Phytogenic feed additives alleviate pathogenic *Escherichia coli*-induced intestinal damage through improving barrier integrity and inhibiting inflammation in weaned pigs

Se Yeon Chang1†, Min Ho Song2†, Ji Hwan Lee1, Han Jin Oh1, Yong Ju Kim1, Jae Woo An1, Young Bin Go1, Dong Cheol Song1, Hyun Ah. Cho1, Seung Yeol Cho3, Dong Jun Kim3, Mi Suk Kim3, Hyeun Bum Kim4* and Jin Ho Cho1*

Abstract

**Background:** This study was conducted to investigate the effects of each phytogenic feed additive (PFA; PFA1, bitter citrus extract; PFA2, a microencapsulated blend of thymol and carvacrol; PFA3, a mixture of bitter citrus extract, thymol, and carvacrol; PFA4, a premixture of grape seed, grape marc extract, green tea, and hops; PFA5, fenugreek seed powder) on the growth performance, nutrient digestibility, intestinal morphology, and immune response in weaned pigs infected with *Escherichia coli* (*E. coli*).

**Results:** A total of 63 4-week-old weaned pigs were placed in individual metabolic cages and assigned to seven treatment groups. The seven treatments were as follows: 1) NC; basal diet without *E. coli* challenge, 2) PC; basal diet with *E. coli* challenge, 3) T1; PC + 0.04% PFA1, 4) T2; PC + 0.01% PFA2, 5) T3; PC + 0.10% PFA3, 6) T4; PC + 0.04% PFA4, 7) T5; PC + 0.10% PFA5. The experiments lasted in 21 d, including 7 d before and 14 d after the first *E. coli* challenge. In the *E. coli* challenge treatments, all pigs were orally inoculated by dividing a total of 10 mL of *E. coli* F18 for 3 consecutive days. The PFA-added groups significantly increased (*P* < 0.05) average daily gain and feed efficiency and decreased (*P* < 0.05) the fecal score at d 0 to 14 post-inoculation (PI). Tumor necrosis factor α was significantly lower (*P* < 0.05) in the PFA-added groups except for T1 in d 14 PI compared to the PC treatment. The T3 had a higher (*P* < 0.05) immunoglobulin G and immunoglobulin A concentration compared to the PC treatment at d 7 PI. Also, T3 showed significantly higher (*P* < 0.05) villus height:crypt depth and claudin 1 expression in ileal mucosa, and significantly down-regulated (*P* < 0.05) the expression of calprotectin compared to the PC treatment.

**Conclusions:** Supplementation of PFA in weaned pigs challenged with *E. coli* alleviated the negative effects of *E. coli* and improved growth performance. Among them, the mixed additive of bitter citrus extract, thymol, and carvacrol...
Background
Post-weaning diarrhea (PWD) results in increased dehydration, mortality, and lowered growth performance in weaned pigs [1]. PWD is considered the main cause of economic loss in the swine industry because it destabilizes the health status of pigs, reduces production efficiency, and increases production costs as a result [2, 3]. PWD is caused by significant changes in gastrointestinal physiology, microbiology, and immunology due to weaning stress, the biggest stressor in piglets [4]. Pathogenic Escherichia coli (E. coli) is known to be a major cause of PWD [5]. E. coli damages the intestinal epithelium, weakening mucosal and cellular barrier functions and increasing the adhesion of pathogenic bacteria to the mucosal layer [4, 6].

Phytogenic feed additives (PFA) contain various physiologically active ingredients such as alkaloids, flavonoids, saponins, tannins phenolics, thymol, and allicin, and have positive activity including antibacterial, immune-modulating, antioxidant, and growth-promoting effects in animals [7, 8]. When weaned pigs challenged with E. coli were fed a blended plant feed additive containing naringin flavonoids, intestinal damage was prevented by reducing the main acute phase protein of pigs and better controlling the inflammatory response [9]. Also, the addition of essential oils to the diet of weaned pigs challenged with E. coli increased growth performance and the apparent total tract digestibility (ATTD) of nutrients and decreased the incidence of diarrhea [10].

However, no studies have compared the effects of different PFA supplements in weaned pigs infected with E. coli at the same time. Therefore, this study was conducted to investigate the effects of individual PFAs on growth performance, nutrient digestibility, intestinal morphology, and immune response in weaned pigs infected with E. coli, which is a principal causative agent of PWD, and then identify the PFA effective against PWD.

Materials and methods
Test phytogenic feed additives
Five types of PFA were used in this study. PFA1 is composed of bitter citrus extract (BioFlavex GC, HTBA, Beniel, Spain) and contains 25~27% naringin and 11~15% neohesperidin. PFA2 is a microencapsulated blend of thymol and carvacrol (Avipower 2, VetAgro SpA, Reggio, Emilia, Italy), containing 7% of thymol and 7% of carvacrol. PFA3 is a mixture of PFA1, PFA2 and excipient in a ratio of 4:1:5. It contains 0.7% thymol, 0.7% carvacrol, 10~10.8% naringin and 4.4~6% neohesperidin. PFA 4 is a premixture of grape seed and grape marc extract, green tea, and hops (AntaOx Flavosyn, DR. Eckel GmbH, Niederzissen, Germany). It contains more than 10% of flavonoids. PFA5 is composed of fenugreek seed powder (Fenugreek Seed Powder, P&D Export, Jaguar, India) and contains 12% saponin. All PFAs used in this study was obtained by a commercial company (Eugene-Bio, Suwon, South Korea).

Bacterial strains and culture
Shiga toxin-producing E. coli F18 was provided in stock form. The F18 E. coli expressed heat labile toxin (LT) and shiga toxin type 2e (stx2e). Ten microliters of thawed E. coli stock was inoculated into 10 mL of nutrient broth and cultured at 37°C for 24h, and then subcultured. Thereafter, the subcultured E. coli was smeared on MacConkey agar to confirm the bacterial enumeration. A final concentration of 1.2 × 10^{10} CFU/mL was used in this study.

Animals, treatments and experimental design
A total of 63 4-week-old crossbred weanling pigs [(Landrace × Yorkshire) × Duroc] with initial body weight (BW) of 8.03±0.43 kg were used in this study. All pigs were assigned to a completely randomized seven treatment groups based on the initial BW. There was one pig in a cage and nine replicate cages per treatment. Pigs were individually placed in 45 cm × 55 cm × 55 cm stainless steel metabolism cages in an environmentally controlled room. Pigs were housed in individual pens for 21 d, including 7 d before and 14 d after the first E. coli challenge (d 0). Dietary treatments were as follow: 1) NC (negative control; basal diet without E. coli challenge), 2) PC (positive control; basal diet with E. coli challenge), 3) T1 (PC + 0.04% PFA1), 4) T2 (PC + 0.01% PFA2), 5) T3 (PC + 0.10% PFA3), 6) T4 (PC + 0.04% PFA4), 7) T5 (PC + 0.10% PFA5). All diets were formulated to meet or exceed the NRC requirement (Table 1) [11]. All treatment groups were fed the experimental diet for 21 d, including 7 d of adaptation. 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.

In the E. coli challenge treatments, all pigs were orally inoculated by dividing a total of 10 mL of E. coli F18 for 3 consecutive days from d 0 post-inoculation (PI) after 7 d of adaptation.
Sampling and measurements

Growth performance and fecal score

Pigs were weighed individually at the beginning (d − 7), d 0 before inoculation, and d 7, 14 PI. Feed intake (FI) was recorded daily the diet supply amount and remaining amount. Average daily gain (ADG), average daily feed intake (ADFI), and feed efficiency (G:F) were calculated for each interval from d − 7 to 0, d 0 to 7 PI, d 7 to 14 PI and d 0 to 14 PI. The fecal scores were individually recorded at 08:00 and 17:00 by the same person during the entire experimental period. The fecal score was assigned as follows: 0, Normal feces; 1, Soft feces; 2, Mild diarrhea; 3, Severe diarrhea. The fecal score of each pig was calculated as an average within the period before and after the E. coli challenge.

Nutrient digestibility

Chromium oxide (Cr₂O₃, 2 g/kg) was added to the diets as an indigestible marker to measure digestibility [12]. Pigs were fed diets mixed with chromium oxide for 4 consecutive days from d 4 and d 11, fresh excreta samples were collected in that period. At the same time, 9 replications of these feed samples were collected. Fresh fecal and feed samples were stored in a freezer at −20 °C immediately after collection. At the end of the experiment, fecal samples were dried at 70 °C for 72 h and then crushed on a 1-mm screen. The procedures utilized for the determination of dry matter (DM) and crude protein (CP) digestibility were conducted with the methods by the AOAC [13]. Chromium levels were determined via UV absorption spectrophotometry (UV-1201, Shimadzu, Kyoto, Japan) using the Williams et al. [14] method. For calculating the ATTD of the nutrients, we used the following equation: Digestibility = 1 − [(Nf × Cd)/(Nd × Cf)] × 100, where Nf = concentration of nutrient in fecal, Nd = concentration of nutrient in the diet, Cd = concentration of chromium in the diet, and Cf = concentration of chromium in the fecal.

Complete blood count and measurement of serum immunoglobulin, interleukin and TNF-α

Blood samples were collected from the jugular vein of all pigs before the E. coli challenge (d 0), and on d 7 and 14 PI. At the time of collection, blood samples were collected into vacuum tubes containing K₃EDTA for complete blood count analysis, and nonheparinized tubes for serum analysis, respectively. After collection, serum samples were centrifuged at 3000 × g for 20 min at 4 °C. Thereafter, the blood sample tubes were stored in −20 °C refrigerator until analysis. The white blood cell (WBC), basophil, neutrophil, and lymphocyte levels in the whole blood were measured using an automatic blood analyzer (ADVIA 120, Bayer, NY, USA). Immunoglobulin G (IgG) and immunoglobulin A (IgA) levels were gauged using an automatic biochemistry blood analyzer (Hitachi 747; Hitachi, Tokyo, Japan). Interleukin-6 (IL-6) and tumor necrosis factor α (TNF-α) concentrations were determined using commercially available ELISA kits (Quantikine, R&D systems, Minneapolis, MN, USA) and the absorbance was measured at 450 nm.

Intestinal morphology

At the end of the experiment (d 14), pigs were anesthetized with carbon dioxide gas after blood sampling and euthanized by exsanguination. After euthanization, intestinal tissues of about 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, USA) for intestinal morphology and expression

Table 1 Compositions of basal diets (as-fed-basis)

| Items                        | Content |
|------------------------------|---------|
| Ingredients, %               |         |
| Corn                         | 34.43   |
| Extruded corn                | 15.00   |
| Lactose                      | 10.00   |
| Dehulled soybean meal, 51% CP | 13.50   |
| Soy protein concentrate, 65% CP | 10.00  |
| Plasma powder                | 6.00    |
| Whey                         | 5.00    |
| Soy oil                      | 2.20    |
| Monocalcium phosphate        | 1.26    |
| Limestone                    | 1.40    |
| L-Lysine-HCl, 78%            | 0.06    |
| DL-Methionine, 50%           | 0.15    |
| Choline chloride, 25%        | 0.10    |
| Vitamin premix<sup>a</sup>   | 0.25    |
| Trace mineral premix<sup>c</sup> | 0.25  |
| Salt                         | 0.40    |
| Total                        | 100.00  |
| Calculated value             |         |
| ME, Kcal/kg                  | 3433    |
| CP, %                        | 20.76   |
| Lysine, %                    | 1.35    |
| Methionine, %                | 0.39    |
| Ca                           | 0.82    |
| P                            | 0.65    |
| Analyzed value               |         |
| ME, kcal/kg                  | 3512    |
| CP, %                        | 20.92   |

<sup>a</sup>Abbreviation: CP Crude protein

<sup>b</sup>Provided per kg of complete diet: vitamin A, 11,025 IU; vitamin D₃, 1103 IU; vitamin E, 44 IU; vitamin K, 4 mg; niacin, 50 mg; thiamine, 4 mg; d-pantothenic, 29 mg; choline, 166 mg; and vitamin B₁₂, 33 mg

<sup>c</sup>Provided per kg of complete diet without Zinc: Cu (as CuSO₄·5H₂O), 12 mg; Mn (as MnO₂), 8 mg; I (as KI), 0.28 mg; and Se (as Na₂SeO₃·5H₂O), 0.15 mg

<sup>d</sup>Provided per kg of complete diet: vitamin A, 11,025 IU; vitamin D₃, 1103 IU; vitamin E, 44 IU; vitamin K, 4 mg; niacin, 50 mg; thiamine, 4 mg; d-pantothenic, 29 mg; choline, 166 mg; and vitamin B₁₂, 33 mg
analysis of intestinal tight junction proteins. After cutting the intestine sample, it was dehydrated and dealcloholized. The samples were then installed on slides, treated with paraffin, and stained with hematoxylin and eosin. The slides were examined using an Olympus IX51 inverted phase-contrast microscope. Intestinal morphological measurements included the villus height (VH), crypt depth (CD), and villus height: crypt depth ratio (VH:CD).

**Immunohistochemical staining**

The expression of calprotectin and claudin 1 (CLDN1) was studied by using immunohistochemistry (IHC). The ileal tissue fixed in 10% NBF solution was cut, embedded in a paraffin wax block and sectioned to a thickness of 5μm. After deparaffinizing the paraffin sections and rehydrating the tissues, they were placed in running tap water for 10 min. This section was reacted in 0.03% hydrogen peroxide solution for 15 min, and then dipped in distilled water (DW) for 10 min. Then, the antigen was retrieved and dipped in DW for 10 min, followed by blocking in 4% bovine serum albumin solution for 30 min. The tissue section was then incubated with primary antibodies, anti-calprotectin (1:800, Thermo Fisher Scientific, Waltham, MA, USA) and anti-claudin1 (1:200, Novus Biologicals, Minneapolis, MN, USA). Then, it was incubated with the secondary antibodies, envision anti-mouse (Dako, Santa Clara, CA, USA) and envision anti-rabbit (Dako). The sections were washed with tris-buffered saline-tween and incubated with 3,3’-diaminobenzidine (DAB) to visualize immune complexes. The processed slide sections were slide-scanned through Axio Scan Z1 (Carl Zeiss, Jena, Germany). The completed file was analyzed using Zen Image Analysis, an Axio Scan Z1 program (Zen 3.4 blue edition). Analysis was analyzed by the contrast of DAB of IHC and positive reaction color to total tissue area, and the results were expressed as a total area, a positive area, and the percentage of positive area.

**Statistical analysis**

All data were analyzed via the general linear model procedures of SAS (SAS Institute, Cary, NC, USA), using each pen as the experimental unit. Differences between treatment means were determined using Tukey’s multiple range test. A probability level of \( P < 0.05 \) was indicated to be statistically significant, and a level of \( 0.05 \leq P < 0.10 \) was considered to have such a tendency.

**Results**

**Growth performance**

There was no difference between treatment groups in the initial BW of pigs (Table 2). At d 7 PI, T2 showed a significantly higher \( (P < 0.05) \) BW than the other treatment groups. Compared with the NC treatment, the PC treatment reduced \( (P < 0.05) \) ADG and G:F at d 0 to 7 PI. Also, at d 0 to 7 PI, the PFA-added treatment groups showed higher \( (P < 0.05) \) ADG and G:F compared to the PC treatment. At d 7 to 14 PI, the T3 had a significantly lower \( (P < 0.05) \) ADFI than other treatments, but showed a higher tendency \( (P = 0.073) \) in G:F. Compared with the PC treatment, the PFA-added treatment groups significantly increased ADG and G:F \( (P < 0.05) \) at d 0 to 14 PI, but there was no difference in ADFI.

**Fecal score**

Before *E. coli* inoculation, there was no difference in the fecal score at d - 7 to 0 (Table 3). The fecal score from d 0 to 7 PI was significantly higher \( (P < 0.05) \) in the PC treatment than in other treatment groups. Also, in d 0 to 14 PI, the PC treatment showed significantly higher \( (P < 0.05) \) fecal scores than other treatment groups, and the PFA-added treatment groups showed similar or lower \( (P < 0.05) \) fecal scores to the NC treatment.

**Nutrient digestibility**

At d 7 PI, there were no significant differences in DM and CP digestibility between treatment groups (Table 4). The DM digestibility was significantly higher \( (P < 0.05) \) in the PFA-added treatment groups at d 14 PI compared to the PC treatment. No difference was observed in CP digestibility at d 14 PI among treatment groups.

**Blood profile**

WBC, basophil, neutrophil, and lymphocyte levels did not differ significantly between treatment groups on d 0 before *E. coli* inoculation (Table 5). The number of WBCs in whole blood was significantly lower \( (P < 0.05) \) in T2 and NC treatment at d 7 PI compared to other treatment groups. Also, T2 had a significantly lower percentage \( (P < 0.05) \) of neutrophils and a significantly higher percentage \( (P < 0.05) \) of lymphocytes at d 7 PI. The neutrophils and lymphocytes of the PFA-added treatment groups except for T1 were recovered to the NC treatment level at d 14 PI, and among them, T2 showed significantly similar values \( (P < 0.05) \) to the NC treatment.

**Serum immunoglobulin**

In serum IgG, IgA, TNF-α, and IL-6, there was no significant difference between treatment groups at d 0 before *E. coli* inoculation (Table 6). At d 7 PI, T3 had a higher IgG and IgA concentration \( (P < 0.05) \) compared to PC treatment. TNF-α was significantly lower \( (P < 0.05) \) in the PFA-added treatment groups than in the PC treatment at d 7 PI, among which T3 and T5
were the lowest ($P<0.05$). At d 7 PI, T3 showed the lowest IL-6 ($P<0.05$) compared to other treatments. At d 14 PI, the IgG concentration showed a tendency for T3 to be higher ($P=0.077$) than the PC treatment. TNF-α was significantly lower ($P<0.05$) in the PFA-added treatment groups except for T1 in d 14 PI compared to the PC treatment.

### Intestinal morphology

VH in T3 and NC treatment was significantly higher ($P<0.05$) than in PC treatment (Table 7; Fig. 1). In CD, the PFA-added treatment groups showed a tendency to be numerically lower ($P=0.067$) than those of the PC treatment. VH:CD showed significantly higher values ($P<0.05$) in T3 than PC treatment.
Tight junction

T3 and NC treatment significantly down-regulated (P < 0.05) the expression of calprotectin in the ileum mucosa compared to other treatments (Table 8). The expression of CLDN1 in the ileal mucosa was significantly higher (P < 0.05) in the PFA-added treatment groups than in the PC treatment, and the T3 showed the highest (P < 0.05) among the PFA-added treatment groups.

Discussion

E. coli-induced PWD is a common intestinal disease in weaned pigs, causing economic loss by reducing growth performance and increasing mortality [15]. This disease usually occurs in the early weaning of pigs at 3 to 4 weeks of age, and the symptoms appear between 3 and 10 d after weaning [16, 17]. In the results of this study, after the E. coli challenge, crypt depth and the expression of calprotectin were increased, and the villus height was decreased compared to the NC treatment. Also, 61% of pigs in this study had diarrhea for 7 d after the E. coli challenge. These observations are consistent with those seen in weaned pigs infected with E. coli in previous studies [18–20]. Therefore, these obvious clinical signs and symptoms indicated that the pigs were successfully infected with E. coli.

Our study was performed to assess the effects of several PFAs added to the diets of E. coli-infected weaned pigs and to determine which PFAs were effective for PWD. The results of this study indicated that all PFAs had a positive effect on the growth performance of E. coli-infected weaned pigs. The findings suggest that PFAs may help weaned pigs cope with stress during the post-weaning phase when impaired pig growth performance

| Table 4 Effect of PFAs on nutrient digestibility in weaned pigs challenged with E. coli
| Items | NC | PC | T1 | T2 | T3 | T4 | T5 | SEM | P-value
|-------|----|----|----|----|----|----|----|-----|-------|
| d 7 Pl DM, % | 82.48 | 81.97 | 81.93 | 81.21 | 82.40 | 81.44 | 81.74 | 0.499 | 0.521 |
| CP, % | 73.86 | 72.61 | 73.05 | 74.03 | 72.99 | 73.23 | 73.40 | 0.551 | 0.558 |
| d 14 Pl DM, % | 79.21<sup>ab</sup> | 75.62<sup>c</sup> | 79.28<sup>ab</sup> | 78.80<sup>b</sup> | 81.78<sup>a</sup> | 78.38<sup>bc</sup> | 78.83<sup>b</sup> | 0.678 | 0.001 |
| CP, % | 77.01 | 75.60 | 76.05 | 76.88 | 76.46 | 76.23 | 76.28 | 0.460 | 0.375 |

<sup>ab</sup>Marks with different letters are significantly differ (P < 0.05)

| Table 5 Effect of PFAs on blood profile in weaned pigs challenged with E. coli
| Items | NC | PC | T1 | T2 | T3 | T4 | T5 | SEM | P-value
|-------|----|----|----|----|----|----|----|-----|-------|
| d 0 WBC, 10<sup>3</sup>/μL | 18.68 | 19.69 | 19.93 | 18.65 | 19.85 | 20.22 | 18.59 | 1.123 | 0.882 |
| Basophil, % | 0.50 | 0.47 | 0.47 | 0.50 | 0.53 | 0.57 | 0.57 | 0.039 | 0.493 |
| Neutrophil, % | 54.23 | 56.13 | 53.57 | 54.43 | 53.53 | 57.30 | 54.67 | 1.515 | 0.542 |
| Lymphocyte, % | 38.47 | 36.13 | 38.33 | 39.53 | 37.47 | 37.67 | 39.40 | 1.359 | 0.608 |
| d 7 Pl WBC, 10<sup>3</sup>/μL | 19.21<sup>b</sup> | 24.44<sup>ab</sup> | 22.76<sup>b</sup> | 21.85<sup>b</sup> | 24.10<sup>ab</sup> | 28.85<sup>a</sup> | 23.57<sup>ab</sup> | 1.532 | 0.004 |
| Basophil, % | 0.77 | 0.60 | 0.67 | 0.60 | 0.73 | 0.73 | 0.73 | 0.052 | 0.128 |
| Neutrophil, % | 43.13<sup>b</sup> | 57.30<sup>a</sup> | 48.17<sup>b</sup> | 42.33<sup>b</sup> | 50.43<sup>b</sup> | 47.97<sup>b</sup> | 49.30<sup>b</sup> | 2.039 | 0.001 |
| Lymphocyte, % | 46.07<sup>ab</sup> | 31.97<sup>c</sup> | 41.93<sup>ab</sup> | 48.47<sup>a</sup> | 42.13<sup>ab</sup> | 39.30<sup>bc</sup> | 42.20<sup>ab</sup> | 2.045 | 0.001 |
| d 14 Pl WBC, 10<sup>3</sup>/μL | 13.91<sup>c</sup> | 18.11<sup>b</sup> | 21.51<sup>b</sup> | 18.86<sup>bc</sup> | 21.75<sup>b</sup> | 23.85<sup>a</sup> | 22.37<sup>ab</sup> | 1.302 | 0.001 |
| Basophil, % | 0.60<sup>ab</sup> | 0.70<sup>ab</sup> | 0.47<sup>ab</sup> | 0.57<sup>ab</sup> | 0.50<sup>b</sup> | 0.47<sup>b</sup> | 0.80<sup>a</sup> | 0.082 | 0.044 |
| Neutrophil, % | 34.50<sup>bc</sup> | 42.37<sup>ab</sup> | 49.60<sup>a</sup> | 30.87<sup>c</sup> | 39.43<sup>bc</sup> | 42.87<sup>ab</sup> | 36.67<sup>bc</sup> | 2.016 | 0.001 |
| Lymphocyte, % | 57.23<sup>a</sup> | 46.20<sup>c</sup> | 45.87<sup>c</sup> | 56.20<sup>ab</sup> | 51.57<sup>bc</sup> | 47.50<sup>bc</sup> | 47.00<sup>c</sup> | 2.071 | 0.001 |

<sup>a</sup>Marks with different letters are significantly differ (P < 0.05)
most commonly occurs. Naringin and neohesperidin are the major antioxidants in citrus fruits, with naringin, in particular, reported to have anti-inflammatory and antioxidant properties [21, 22]. Through this action, ADG and the feed efficiency of weaned pigs were improved when naringin was added to the diet of weaned pigs in our study, as well as in a previous study [23]. According to Windisch et al. [24], essential oils, including thymol and carvacrol, might enhance the activity of digestive enzymes and hence, increase nutritional absorption, resulting in a higher feed efficiency. This is consistent with the results of the current study. Also, the addition of essential oils reduced the incidence of diarrhea by 50% [25]. Previous studies reported that both thymol and carvacrol had antibacterial and anti-inflammatory effects [26, 27]. According to Ouwehand et al. [28], beneficial microorganisms like Lactobacilli and Bifidobacteria were less susceptible to the antibacterial activity of essential oils than potentially harmful bacteria like E. coli and Salmonella. For this reason, in this study, it seems that a microencapsulated blend of thymol and carvacrol reduced the fecal scores by causing the positive modulation of intestinal microflora. Although no previous studies on bitter citrus extract, thymol, and carvacrol mixed additives have been conducted, the results in the present study suggest that the positive effects such as antibacterial, anti-inflammatory, and antioxidant properties in the components of each additive may have alleviated the adverse effects of E. coli challenge. In particular, this mechanism can explain the improvement of the T3 group’s growth performance and fecal scores.

In the case of nutrient digestibility, there was no significant difference between the treatment groups 7 d after the E. coli challenge. However, after 14 d, DM digestibility was higher in the PFA-added treatment groups than in the PC treatment. The enhanced digestive capacity of the small intestine could be attributed to PFA, which stabilizes microbial eubiosis in the gut [29].

### Table 6

| Items             | NC  | PC  | T1  | T2  | T3  | T4  | T5  | SEM  | P-value |
|-------------------|-----|-----|-----|-----|-----|-----|-----|------|---------|
| IgG, mg/dL        | 219.67 | 215.00 | 220.33 | 215.33 | 216.67 | 219.33 | 218.67 | 9.251 | 0.999    |
| lGA, mg/dL        | 1.67  | 1.67 | 1.33 | 1.33 | 1.67 | 1.67 | 1.33 | 0.167 | 0.350    |
| TNF-α, pg/mL      | 43.80 | 43.08 | 44.20 | 40.11 | 41.53 | 42.39 | 41.09 | 1.874 | 0.704    |
| IL-6, pg/mL       | 50.93 | 53.30 | 52.43 | 55.30 | 51.20 | 52.37 | 50.33 | 5.217 | 0.996    |

### Table 7

| Items             | NC  | PC  | T1  | T2  | T3  | T4  | T5  | SEM  | P-value |
|-------------------|-----|-----|-----|-----|-----|-----|-----|------|---------|
| VH, μm            | 376.88a | 324.45b | 334.45ab | 351.12ab | 371.27a | 360.95ab | 334.93ab | 10.453 | 0.004    |
| CD, μm            | 141.55 | 175.57 | 165.69 | 157.20 | 148.86 | 150.40 | 174.68 | 9.115 | 0.067    |
| VH:CD              | 2.69a | 1.89c | 2.07abc | 2.30abc | 2.59ab | 2.44abc | 1.90bc | 0.147 | 0.001    |

**Abbreviations:** NC Basal diet without E. coli challenge (negative control), PC Basal diet with E. coli challenge (positive control), T1 PC + 0.1% PFA1, T2 PC + 0.1% PFA2, T3 PC + 0.1% PFA3, T4 PC + 0.04% PFA4, T5 PC + 0.10% PFA5, PI Post-inoculation, lIgG Immunoglobulin G, lIgA Immunoglobulin A, TNF-α Tumor necrosis factor α, IL-6 Interleukin-6, SEM Standard error of mean

*Means with different letters are significantly differ (P < 0.05)
studies showed that polyphenols helped digestion by reducing inflammation and increasing the synthesis of digestive enzymes [30]. PWD-induced weaned pigs fed fenugreek extract showed higher DM digestibility than pigs fed the basal diet after an E. coli challenge [29]. In the present study, the T3 group fed a mixture of bitter citrus extract, thymol and carvacrol showed the highest DM digestibility on 14 d after the E. coli challenge. This is considered to be a synergistic effect of the addition of each PFA. There was no significant difference in CP digestibility between the treatment groups at both d 7 PI and d 14 PI. However, when CP digestibility was viewed only as a numerical value, and not a statistical difference, supplementation with thymol and carvacrol showed a digestibility similar to or higher than that of the NC treatment that was not subjected to an E. coli challenge.

An increase in the total number of WBCs indicates the presence of systemic inflammation [32]. Lymphocytes in the whole blood provide specific cellular and humoral immune responses, whereas neutrophils serve as a first-line defense against bacterial infections [17]. Their ratio is commonly utilized as a biomarker to diagnose systemic inflammation severity [32]. According to Liu et al. [33], an E. coli challenge could induce systemic inflammation in weaned pigs by increasing the total number of WBCs and neutrophils. The present study also confirmed that the E. coli challenge increased WBCs and neutrophils and decreased lymphocytes at both d 7 PI and d 14 PI. Among them, supplementation with thymol and carvacrol showed the same or significantly improved WBCs, neutrophil, and lymphocyte counts at d 7 PI, and neutrophil and lymphocyte counts at d 14 PI compared to the NC treatment not infected with E. coli. This suggests that the addition of thymol and carvacrol to weaned pigs infected with E. coli could attenuate systemic inflammation caused by an E. coli infection. Similarly, in pigs challenged with lipopolysaccharide (LPS) from E. coli, the

---

**Table 8** Effect of PFAs on expression of tight junction proteins in weaned pigs challenged with E. coli

| Items      | NC   | PC   | T1      | T2     | T3     | T4     | T5     | SEM   | P-value |
|------------|------|------|---------|--------|--------|--------|--------|-------|---------|
| Calprotectin, % | 0.033<sup>c</sup> | 0.100<sup>a</sup> | 0.084<sup>ab</sup> | 0.066<sup>abc</sup> | 0.042<sup>c</sup> | 0.057<sup>bc</sup> | 0.062<sup>abc</sup> | 0.004  | 0.001   |
| CLDN1, %   | 18.24<sup>a</sup> | 8.48<sup>d</sup> | 13.86<sup>c</sup> | 14.42<sup>c</sup> | 17.72<sup>ab</sup> | 15.63<sup>bc</sup> | 15.78<sup>abc</sup> | 0.440  | 0.001   |

<sup>1</sup>Abbreviations: NC Basal diet without E. coli challenge (negative control), PC Basal diet with E. coli challenge (positive control), T1 PC + 0.04% PFA1, T2 PC + 0.01% PFA2, T3 PC + 0.10% PFA3, T4 PC + 0.04% PFA4, T5 PC + 0.10% PFAS, PI Post-inoculation, CLDN1 Claudin 1, SEM Standard error of mean

<sup>a-d</sup>Means with different letters are significantly differ (<i>P</i> < 0.05)
addition of essential oils showed improved WBCs and neutrophil results compared to pigs fed the basal diet after the LPS challenge due to the anti-inflammatory response to essential oils [34].

Serum IgA and IgG are important immunoglobulins in humoral immunity [35]. In particular, IgG has been found in high amounts in the serum in response to external infection, with a lengthy half-life [36]. Due to weaning stress and the immaturity of the piglet immune system, a reduction in serum IgG concentrations normally occurs during the weaning phase [37]. The study results showed that the *E. coli* challenge significantly decreased serum IgG and IgA concentrations at d 7 PI compared to the NC treatment, and showed a decreased tendency at d 14 PI. Furthermore, the feeding of a mixture of bitter citrus extract, thymol and carvacrol to pigs infected with *E. coli* showed significantly higher IgG and IgA concentrations at both d 7 PI and d 14 PI compared to PC treatment. Ahmed et al. [38] reported that the feeding of essential oils in the *E. coli* challenge condition increased the IgG levels of weaned pigs, consistent with the results of the present study. Thymol contained in essential oils can increase goblet cells in the animal ileum and inhibit the growth of pathogenic bacteria [39, 40]. Also, the addition of essential oils could improve immunity by regulating the humoral immune system of weaned pigs [41]. The mechanism by which PFA increases IgG levels requires further study, but it has been suggested that active phytotherapeutic (flavonoids and polyphenols) molecules may act as additional ligands of Fc receptors to bind IgG and stimulate immune responses [42]. Cytokines are also involved in the maintenance of immune homeostasis [35]. The pro-inflammatory cytokines IL-6 and TNF-α are frequently used as potential markers in weaning pigs to diagnose pathogenic infections [43]. TNF-α and IL-6 impair animal performance by impairing immunity and nutrient metabolism [44]. This could emphasize the crucial role of cytokines in the regulation of immune and inflammatory responses. In the present study, the *E. coli* challenge significantly increased TNF-α at both d 7 PI and d 14 PI. Also, the *E. coli* challenge increased IL-6 at d 7 PI and showed a high trend at d 14 PI. However, both TNF-α and IL-6 were significantly lower in the T3 group fed a mixture of bitter citrus extract, thymol, and carvacrol than in the PC treatment, suggesting that this PFA positively modulated the immune and inflammatory responses. TNF-α levels in the blood have been linked to gut inflammatory disease, and a previous study found that a combination of carvacrol and thymol lowered *TNF-α* mRNA expression in the intestine of weaned pigs, leading to improved gut health and growth performance [45]. Therefore, the present results suggest that the addition of a mixture of bitter citrus extract, thymol, and carvacrol could suppress the inflammatory response caused by *E. coli* in weaned pigs and have a beneficial effect on health status.

Intestinal morphology is commonly assessed by measuring VH, CD, and the VH:CD ratio [46]. Intestinal morphology reveals some information on gut health. A shortening of the villus and deeper crypts may decrease the surface area for nutrient absorption. Therefore, the VH:CD ratio value is utilized as a useful measure of nutrient digestion and absorption [47]. The *E. coli* inoculum used in the present study expresses LT and Stx2e, which could induce intestinal morphological lesions such as villus atrophy and leaky gut in weaned pigs [33, 48]. These lesions impair nutrient absorption and are a major cause of reduced growth performance [49, 50]. In this study, the *E. coli* challenge also reduced the VH:CD ratio, indicating the deterioration of intestinal health. However, supplementation with a mixture of bitter citrus extract, thymol, and carvacrol improved VH, CD, and the VH:CD ratio in pigs compared to those in the PC treatment. Essential oils reduce the number of pathogenic bacteria in the gut through their antibacterial action [28]. It has been reported that essential oils can improve epithelial cell proliferation to build intestinal villus and improve intestinal morphology [51]. In addition, the antioxidant effect could decrease the intestinal damage caused by oxidative stress and improve the intestinal morphology of weaned pigs [52]. Therefore, the improvement in intestinal morphology in the T3 group was thought to be due to the complex antibacterial and antioxidant actions of thymol, carvacrol, and bitter citrus extract.

The intestinal epithelia, especially the internal tight junctions such as occludin and CLDN1 between enterocytes, are critical in maintaining intestinal permeability [53]. Enteric infections and endotoxin translocation can increase the permeability of the intestinal epithelium by altering tight junctions [54]. A disruption in intestinal barrier function might also be caused by a disruption in the expression of tight junction proteins [1, 55]. As a result of the diminished intestinal integrity, bacterial translocation is enhanced, and cellular pro-inflammatory responses stimulated by foreign invading bacteria might be increased [15, 56, 57]. Calprotectin mainly plays a role in preventing the binding of bacteria to mucosal epithelial cells through zinc competition and is used as an indicator to quantify the extent of intestinal inflammation in infectious and inflammatory diseases [19, 58]. In the current study, supplementation with bitter citrus extract, thymol, and carvacrol down-regulated the expression of calprotectin and up-regulated the expression of CLDN1 in the ileal mucosa on d 14 PI. A previous study reported that feeding guava leaf extract containing naringin to weaned pigs
challenged with *E. coli* improved intestinal permeability and mucosal damage caused by *E. coli* and improved tight junction integrity [59]. The down-regulation of calprotectin could be seen as an indicator of attenuated intestinal inflammation caused by PFA. Therefore, these results indicated that supplementation with bitter citrus extract, thymol, and carvacrol helped to maintain normal intestinal integrity and immune functions, enhancing disease resistance and the performance of *E. coli*-challenged pigs.

**Conclusion**

In weaned pigs infected with *E. coli*, the addition of PFA alleviated the negative effects of *E. coli* and improved growth performance. When supplemented with thymol and carvacrol, the antibacterial and anti-inflammatory effects of thymol and carvacrol have been shown to prevent diarrhea. When a mixture of bitter citrus extract, thymol, and carvacrol was fed, immunity, intestinal morphology, and tight junction expression were all improved. Therefore, when a mixture of bitter citrus extract, thymol, and carvacrol is added, each PFA effect seems to be added and synergistic, and it is considered that this mixed additive is the most effective PFA among the five additives used in the study. However, it seems that additional studies are needed on the proper amount of each additive and the basic mechanism of PFA against PWD.

**Abbreviations**

ADFI: Average daily feed intake; ADG: Average daily gain; ATTD: Apparent total tract digestibility; BW: Body weight; CD: Crypt depth; CLDN1: Claudin 1; CP: Crude protein; DAB: 3,3′-diaminobenzidine; DM: Dry matter; DW: Distilled water; *E. coli*: *Escherichia coli*; FI: Feed intake; G:F: Feed efficiency; IgA: Immunoglobulin A; IgG: Immunoglobulin G; IHC: Immunohistochemistry; IL-6: Interleukin-6; LPS: Lipopolysaccharide; LT: Heat labile toxin; MBF: Neutral buffered formalin; NC: Negative control; PC: Positive control; PFA: Phytogenic feed additive; PI: Post-inoculation; PWD: Post-weaning diarrhea; Stx2e: Shiga toxins type 2e; TNF-α: Tumor necrosis factor α; VH: Villus height; VH:CD: Villus height: crypt depth; WBC: White blood cell.

**Acknowledgements**

Not applicable.

**Authors’ contributions**

CSY and SMH conducted the animal work, laboratory work and wrote most of the manuscript. LJH, OHJ, KYI, AHW, GB, SDC, and CHA helped to conduct animal trials and part of the laboratory work and helped to revise the manuscript. CSY, KDI, and KMS conducted laboratory work on the functionality of the phytotherapeutic feed additives and provided and manufactured Phytoprogenic feed additives used in this study. KHB and CJH was the principal investigator, oversaw the development of the study, and wrote the last version of the manuscript. All authors read and approved the final manuscript.

**Funding**

This work was carried out with the support of “Cooperative Research Program for Agriculture Science & Technology Development (Project No. PJ01622001)” Rural Development Administration, Korea.

**Availability of data and materials**

The datasets from the current study are available from the corresponding author upon reasonable request.

**Declarations**

**Ethics approval and consent to participate**

The protocol for this study was reviewed and approved by the Institutional Animal Care and Use Committee of Chungbuk National University, Cheongju, Korea (approval no. CBUA-1618-21-02).

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare no conflicts of interest associated with this study.

**Author details**

1 Department of Animal Science, Chungbuk National University, Cheongju 28644, South Korea. 2 Division of Animal and Dairy Science, Chungnam National University, Daejeon 34134, South Korea. 3 Eugene-Bio, Suwon 16675, South Korea. 4 Department of Animal Resources Science, Dankook University, Cheonan 31116, South Korea.

**Received:** 22 February 2022 **Accepted:** 3 July 2022

**Published online:** 02 September 2022

**References**

1. Kim K, He Y, Xiong X, Ehrlich A, Li X, Raybould H, et al. 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.
2. Piva A, Morlacchini M, Casadei G, Gatta P, Biagi G, Prandini A. Sodium butyrate improves growth performance of weaned piglets during the first period after weaning. Ital J Anim Sci. 2002;1:35–41.
3. Park JH, Sureshkumar S, Kim IH. Effects of dietary lysylzyme supplementation on growth performance, nutrient digestibility, intestinal microbiota, and blood profiles of weaning pigs challenged with *Escherichia coli*. J Anim Sci Technol. 2021;63:501.
4. Heo JM, Opapeju FO, Pluske JR, Kim JC, Hampson DJ, Nyachoti CM. Gastrointestinal health and function in weaned pigs: a review of feeding strategies to control post-weaning diarrhea without using in-feed antimicrobial compounds. J Anim Physiol Anim Nutr. 2013;97:207–37.
5. Luppi A, Gibellini M, Gint V, Vangroenweghe F, Vandenbroucke V, Bauerfeind R, et al. Prevalence of virulence factors in enterotoxigenic *Escherichia coli* isolated from pigs with post-weaning diarrhea in Europe. Porc Health Manag. 2016;2:20.
6. 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;51:215–36.
7. Yang C, Chowdhury MA, HUO Y, Gong J. Phytoprogenic compounds as alternatives to in-feed antibiotics: potentials and challenges in application. Pathogens. 2015;4(1):137–56.
8. Upadhyaya SD, Kim IH. Efficacy of phytoprogenic feed additive on performance, production and health status of monogastric animals–a review. Anim Sci Technol. 2017;17:929–48.
9. Montoya D, D’Angelo M, Martin-Orue SM, Rodriguez-Sorrento A, Saladrigas-García M, Arauco C, et al. Effectiveness of two plant-based in-feed additives against an *Escherichia coli* F4 oral challenge in weaned piglets. Animals. 2021;11:2024.
10. Tian Q, Piao X. Essential oil blend could decrease diarrhea prevalence by improving antioxidative capability for weaned pigs. Animals. 2019;9:847.
11. National Research Council (NRC). Nutrient requirements of swine. 11th ed. Washington: National Academy Press, 2012.
12. Mun D, Kyong H, Kong M, Ryu S, Iang KB, Bae J, et al. Effects of Bacillus-based probiotics on growth performance, nutrient digestibility, and intestinal health of weaned pigs. J Anim Sci Technol. 2021;63:1314.
13. AOAC, editor. Official methods of analysis. 18th ed. Gaithersburg: AOAC International, 2005.
14. Williams CH, David DJ, Iismaa O. The determination of chromic oxide in faces samples by atomic absorption spectrophotometry. J Agric Sci. 1962;59:381–5.

15. Almeida JAS, Liu Y, Song M, Lee JJ, Gaskins HR, Maddox CW, et al. Escherichia coli challenge and one type of smectite alter intestinal barrier of pigs. J Anim Sci Biotechnol. 2013;4:52.

16. Nagy B, Fedele PZ. Enterotoxicogenic Escherichia coli in veterinary medicine. Int J Med Microbiol. 2005;295:443–54.

17. He Y, Kim K, Kovanda L, Jinno C, Song M, et al. Bacillus subtilis: a potential growth promoter in weaned pigs in comparison to carboxad. J Anim Sci. 2020;98:9.

18. Kim K, He Y, Jinno C, Kovanda L, Li X, Song M, et al. Trace amounts of antibiotic exacerbated diarrhea and systemic inflammation of weaned pigs infected with a pathogenic Escherichia coli. J Anim Sci. 2021;99:3.

19. Tang ZR, Deng H, Zhang XL, Zen Y, Xiao DF, Sun WZ, et al. Effects of orally administering the antimicrobial peptide butorifolin II on small intestinal mucosal membrane integrity, the expression of tight junction proteins and protective factors in weaned piglets challenged by enterotoxigenic Escherichia coli. Anim Feed Sci Technol. 2013;186:177–85.

20. Sun Y, Duarte ME, Kim SW. Dietary inclusion of multipurpose probiotics to reduce the severity of post-weaning diarrhea caused by Escherichia coli F18+ in pigs. Anim Nutr. 2021;7:326–33.

21. Lipinski K, Mazar A, Antoszkiewicz Z, Purwin C. Polyphenols in monogastric nutrition—a review. Anim Feed Sci Technol. 2017;217:41–58.

22. Goleomyts M, Kartsonas N, Charismiadou MA, Symeon GK, Simitzi PS, Deligeorgis SG. The influence of naringin or hesperidin dietary supplementation on broiler meat quality and oxidative stability. PLoS One. 2015;10:e0141652.

23. Goodarzi Boroojeni F, Männer K, Zentek J. The impacts of polyphenols in regulation of heat shock proteins and gut microflora. Livest Sci. 2007;109:157–60.

24. Windisch W, Schedle K, Plitzner C, Kroismayr A. Use of phytogenic products as feed additives for swine and poultry. J Anim Sci. 2008;86(suppl 14):E140–8.

25. Li SY, Ru YJ, Liu M, Xu B, Pétron A, Shi XG. The effect of essential oils on performance, immunity and gut microbial population in weaner pigs. Livest Sci. 2012;145:19–23.

26. Michiels J, Missotten J, Van Hoeck A, Oynon A, Fremout D, De Smet S, Dierick N. In vitro dose–response of carvacrol, thymol, eugenol and \(-\)cinnamaldehyde and their functional traits of the gut in piglets after weaning. Arch Anim Nutr. 2018;72:178–89.

27. Ouwehand AC, Kettunen H, Schulze H, Rautonen N. In vitro activity of essential oils towards intestinal microbes. Reprod Nutr Dev. 2006;46:5110.

28. Mohana Devi S, Lee SI, Kim LH. Effect of phytotherapies on growth performance, fecal score, blood profiles, fecal noxious gas emission, digestibility, and intestinal morphlogy of weaning pigs challenged with Escherichia coli K88. PLoJ Vet Sci. 2015;18:557–64.

29. Hussain T, Wang J, Murtaza G, Metwally E, Yang H, Kalhoro MS, et al. The role of polyphenols in regulation of heat shock proteins and gut microbiota in weaning stress. Oxidative Med Cell Longev. 2021;2021:667444.

30. Platel K, Srinivasan K. Digestive stimulant action of spices: a myth or reality? Indian J Med Res. 2004;119:167–79.

31. Gordon-Smith T. Structure and function of red and white blood cells. Medicine. 2009;37:119–24.

32. Liu Y, Song M, Che TM, Almeida JAS, Lee JJ, Bravo D, et al. Dietary plant extracts alleviate diarrhea and alter immune responses of weaned pigs experimentally infected with a pathogenic Escherichia coli. J Anim Sci. 2013;91:5294–306.

33. Kwak WG, Song MH, Lee DH, Yun W, Lee JH, Lee CH, et al. The effects of microencapsulated compounds supplementation on growth performance, immune cells, and rectal temperature in weaned pigs by lipopolysaccharides. Can J Anim Sci. 2019;99:505–13.

34. Han Y, Zhan T, Tang C, Zhao Q, Dansou DM, Yu Y, et al. Effect of replacing in-feed antibiotic growth promoters with a combination of egg immunoglobulins and phytochemicals on the performance, serum immunity, and intestinal health of weaned pigs challenged with Escherichia coli K88. Animals. 2021;11:1292.

35. Horton R, Vidarsson G. Antibodies and their receptors: different potential roles in mucosal defense. Front Immunol. 2021;13:200.

36. Klobasa F, Butler IE, Werhahn E, Habe F, Mayer F. Maternal-neonatal immunoregulation in swine. II. Influence of multiplicity of de novo immunoglobulin synthesis by piglets. Vet Immunol Immunopathol. 1986;11:49–59.

37. Ahmed ST, Hassain ME, Kim GM, Hwang JA, Ji H, Yang CJ. Effects of resveratrol and essential oils on growth performance, immunity, digestibility and fecal microbial shedding in challenged piglets. Asian Australas J Anim Sci. 2013;26:683–90.

38. Zhang W, Zhu YH, Zhou D, Wu Q, Song D, Dicksveld J, et al. Oral administration of a select mixture of Bacillus probiotics affects the gut microbiota and goblet cell function following Escherichia coli challenge in newly weaned pigs of genotype MUC4 that are supposed to be enterotoxigenic E. coli F4ab/ac receptor negative. Appl Environ Microbiol. 2017;83:e02747–16.

39. Heidegaard CJ, Strube ML, Hansen MB, Lindved BK, Likhme A, Boye M, et al. Natural pig plasma immunoglobulins have anti-bacterial effects: potential for use as feed supplement for treatment of intestinal infections in pigs. PLoS One. 2016;11:e0147373.

40. Tan BF, Lim T, Boontam W. Effect of dietary supplementation with essential oils and a Bacillus probiotic on growth performance, diarrhoea and blood metabolites in weaned pigs. Anim Prod Sci. 2020;61:64–71.

41. Nimmerjahn F, Ravetch JV. Antibody-mediated modulation of immune responses. Immunol Rev. 2010;236:265–75.

42. Zhang L, Xu YQ, Liu HY, Lai T, Ma JL, Wang JF, et al. Evaluation of Lactobacillus rhamnosus GG using an Escherichia coli K88 model of piglet diarrhoea: effects on diarrhoea incidence, faecal microflora and immune responses. Vet Microbiol. 2010;141:142–8.

43. Spurlock ME. Regulation of metabolism and growth during immune challenge: an overview of cytokine function. J Anim Sci. 1997;75:1773–83.

44. Wei HK, Xue HX, Zhou ZX, Peng J. A carvacrol–thymol blend decreased intestinal oxidative stress and influenced selected microbes without changing the messenger RNA levels of right intestine protein in jejunal mucosa of weaned pigs. Animal. 2017;11:193–201.

45. Liu Y, Chen F, Odlie J, Lin X, Jacobis SK, Zhu H, et al. Fish oil enhances intestinal integrity and inhibits TLR4 and NOD2 signalling pathways in weaned pigs after LPS challenge. J Nutr. 2012;142:2017–24.

46. Montagane L, Cavaney FS, Hampson DJ, Lalles JP, Plumke JR. Effect of diet composition on postweaning colibacillosis in piglets. J Anim Sci. 2004;82:2364–74.

47. Sonntag AK, Biesalszewska M, Melamm A, Dierksen N, Schierack P, Wieler LH, et al. Shiga toxin 2e-producing Escherichia coli isolates from humans and pigs differ in their virulence profiles and interactions with intestinal epithelial cells. Appl Environ Microbiol. 2005;71:8855–63.

48. Rose R, Whipp SC, Moon HW. Effects of Escherichia coli heat-stable enterotoxin b on small intestinal villi in pigs, rabbits, and lambs. Vet Pathol. 1987;24:71–9.

49. Dubreuil JD. Escherichia coli STb toxin and colibacillosis: knowing is half the battle. FEMS Microbiol Lett. 2008;278:137–45.

50. Mourão JL, Pinheiro V, Alves A, Guedes CM, Pinto L, Saavedra MJ, et al. Effect of mannan oligosaccharides on the performance, intestinal morphology and fecal fermentation of fattening rabbits. Anim Feed Sci Technol. 2006;126:107–20.

51. Han X, Piao XS, Zhang HY, Li PF, Yi JQ, Zhang Q, et al. Forsythia suspensa extract has the potential to substitute antibiotic in broiler chicken. Asian Australas J Anim Sci. 2012;25:569–76.

52. Gao Y, Han F, Huang X, Rong Y, Yi H, Wang Y. Changes in gut microbial populations, intestinal morphology, expression of tight junction proteins, and cytokine production between two pig breeds after challenge with Escherichia coli K88: a comparative study. J Anim Sci. 2013;91:5614–25.

53. Moreno M, Pérez-Bosque A. Dietary plasma proteins, the intestinal immune system, and the barrier functions of the intestinal mucosa. J Anim Sci. 2009;87(Suppl 14):E92–100.

54. Dubreuil JD. Enterotoxigenic Escherichia coli targeting intestinal epithelial tight junctions: an effective way to alter the barrier integrity. Microbiol. 2017;113:129–34.

55. Lessard M, Dupuis M, Gagnon N, Nadeau E, Matte JJ, Goulet J, et al. Administration of Pedicoccus acidilactici or Saccharomyces cerevisiae boulardii modulates development of porcine mucosal immunity and
reduces intestinal bacterial translocation after *Escherichia coli* challenge. J Anim Sci. 2009;87:922–34.

57. Ashida H, Ogawa M, Kim M, Mimuro H, Sasakawa C. Bacteria and host interactions in the gut epithelial barrier. Nat Chem Biol. 2012;8:36–45.

58. Kalla R, Kennedy NA, Ventham NT, Boyapati RK, Adams AT, Nimmo ER, et al. Serum calprotectin: a novel diagnostic and prognostic marker in inflammatory bowel diseases. Am J Gastroenterol. 2016;111:1796–805.

59. Wang D, Zhou L, Zhou H, Hu H, Hou G. Chemical composition and protective effect of guava (*Psidium guajava* L.) leaf extract on piglet intestines. J Sci Food Agric. 2021;101:2767–78.