serves as the primary antibody in intestinal secretions, agglutinating bacteria and preventing their adhesion to enterocyte surfaces through a non-inflammatory defense mechanism that preserves tissue integrity [41]. The increased serum concentrations of IgA and IgG observed in supplemented groups indicate strong humoral immune activation. These results are consistent with prior research showing that

parabiotic supplementation balanced inflammatory responses and enhanced mucosal immunity in animals challenged by pathogens [42].

The VH and CD represent characteristic histopathological changes following enteric infection, resulting from enterocyte death and compensatory proliferation of intestinal stem cells attempting to regenerate damaged epithelium [33, 34]. Short-chain fatty acids produced through BS fermentation of resistant starch and dietary fiber, serve as the primary energy source for colonocytes while simultaneously stimulating epithelial cell proliferation and differentiation [43, 44]. Beyond metabolic support, butyrate exhibits anti-inflammatory properties through inhibition of NF-κB signaling and histone deacetylase activity, reducing inflammatory damage to intestinal mucosa [45, 46]. The immunomodulatory polysaccharide β-glucan further contributes to morphological preservation by enhancing intestinal stem cell activity and accelerating epithelial turnover, facilitating rapid replacement of damaged enterocytes [30]. In the present study, CD was reduced in supplemented groups compared to the NC, resulting in improved VH:CD ratios. The maintained ratio indicates balanced cellular proliferation and differentiation, suggesting that supplementation supports physiological tissue homeostasis rather than pathological compensatory hyperplasia. These morphological improvements are consistent with previous reports showing that combined probiotic and prebiotic supplementation preserved intestinal structure and function in weaned pigs challenged with enteric pathogens [24].

TJs constitute the primary structural components of intestinal barrier function, regulating paracellular permeability and preventing bacterial translocation into systemic circulation [47, 48]. CLDN1, a critical transmembrane protein within TJ complexes, determines ion selectivity and establishes the fundamental tightness of the barrier [49]. ETEC infection disrupts TJ integrity through multiple pathways including direct toxin effects on cytoskeletal organization, inflammatory cytokine-induced activation of myosin light chain kinase leading to junction disassembly, and oxidative stress-mediated protein degradation [50, 51]. The mechanisms by which probiotic and parabiotic interventions preserve TJ function involve activation of protective signaling pathways including protein kinase C and AMP-activated protein kinase, which stabilize tight junction assembly and reduce protein turnover [50, 52]. In the present study, CLDN1 gene expression was significantly upregulated in supplemented groups compared to the NC, providing molecular evidence for enhanced barrier function. The maintained barrier integrity likely contributed to reduced bacterial translocation and systemic endotoxemia, as reflected by normalized blood inflammatory markers. This result is consistent

with previous studies demonstrating that probiotic supplementation increased TJ expression and improved barrier function in ETEC challenged pigs and intestinal epithelial cell models [37, 50].

Short-chain fatty acids generated from the butyrate synthesis of resistant starch and dietary fiber serve as the main energy source for colonocytes, while also promoting epithelial cell proliferation and differentiation [43, 44]. In addition to providing metabolic support, butyrate possesses anti-inflammatory properties by inhibiting NF-κB signaling and histone deacetylase activity, which helps to mitigate inflammatory damage to the intestinal mucosa [45, 46]. The immunomodulatory polysaccharide β-glucan further aids in maintaining morphology by enhancing intestinal stem cell activity and accelerating epithelial turnover, facilitating the swift replacement of damaged enterocytes [30]. In this study, CD was lower in the supplemented groups compared to the negative control (NC), leading to improved VH:CD ratios. This maintained ratio suggests a balanced approach to cellular proliferation and differentiation, indicating that supplementation fosters physiological tissue homeostasis rather than pathological compensatory hyperplasia. These morphological enhancements align with previous findings that combined probiotic and prebiotic supplementation preserved intestinal structure and function in weaned pigs challenged with enteric pathogens [24].

TJs are crucial structural components of the intestinal barrier, regulating paracellular permeability and preventing bacterial translocation into systemic circulation [47, 48]. CLDN1, an essential transmembrane protein within TJ complexes, determines ion selectivity and establishes the barrier's fundamental tightness [49]. ETEC infection disrupts TJ integrity through various mechanisms, including direct toxin effects on cytoskeletal organization, inflammatory cytokine-induced activation of myosin light chain kinase leading to junction disassembly, and oxidative stress-mediated protein degradation [50, 51]. Probiotic and parabiotic interventions preserve TJ function by activating protective signaling pathways, such as protein kinase C and AMP-activated protein kinase, which stabilize tight junction assembly and reduce protein turnover [50, 52]. In this study, CLDN1 gene expression was significantly upregulated in the supplemented groups compared to the NC, providing molecular evidence for enhanced barrier function. The preserved integrity of the barrier likely contributed to reduced bacterial translocation and systemic endotoxemia, as indicated by normalized blood inflammatory markers. These findings are consistent with previous research showing that probiotic supplementation increased TJ expression and improved barrier function in ETEC-challenged pigs and intestinal epithelial cell models [37, 50]. The preservation of alpha diversity indices across treatment groups suggests that supplementation modulated, rather than disrupted, the microbial community structure. This is functionally

significant because excessive disturbances in commensal communities can impair colonization resistance and metabolic functions crucial for host health [53]. An ideal intervention strategy focuses on selectively enhancing beneficial taxa while suppressing potential pathogens. The genus *Blautia* includes important butyrate-producing bacteria that convert dietary fiber and resistant starch into short-chain fatty acids. These fatty acids play multiple roles, such as providing energy to colonocytes, signaling anti-inflammatory responses, and enhancing intestinal barrier function [46, 54]. The observed increase in *Blautia* abundance in supplemented groups suggests an enhanced capacity for beneficial metabolite production. By inhibiting α-glucosidase, DNJ reduces small-intestinal hydrolysis of dietary α-glucans and shifts fermentable carbohydrate to the hindgut, where it can support SCFA-producing genera; this is consistent with the higher Blautia abundance in the supplemented groups [55]. Conversely, the decline in certain Bacteroidota members may reflect a reduction in inflammation-associated taxa, as specific Bacteroidota species tend to proliferate during intestinal inflammation and can contribute to barrier dysfunction in pathological conditions [56]. Beta diversity analysis revealed treatment-specific clustering patterns, indicating that supplementation led to reproducible and consistent shifts in microbiota, rather than random or variable effects. These compositional changes likely contributed to functional outcomes such as increased short-chain fatty acid production, enhanced metabolic capacity, and stronger colonization resistance against enteric pathogens. The selective nature of these changes, which preserved overall diversity while modulating specific taxa, aligns with previous research showing that probiotic and prebiotic supplementation can strategically shape gut microbiota composition in weaned pigs [24, 57]. Importantly, fecal and intestinal SCFA concentrations, as well as inflammation-related metabolites, were not directly quantified in this study. The associations drawn here between the increase in Blautia, the decrease in Bacteroidota, and downstream SCFA production or inflammatory status are therefore based on the established metabolic functions of these taxa in the literature rather than on direct biochemical measurements in our cohort. These mechanistic interpretations should be regarded as hypothesis-generating and require confirmation through targeted metabolomic and functional analyses in future studies.

REFERENCES

1. Tang X, Xiong K, Fang R, Li M. Weaning stress and intestinal health of piglets: A review. Front Immunol. 2022;13:1042778. https://doi.org/10.3389/fimmu.2022.1042778
2. Gresse R, Chaucheyras-Durand F, Fleury MA, Van de Wiele T, Forano E, Blanquet-Diot S. Gut microbiota dysbiosis in postweaning piglets: Understanding the keys to health. Trend Microbiol. 2017;*25*(10):851-73. https://doi.org/10.1016/j.tim.2017.05.004
3. Beaumont M, Cauquil L, Bertide A, Ahn I, Barilly C, Gil L, Canlet C, Zemb O, Pascal G, Samson A, Combes S. Gut microbiota-derived metabolite signature in suckling and weaned piglets. J Proteome Res. 2021;20:982–94. https://doi.org/10.1021/acs.jproteome.0c00745
4. Matsumoto H, Miyagawa M, Takahashi S, Shima R, Oosumi T. Improvement of the enterotoxigenic Escherichia coli infection model for post-weaning diarrhea by controlling for bacterial adhesion, pig breed and MUC4 genotype. Vet Sci. 2020;7(3):106. https://doi.org/10.3390/vetsci7030106
5. Smith MG, Jordan D, Chapman TA, Chin JC, Barton MD, Do TN, Fahy VA, Fairbrother, J.M., Trott, D. J. Antimicrobial resistance and virulence gene profiles in multi-drug resistant enterotoxigenic Escherichia coli isolated from pigs with post-weaning diarrhoea. Vet Microbiol. 2010;145(3-4):299-307. https://doi.org/10.1016/j.vetmic.2010.04.004
6. Ali MS, Lee EB, Hsu WH, Suk K, Sayem SAJ, Ullah HA, Lee SJ, Park SC. Probiotics and postbiotics as an alternative to antibiotics: An emphasis on pigs. Pathogens. 2023;12(7):874. https://doi.org/10.3390/pathogens12070874
7. Li Z, Ali S, Arain MA, Hassan FU, Nabi F, Akhtar MU, Yuan H. Postbiotics and Parabiotics: A Viable Health Promoting Alternative for Poultry Industry—A Comprehensive Review. Probiotics Antimicrob Proteins. 2025;1-22. https://doi.org/10.1007/s12602-025-10708-x
8. Hosseini SH, Farhangfar A, Moradi M, Dalir-Naghadeh B. Beyond probiotics: Exploring the potential of postbiotics and parabiotics in veterinary medicine. Res Vet Sci. 2024;167:105133. https://doi.org/10.1016/j.rvsc.2023.105133.
9. Choi EJ, Iwasa M, Han KI, Kim WJ, Tang Y, Hwang YJ, Chae JR, Han WC, Shin YS, Kim E. K. Heat-killed Enterococcus faecalis EF-2001 ameliorates atopic dermatitis in a murine model. Nutrients. 2016;8(3):146. https://doi.org/10.3390/nu8030146

10. Markowiak P, Śliżewska K. The role of probiotics, prebiotics and synbiotics in animal nutrition. Gut Pathogens. 2018;10:21. https://doi.org/10.1186/s13099-018-0250-0
11. Cui Y, Meng W, He F, Chen Z, Liu H, Li D. Heat-killed Bacillus subtilis concerning broilers' performance, cecal architecture and microbiota. Front Microbiol. 2025;16:1606352.
12. Akter S, Park JH, Jung HK. Potential health-promoting benefits of paraprobiotics, inactivated probiotic cells. J Microbiol Biotechnol. 2020;30(4):477. https://doi.org/10.4014/jmb.1911.11019
13. Dang DX, Choi SY, Choi YJ, Lee JH, Castex M, Chevaux E, Saornil D, Laguna FBL, Kim IH. Probiotic, paraprobiotic, and hydrolyzed yeast mixture supplementation has comparable effects to zinc oxide in improving growth performance and ameliorating post-weaning diarrhea in weaned piglets. Probiotics and Antimicrobial Proteins. 2024;16(1):249-58. https://doi.org/10.1007/s12602-022-10008-8
14. NRC. (2012). Nutrient requirements of swine. 11th ed. National Academy Press, Washington, DC.
15. Zhao PY, Jung JH, Kim IH. Effect of mannan oligosaccharides and fructan on growth performance, nutrient digestibility, blood profile, and diarrhea score in weanling pigs. J Anim Sci. 2012;90:833-9. https://doi.org/10.2527/jas.2011-3921
16. AOAC [Association of Official Analytical Chemists] International. Official methods of analysis of AOAC International. 18th ed. Washington, DC: AOAC International; 2007.
17. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using realtime quantitative PCR and the 2− ΔΔCT method. Methods. 2001;25:402–8. https://doi.org/10.1006/meth.2001.1262
18. Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet CC, Al-Ghalith GA, et al. Reproducible, interactive, scalable, and extensible microbiome data science using QIIME 2. Nat Biotechnol. 2019;37:852–7. https;//doi.org/10.1038/s41587-019-0209-9
19. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes, S.P. DADA2. High-resolution sample inference from Illumina amplicon data. Nat Methods. 2016;13:581–3. https;//doi.org/10.1038/nmeth.3869
20. Fairbrother JM, Nadeau É, Gyles CL. *Escherichia coli* in postweaning diarrhea in pigs: An update on bacterial types, pathogenesis, and prevention strategies. Anim Health Res Rev.

2005;6(1):17-39. https://doi.org/10.1079/AHR2005105

21. Zhu L, Shimada T, Chen R, Lu M, Zhang Q, Lu W, Yin M, Enomoto T, Cheng L. Effects of lysed *Enterococcus faecalis* FK-23 on experimental allergic rhinitis in a murine model. J Biomed Res. 2012;26(3):186-194. https;//doi.org/10.7555/JBR.26.20120023
22. Taverniti V, Guglielmetti S. The immunomodulatory properties of probiotic microorganisms beyond their viability (ghost probiotics: Proposal of paraprobiotic concept). Genes Nutr. 2011;6(3):261-74. https://doi.org/10.1007/s12263-011-0218-x
23. He Y, Kim K, Kovanda L, Jinno C, Song M, Chase J, Li X, Tan B, Liu Y. *Bacillus subtilis*: A potential growth promoter in weaned pigs in comparison to carbadox. J Anim Sci. 2020;98(9):skaa290. https://doi.org/10.1093/jas/skaa290
24. Ding H, Zhao X, Ma C, Gao Q, Yin Y, Kong X., He, J. Dietary supplementation with Bacillus subtilis DSM 32315 alters the intestinal microbiota and metabolites in weaned piglets. J Appl Microbiol. 2021;130(1):217-32. https://doi.org/10.1111/jam.14767
25. Kim K, He Y, Xiong X, Ehrlich A, Li X, Raybould H, Atwill ER, Maga EA, Jørgensen J, Liu Y. Dietary supplementation of Bacillus subtilis influenced intestinal health of weaned pigs experimentally infected with a pathogenic E. coli. J Anim Sci Biotechnol. 2019;10:52. https://doi.org/10.1186/s40104-019-0364-3
26. Park YS, Lee JY, Hwang KY, Kim K. Production of α-glucosidase inhibitor and 1-deoxynojirimycin by Bacillus subtilis MORI. Microbiol Biotechnol Lett. 2021;49(4):566–75. https://doi.org/10.48022/mbl.2111.11008
27. Stavale EJ, Vu H, Sampath A, Ramstedt U, Warfield KL. In vivo therapeutic protection against influenza A (H1N1) oseltamivir-sensitive and resistant viruses by the iminosugar UV-4. PLoS One. 2015;10(3):e0121662. https://doi.org/10.1371/journal.pone.0121662
28. Dubreuil JD, Isaacson RE, Schifferli DM. Animal enterotoxigenic Escherichia coli. EcoSal Plus. 2016;7(1). https://doi.org/10.1128/ecosalplus.esp-0006-2016
29. Spring P, Wenk C, Dawson KA, Newman KE. The effects of dietary mannanoligosaccharides on cecal parameters and the concentrations of enteric bacteria in the ceca of salmonella-challenged broiler chicks. Poult Sci. 2000;79(2):205-11. https://doi.org/10.1093/ps/79.2.205
30. Vetvicka V, Oliveira C. β(1-3)(1-6)-D-glucans modulate immune status in pigs: Potential importance for efficiency of commercial farming. Ann Transl Med. 2014;2(2):16.

https://doi.org/10.3978/j.issn.2305-5839.2014.01.04

31. Hahn TW, Lohakare JD, Lee SL, Moon WK, Chae BJ. Effects of supplementation of β-glucans on growth performance, nutrient digestibility, and immunity in weanling pigs. J Anim Sci. 2006;84(6):1422-8. https://doi.org/10.2527/2006.8461422x
32. Ramachandran R, Chalasani AG, Lal R, Roy U. A broad-spectrum antimicrobial activity of *Bacillus subtilis* RLID 12.1. Sci World J. 2014:968487. https://doi.org/10.1155/2014/968487
33. Gresse R, Chaucheyras-Durand F, Fleury MA, Van de Wiele T, Forano E, Blanquet-Diot S. Gut microbiota dysbiosis in postweaning piglets: understanding the keys to health. Trend Microbiol. 2017;25(10):851-73. https://doi.org/10.1016/j.tim.2017.05.004
34. Luise D, Lauridsen C, Bosi P, Trevisi P. Methodology and application of Escherichia coli F4 and F18 encoding infection models in post-weaning pigs. J Anim Sci Biotechnol. 2019;10:53. https://doi.org/10.1186/s40104-019-0352-7
35. Gebhardt JT, Tokach MD, Dritz SS, DeRouchey JM., Woodworth JC, Goodband RD, Henry SC. Postweaning mortality in commercial swine production. II: Review of infectious contributing factors. Transl Anim Sci. 2020;4(2):txaa052. https://doi.org/10.1093/tas/txaa068
36. Pluske JR, Hampson DJ, Williams IH. Factors influencing the structure and function of the small intestine in the weaned pig: A review. Livest Prod Sci. 1997;51(1-3):215-36. https://doi.org/10.1016/S0301-6226(97)00057-2
37. Wang H, Zhang C, Wu G, Sun Y, Wang B, He B, Dai Z, Wu Z. Glutamine enhances tight junction protein expression and modulates corticotropin-releasing factor signaling in the jejunum of weanling piglets. J Nutr. 2018;148(1):1-8. https://doi.org/10.3945/jn.114.202515
38. Itoh T, Fujimoto Y, Kawai Y, Toba T, Saito T. Immunomodulatory effects of heat-killed *Enterococcus faecalis* TH10 on murine macrophage cells. Microbiol Open. 2012;1(4):373-83. https://doi.org/10.1002/mbo3.41
39. Piao X, Li S, Sui X, Guo L, Liu X, Li H, Gao L, Cai S, Li Y, Wang T, Liu B. 1-Deoxynojirimycin (DNJ) ameliorates indomethacin-induced gastric ulcer in mice by affecting NF-κB signaling pathway. Front Pharmacol. 2018;9:372. https://doi.org/10.3389/fphar.2018.00372
40. Strugnell RA, Wijburg OL. The role of secretory antibodies in infection immunity. Nat Rev Microbiol. 2010;8(9):656-67. https://doi.org/10.1038/nrmicro2384

41. Mantis NJ, Rol N, Corthésy B. Secretory IgA's complex roles in immunity and mucosal homeostasis in the gut. Mucosal Immunol. 2011;4(6):603-11. https://doi.org/10.1038/mi.2011.41

42. Wang J, Zeng Y, Wang S, Liu H, Zhang D, Zhang W, Wang Y, Ji H. Next-generation probiotic candidates targeting intestinal health in weaned piglets: Both live and heat-killed *Akkermansia muciniphila* prevent pathological changes induced by enterotoxigenic *Escherichia coli* in the gut. Anim Nutr. 2024. https://doi.org/10.1016/j.aninu.2024.01.005

43. Rivière A, Selak M, Lantin D, Leroy F, De Vuyst L. Bifidobacteria and butyrate-producing colon bacteria: Importance and strategies for their stimulation in the human gut. Front Microbiol. 2016;7:979. https://doi.org/10.3389/fmicb.2016.00979

44. Kim CH, Park J, Kim M. Gut microbiota-derived short-chain fatty acids, T cells, and inflammation. Immune Network. 2014;14(6):277-88. http://dx.doi.org/10.4110/in.2014.14.6.277

45. Furusawa Y, Obata Y, Fukuda S, Endo TA, Nakato G, Takahashi D, Nakanishi Y, Uetake C, Kato K, Kato T, Takahashi M, Fukuda NN, Murakami S, Miyauchi E, Hino S, Atarashi K, Onawa S, Fujimura Y, Lockett T, Clarke JM, Topping DL, Tomita M, Hori S, Ohara O, Morita T, Koseki H, Kikuchi J, Honda K, Hase K, Ohno H. Commensal microbe-derived butyrate induces the differentiation of colonic regulatory T cells. Nat. 2013;504(7480):446-50. https://doi.org/10.1038/nature12721

46. Fusco W, Lorenzo MB, Cintoni M, Porcari S, Rinninella E, Kaitsas F, Lener E, Mele MC, Gasbarrini A, Collado MC, Cammarota G, Ianiro G. Short-chain fatty-acid-producing bacteria: key components of the human gut microbiota. Nutr. 2023;15(9):2211. https://doi.org/10.3390/nu15092211

47. Turner JR. Intestinal mucosal barrier function in health and disease. Nat Rev Immunol. 2009;9(11):799-809. https://doi.org/10.1038/nri2653

48. Otani T, Furuse M. Tight junction structure and function revisited. Trends in Cell Biol. 2020;30(10):805-17. https://doi.org/10.1016/j.tcb.2020.08.004

49. Günzel D, Yu AS. Claudins and the modulation of tight junction permeability. Physiol Rev. 2013;93(2):525-69. https://doi.org/10.1152/physrev.00019.2012

50. Yang F, Wang A, Zeng X, Hou C, Liu H, Qiao S. Lactobacillus reuteri I5007 modulates tight

junction protein expression in IPEC-J2 cells with LPS stimulation and in newborn piglets under normal conditions. BMC Microbiol. 2015;15:32. https://doi.org/10.1186/s12866-015-0372-1

51. Guo S, Al-Sadi R, Said HM, Ma TY. Lipopolysaccharide causes an increase in intestinal tight junction permeability in vitro and in vivo by inducing enterocyte membrane expression and localization of TLR-4 and CD14. Am J Pathol. 2013;182(2):375-87. https://doi.org/10.1016/j.ajpath.2012.10.014
52. Anderson RC, Cookson AL, McNabb WC, Park Z, McCann MJ, Kelly WJ, Roy NC. Lactobacillus plantarum MB452 enhances the function of the intestinal barrier by increasing the expression levels of genes involved in tight junction formation. BMC Microbiol. 2010;10:316. https://doi.org/10.1186/1471-2180-10-316
53. Ducarmon QR, Zwittink RD, Hornung BVH, van Schaik W, Young VB, Kuijper EJ. Gut microbiota and colonization resistance against bacterial enteric infection. Microbiol Mol Biol Rev. 2019;83(3):e00007-19. https://doi.org/10.1128/mmbr.00007-19
54. Liu H, Wang J, He T, Becker S, Zhang G, Li D, Ma X. Butyrate: A double-edged sword for health? Adv Nutr. 2018;9(1):21-9. https://doi.org/10.1093/advances/nmx009
55. Li Y, Zhong S, Yu J, Sun Y, Zhu J, Ji D, Wu C. The mulberry-derived 1-deoxynojirimycin (DNJ) inhibits high-fat diet (HFD)-induced hypercholesteremia and modulates the gut microbiota in a gender-specific manner. J Funct Foods. 2019;52:63–72. https://doi.org/10.1016/j.jff.2018.10.034
56. Wexler HM. Bacteroides: The good, the bad, and the nitty-gritty. Clin Microbiol Rev. 2007;20(4):593-621. https://doi.org/10.1128/cmr.00008-07
57. Kim K, Song M, Liu Y, Ji P. Gut microbial shifts by synbiotic combination of *Pediococcus acidilactici* and lactulose in weaned piglets challenged with Shiga toxin-producing *Escherichia coli*. Front Vet Sci. 2022;9:1101869. https://doi.org/10.3389/fvets.2022.1101869.

CONCLUSION

This study demonstrates that dietary supplementation with WB-based parabiotics in combination with BS, β-glucan, and MOS effectively mitigates the adverse effects of ETEC challenge in weaned piglets. The combined formulation at 0.10% inclusion level (FB100) provided superior benefits compared to single parabiotic supplementation, achieving growth performance, nutrient digestibility, and intestinal health parameters comparable to unchallenged control animals. Protective effects were mediated through multiple synergistic mechanisms including immune modulation (reduced IL-6, increased IgA and IgG), enhanced intestinal barrier function (improved villus morphology and CLDN1 expression), reduced inflammatory responses (decreased WBC counts), and beneficial microbiota modulation (reduced *Bacteroidota*, increased *Blautia*).

The dose-dependent improvements observed across treatment groups (FB50 < FB75 < FB100) indicate that the combination of heat-killed *E. faecalis* with viable probiotics and immunogenic polysaccharides provides additive or synergistic benefits through complementary mechanisms of action. These findings support the hypothesis that multi-component functional additives targeting different aspects of intestinal health can more effectively address the complex challenges of the post-weaning period compared to single-component interventions.

Based on these results, dietary supplementation with *E. faecalis*-based combined parabiotics (FB) at 0.10% inclusion level is recommended as an effective strategy to enhance intestinal health, immune function, and growth performance in ETEC challenged weaned piglets. This approach offers a promising alternative to antibiotic growth promoters while providing practical advantages in terms of stability and consistency of efficacy.

CONFLICT OF INTEREST

The authors declare no conflict of interest

ACKNOWLEDMENTS

This research was funded by Winsbio, Korea.

586 **Tables and Figures**

Table 1. Composition of basal diets (as-fed-basis)

Items	Content
Ingredients, %	
EP Corn	46.25
Soybean meal	10.00
Rice	4.00
Soy protein concentrate	2.50
Fermented soybean meal	4.00
Meal protein fat (Whey, Oil, etc)	17.50
Fish meal	1.50
Plasma protein	4.00
Soybean oil	2.40
Lactose	4.00
Ca-formate	1.30
Copra meal	1.50
Vit-premix[1]	0.20
Min-premix[2]	0.25
Methionine	0.10
L-Lysine	0.40
Threonine	0.10
Total	100.00
Calculated value	
NE, kcal/kg	2,600.00
CP, %	19.20
Lysine, %	1.40
Methionine, %	0.40
Ca, %	0.50
P, %	0.45

[1]Provided per kg of complete diet: vitamin A, 9,000 IU; vitamin D_3, 1,620 IU; vitamin E, 35 IU; vitamin B_{12}, 40 μg; vitamin B_2, 6 mg; vitamin B_6, 3 mg; vitamin K_3, 2 mg; vitamin C, 100 mg; niacin, 50 mg; ca-pantothenate, 20 mg; biotin, 0.2 mg.
[2]Provided per kg of complete diet without zinc: Mn, 35 mg; Zn, 30 mg; Fe, 150 mg; Cu, 50 mg; I, 0.5 mg; Co, 0.15 mg; and Se, 0.2 mg
Abbreviation: EP corn, extruded-pelleted corn; NE, net energy; CP, crude protein

587

588

589

Table 2. Effects of dietary *Enterococcus faecalis*–based parabiotic supplementation, alone or in combination, on growth performance in ETEC challenge weaned pigs

Item	PC	NC	WB1	FB50	FB75	FB100	SEM	*p*-value
BW, kg								
Initial	11.86	11.60	11.61	11.77	11.60	11.68	0.591	1.000
d 7	14.29	13.64	13.71	13.89	13.77	13.97	0.638	0.987
d 14	16.89	15.96	16.11	16.23	16.13	16.46	0.645	0.948
ADG, kg								
d 1 to 7	346.67^{a}	290.36^{b}	300.00^{ab}	302.75^{ab}	310.00^{ab}	327.50^{ab}	10.284	0.024
d 8 to 14	370.95^{a}	332.50^{b}	330.36^{b}	334.29^{b}	336.43^{b}	355.00^{ab}	5.982	0.002
d 1 to 14	358.81^{a}	311.43^{c}	315.18^{bc}	318.52^{bc}	323.21^{bc}	341.25^{ab}	6.324	0.001
ADFI, kg								
d 1 to 7	525.48	513.93	516.75	512.68	516.07	515.89	15.462	0.996
d 8 to 14	600.00	600.00	592.50	595.00	580.00	583.93	15.619	0.923
d 1 to 14	562.74	556.96	554.63	553.84	548.04	549.91	15.239	0.990
FCR								
d 1 to 7	1.52^{c}	1.77^{a}	1.733^{ab}	1.70^{ab}	1.67^{abc}	1.57^{bc}	0.037	0.003
d 8 to 14	1.62	1.81	1.73	1.78	1.72	1.65	0.052	0.097
d 1 to 14	1.57^{b}	1.79^{a}	1.73^{ab}	1.74^{ab}	1.70^{ab}	1.61^{b}	0.038	0.012

PC, basal diet; NC, basal diet + ETEC challenge; WB1, NC + 0.10% *Enterococcus faecalis* EF 2001 1.0×10^{10} cells/g; FB50, NC + 0.05% immune FB-110; FB75, NC + 0.075% immune FB-110; FB100, NC + 0.10% immune FB-110; BW, body weight; ADG, average daily gain; ADFI, average daily feed intake; FCR, feed conversion ratio; SEM, standard error of mean.

[a-c] Means within a row with different letters are significantly different at $p < 0.05$.

590

591

592

593

Table 3. Effects of dietary *Enterococcus faecalis*–based parabiotic supplementation, alone or in combination, on nutrient digestibility in ETEC challenge weaned pigs

Items	PC	NC	WB1	FB50	FB75	FB100	SEM	*p*-value
d 7								
DM	89.01[a]	86.31[c]	87.13[bc]	87.79[ab]	88.08[ab]	88.89[a]	0.288	< 0.001
CP	73.36	71.69	71.66	72.05	72.23	73.10	0.747	0.355
GE	76.68[a]	74.52[b]	74.52[b]	74.67[b]	75.38[ab]	76.70[a]	0.605	0.042
d 14								
DM	90.68[a]	87.85[d]	88.63[cd]	89.14[bc]	89.51[abc]	89.89[ab]	0.258	< 0.001
CP	75.81[a]	73.07[b]	74.84[ab]	74.54[ab]	75.08[ab]	75.69[a]	0.560	0.041
GE	78.41[a]	75.47[b]	77.36[ab]	78.15[a]	77.78[a]	78.37[a]	0.439	0.002

PC, basal diet; NC, basal diet + ETEC challenge; WB1, NC + 0.10% *Enterococcus faecalis* EF 2001 1.0×10^{10} cells/g; FB50, NC + 0.05% immune FB-110; FB75, NC + 0.075% immune FB-110; FB100, NC + 0.10% immune FB-110; DM, dry matter; CP, crude protein; GE, gross energy; SE, standard error of mean.
[a-c] Means within a row with different letters are significantly different at $p < 0.05$.

597

Table 4. Effects of dietary *Enterococcus faecalis*–based parabiotic supplementation, alone or in combination, on blood profiles in ETEC challenge weaned pigs

Items	PC	NC	WB1	FB50	FB75	FB100	SEM	*p*-value
d 1								
RBC (10^6/μL)	6.44	6.42	6.42	6.45	6.48	6.32	0.062	0.629
WBC (10^3/μL)	13.42	13.49	13.48	13.24	13.74	13.95	0.257	0.478
IgA (mg/mL)	0.41	0.44	0.42	0.42	0.42	0.44	0.018	0.870
IgG (mg/mL)	1.47	1.56	1.57	1.67	1.66	1.58	0.061	0.330
IL-6 (pg/mL)	39.32	35.71	35.46	38.15	33.36	38.60	3.206	0.788
d 14								
RBC (10^6/μL)	6.68^{a}	5.58^{d}	5.92^{c}	6.06^{bc}	6.17^{bc}	6.31^{b}	0.073	<0.001
WBC (10^3/μL)	11.67^{d}	21.39^{a}	21.43^{a}	18.13^{b}	15.03^{c}	14.09^{c}	0.549	<0.001
IgA (mg/mL)	0.50^{a}	0.28^{b}	0.33^{b}	0.31^{b}	0.51^{a}	0.45^{a}	0.023	<0.001
IgG (mg/mL)	1.97^{a}	1.59^{c}	1.62^{c}	1.69^{bc}	1.87^{ab}	1.90^{ab}	0.051	0.002
IL-6 (pg/mL)	33.12^{d}	90.49^{a}	85.91^{a}	72.81^{b}	54.85^{c}	44.01^{cd}	2.666	<0.001

PC, basal diet; NC, basal diet + ETEC challenge; WB1, NC + 0.10% *Enterococcus faecalis* EF 2001 1.0×10^{10} cells/g; FB50, NC + 0.05% immune FB-110; FB75, NC + 0.075% immune FB-110; FB100, NC + 0.10% immune FB-110; RBC, red blood cell; WBC, white blood cell; IgA, immunoglobulin A; IgG, immunoglobulin G; IL-6, interleukin-6; SEM, standard error of mean.
a-d Means within a row with different letters are significantly different at $p < 0.05$.

598

599

600

601

602

603

Table 5. Effects of dietary *Enterococcus faecalis*–based parabiotic supplementation, alone or in combination, on intestinal morphology in ETEC challenge weaned pigs

Items	PC	NC	WB1	FB50	FB75	FB100	SEM	*p*-value
Villus height, μm	385.33	329.78	343.10	359.20	373.47	379.70	14.221	0.100
Crypt depth, μm	193.14^{c}	294.23^{a}	253.94^{b}	223.74^{bc}	206.03^{c}	202.58^{c}	8.113	<0.001
Villus width, μm	116.00	132.55	122.35	124.59	121.35	124.19	4.532	0.319
VH: CD ratio	2.01^{a}	1.12^{c}	1.35^{bc}	1.62^{ab}	1.82^{a}	1.88^{a}	0.096	<0.001

PC, basal diet; NC, basal diet + ETEC challenge; WB1, NC + 0.10% *Enterococcus faecalis* EF 2001 1.0×10^{10} cells/g; FB50, NC + 0.05% immune FB-110; FB75, NC + 0.075% immune FB-110; FB100, NC + 0.10% immune FB-110; VH:CD ratio, villus height to crypt depth ratio; SEM, standard error of mean.
a-c Means within a row with different letters are significantly different at $p < 0.05$.

604

605

Table 6. Primer sequences used for the RT-qPCR analysis with the Mucin1, ZO-1, CLDN1, and GAPDH genes

Gene	Primers	Sequence (5'-3')
Glyceraldehyde-3-phosphate dehydrogenase 2 (GAPDH)	Forward	TCGGAGTGAACGGATTTGGC
	Reverse	TGACAAGCTTCCCGTTCTCC
Mucin 1	Forward	CCACAACCTGAAGACACAGT
	Reverse	GACCAGAATACAGACCAGCA
ZO-1	Forward	CTCTGTCCATGCAGATAAGC
	Reverse	AATAGCTCCCTGTGGGATAA
CLDN1	Forward	GCTGGGACTAATAGCCATCT
	Reverse	AAGAGAGCCTGACCAAATTC

606

607

608

Table 7. Effects of dietary *Enterococcus faecalis*–based parabiotic supplementation, alone or in combination, on tight junction in ETEC challenge weaned pigs

Items	PC	NC	WB1	FB50	FB75	FB100	SEM	*p*-value
Claudin-1	1.00[a]	0.42[c]	0.49[bc]	0.50[bc]	0.53[bc]	0.90[ab]	0.094	0.002
ZO-1	1.00	0.85	0.84	0.87	0.89	0.97	0.181	0.987
Mucin 1	1.00	0.85	0.81	0.96	1.09	1.12	0.298	0.966

PC, basal diet; NC, basal diet + *E. coli* challenge; WB1, NC + 0.10% *Enterococcus faecalis* EF 2001 1.0×10^{10} cells/g; FB50, NC + 0.05% immune FB-110; FB75, NC + 0.075% immune FB-110; FB100, NC + 0.10% immune FB-110; ZO-1, zonula occludin-1; SEM, standard error of mean.

a, b Means within a row with different letters are significantly different at $p < 0.05$.

609

610

611

Table 8. Effects of dietary *Enterococcus faecalis*–based parabiotic supplementation, alone or in combination, on microbiome abundance in ETEC challenge weaned pigs

Items	PC	NC	WB1	FB50	FB75	FB100	SEM	p-value
d 1								
Chao 1	854.31	815.51	887.20	797.60	748.83	890.39	73.388	0.723
Shannon	7.53	7.16	7.63	7.49	7.29	7.63	0.250	0.708
Simpson	0.98	0.97	0.99	0.99	0.98	0.98	0.006	0.306
d 14								
Chao 1	987.60	1000.36	984.54	973.33	1003.51	974.15	37.594	0.988
Shannon	7.87	7.99	7.74	7.75	7.86	7.95	0.080	0.314
Simpson	0.99	0.99	0.99	0.99	0.99	0.99	0.003	0.973

PC, basal diet; NC, basal diet + ETEC challenge; WB1, NC + 0.10% *Enterococcus faecalis* EF 2001 1.0×10^{10} cells/g; FB50, NC + 0.05% immune FB-110; FB75, NC + 0.075% immune FB-110; FB100, NC + 0.10% immune FB-110; SEM, standard error of mean.

612

613

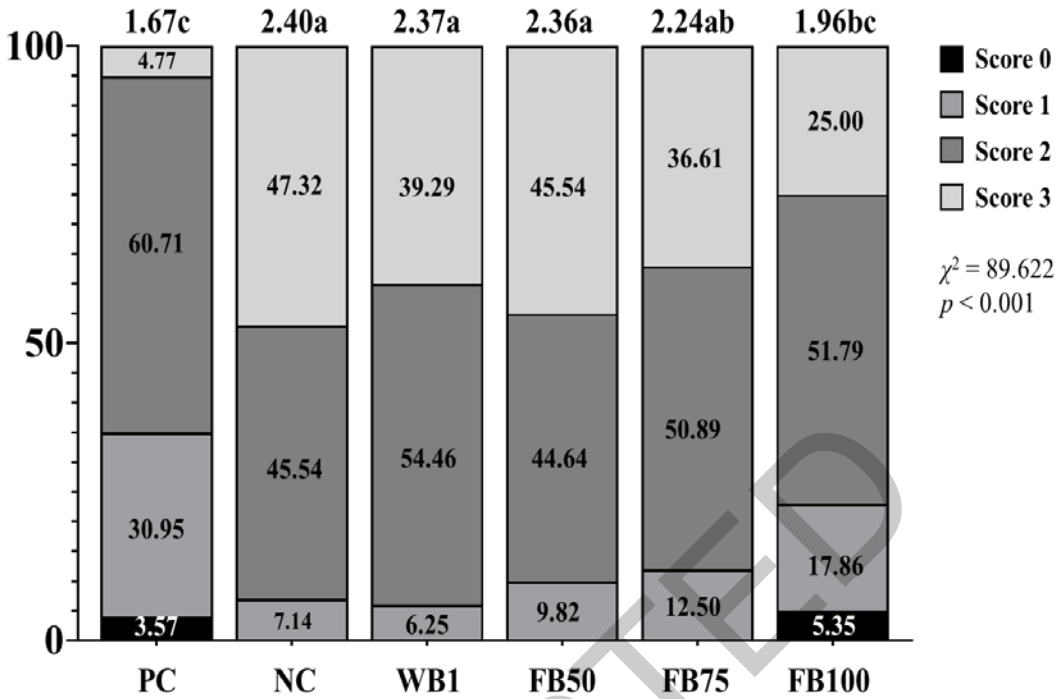

Fig 1. Effects of dietary *Enterococcus faecalis*–based parabiotic supplementation, alone or in combination, on fecal score in ETEC challenge weaned pigs. Normal feces are Score 0, soft feces are Score 1, mild diarrhea is Score 2 and severe diarrhea are Score 3. χ^2= 89.622, $p < 0.001$. PC, basal diet; NC, basal diet + ETEC challenge; WB1, NC + 0.10% *Enterococcus faecalis* EF 2001 1.0×10^{10} cells/g; FB50, NC + 0.05% immune FB-110; FB75, NC + 0.075% immune FB-110; FB100, NC + 0.10% immune FB-110.

a-c Means within a row with different letters are significantly different at $p < 0.05$.

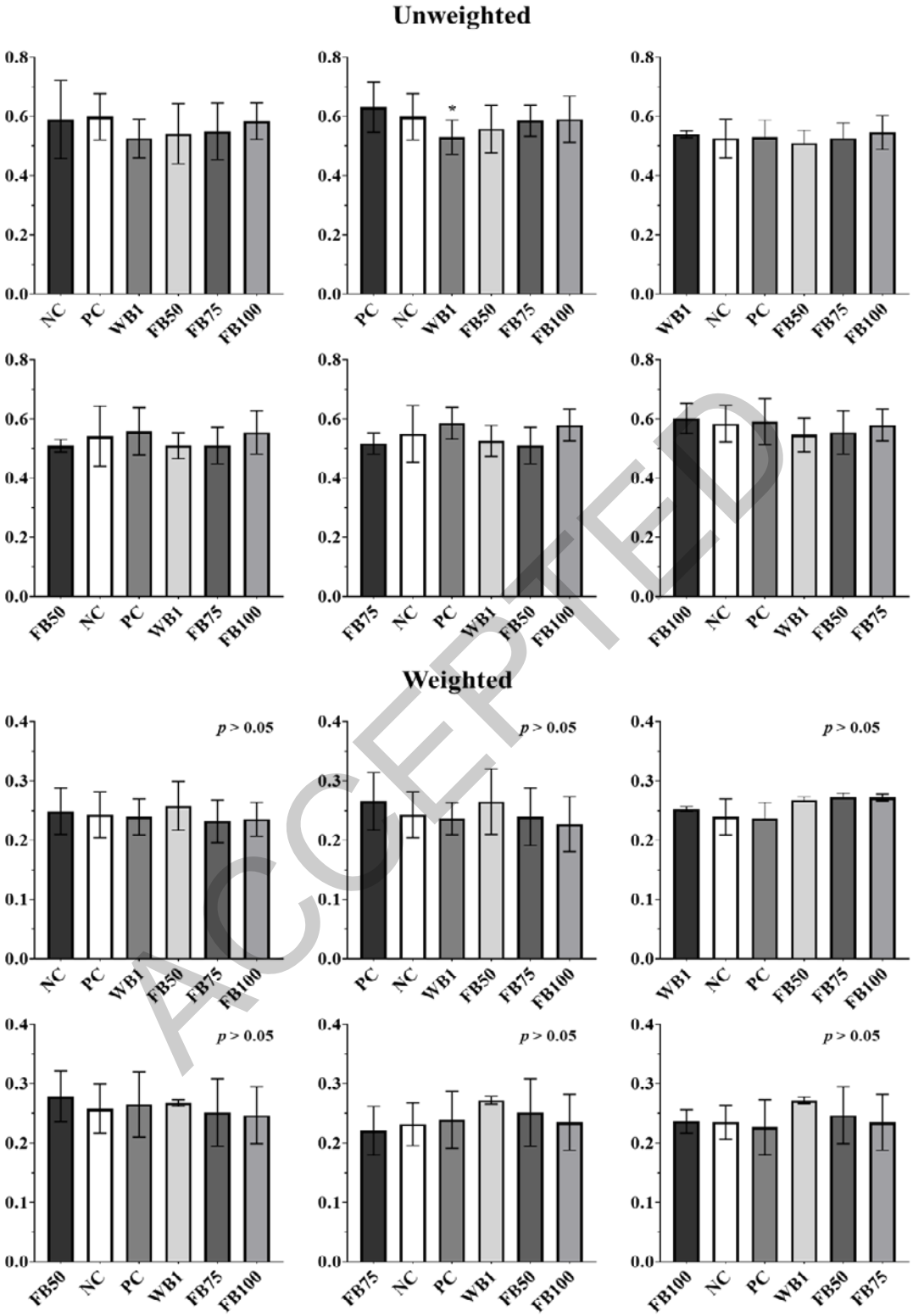

Fig. 2. Effects of dietary *Enterococcus faecalis*–based parabiotic supplementation, alone or in combination, on b-diversity in ETEC challenge weaned pigs (d 1). PC, basal diet; NC, basal diet + ETEC challenge; WB1, NC + 0.10% *Enterococcus faecalis* EF 2001 1.0×10^{10} cells/g; FB50, NC + 0.05% immune FB-110; FB75, NC + 0.075% immune FB-110; FB100, NC + 0.10% immune FB-110; SEM, standard error of mean.

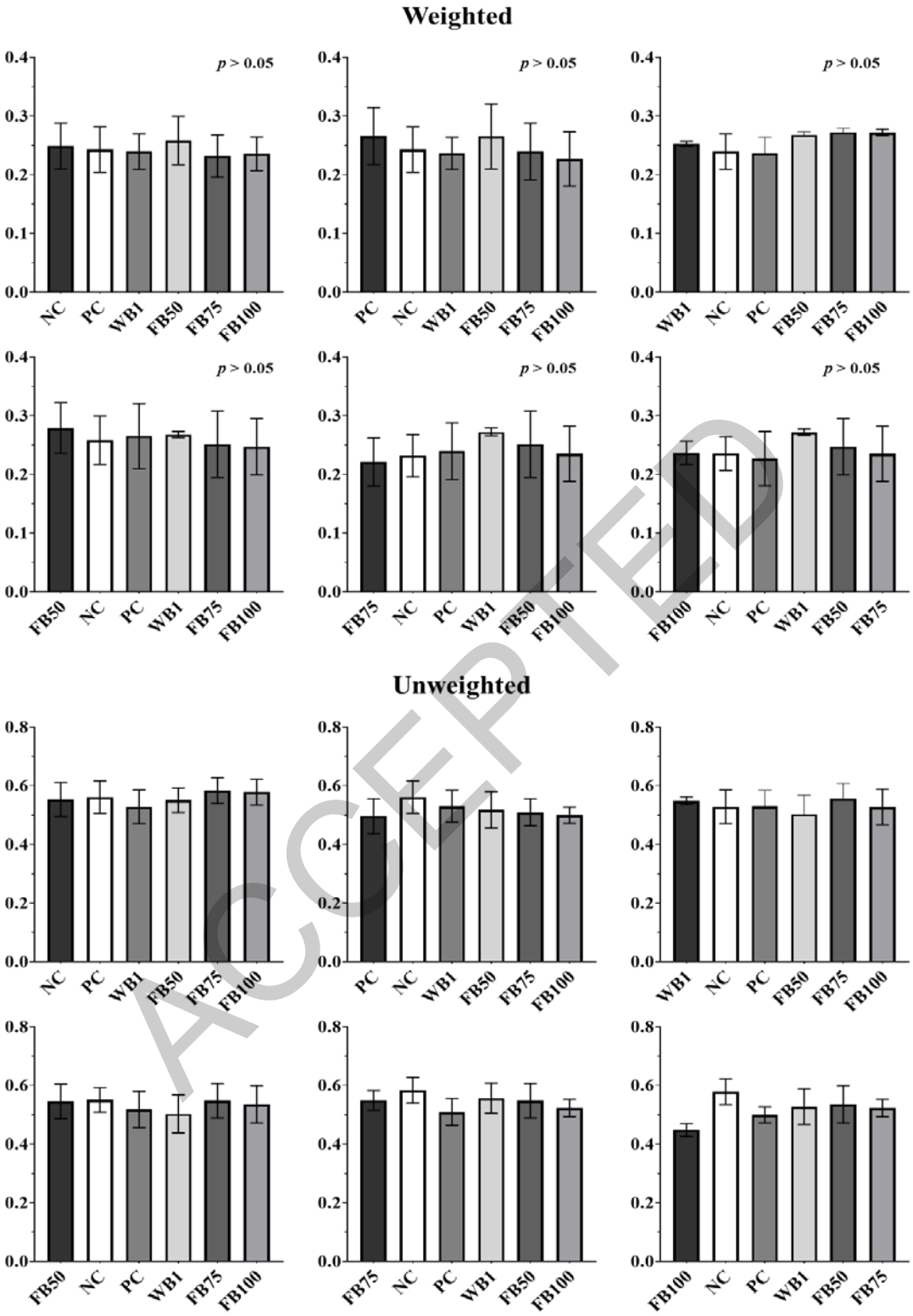

Fig. 3. Effects of dietary *Enterococcus faecalis*–based parabiotic supplementation, alone or in combination, on b-diversity in ETEC challenge weaned pigs (d 14). PC, basal diet; NC, basal diet + ETEC challenge; WB1, NC + 0.10% *Enterococcus faecalis* EF 2001 1.0×10^{10} cells/g; FB50, NC + 0.05% immune FB-110; FB75, NC + 0.075% immune FB-110; FB100, NC + 0.10% immune FB-110; SEM, standard error of mean.

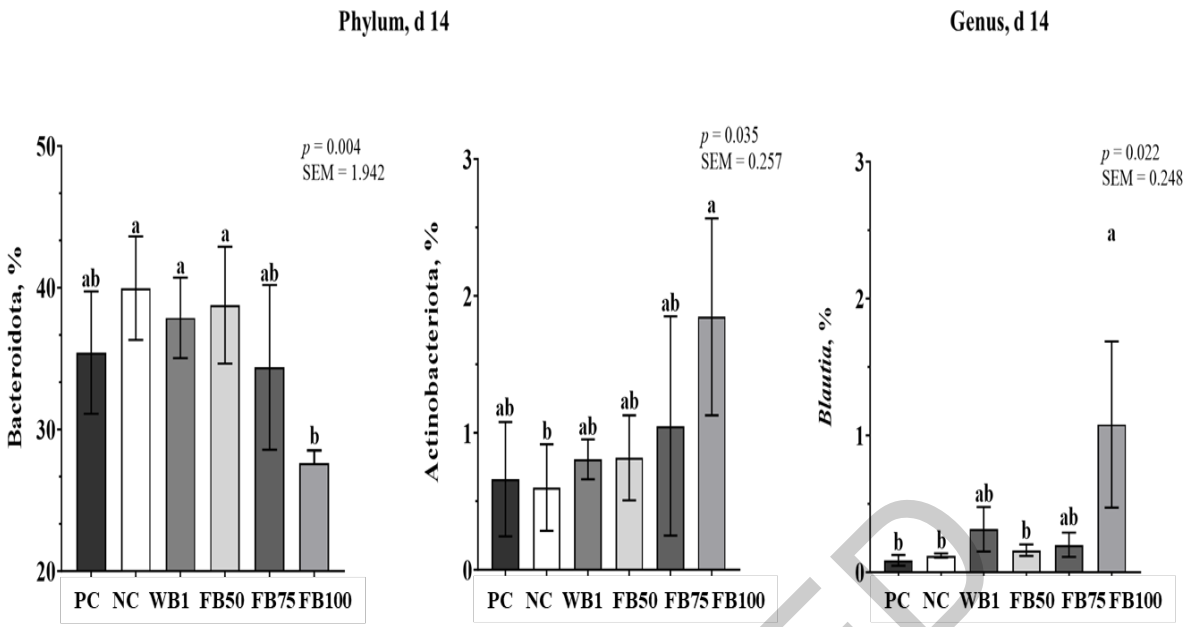

Fig 4. Effects of dietary *Enterococcus faecalis*–based parabiotic supplementation, alone or in combination, on fecal microbiota in ETEC challenge weaned pigs. PC, basal diet; NC, basal diet + ETEC challenge; WB1, NC + 0.10% *Enterococcus faecalis* EF 2001 1.0 × 10^{10} cells/g; FB50, NC + 0.05% immune FB-110; FB75, NC + 0.075% immune FB-110; FB100, NC + 0.10% immune FB-110; SEM, standard error of mean.