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# Effects of pomegranate (*Punica granatum* L.) peel on the growth performance and intestinal microbiota of broilers challenged with *Escherichia coli*

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**ABSTRACT** The effects of pomegranate peel on the growth performance, intestinal morphology, and the cecal microbial community were investigated in broilers challenged with avian pathogenic *Escherichia coli* (**APEC**) O78. A total of 240 one-day-old chicks (120 males and 120 females) were randomly and evenly allotted into 4 treatment groups (each with 6 biological replicates each of 10 chicks), i.e., negative control (**NC**), positive control (**PC**), and 2 experimental groups treated with 0.2% fermented pomegranate peel (**FP**) and 0.2% unfermented pomegranate peel (**UFP**), respectively, with PC, FP, and UFP groups challenged with APEC O78 ($5 \times 10^8$ CFU) on day 14. Results showed that the challenge of APEC O78 decreased the body weight (**BW**) and average daily gain (**ADG**) of broilers from 1 to 28 d ($P < 0.01$). These broilers exhibited more pathological conditions in the heart and liver and higher mortality rates in 28 d compared to the NC group. Diet supplemented with pomegranate peel (either fermented or unfermented) significantly increased BW, ADG, and the villus height/crypt depth ratio (**VCR**) of small intestine in 28 d compared to the NC group ($P < 0.05$). Results of the taxonomic structure of the gut microbiota showed that compared to the NC group, the APEC challenge significantly decreased the relative abundance of *Bacteroidetes* and increased the relative abundance of *Firmicutes* ($P < 0.01$). Compared to the PC group, the relative abundance of *Ruminococcus_torques_group* in FP group was increased, while the relative abundance of *Alistipes* was decreased. In summary, our study showed that the dietary supplementation of pomegranate peel could maintain the intestinal microbiota at a state favorable to the host, effectively reduce the abnormal changes in the taxonomic structure of the intestinal microbiota, and improve the growth performance in broilers treated with APEC.

**Key words:** pomegranate peel, broiler, *Escherichia coli*, growth performance, intestinal microbiota

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## INTRODUCTION

The *avian pathogenic Escherichia coli* (**APEC**) has been commonly found in and closely associated with the intestinal microbiota of broilers. The diseases associated with APEC are primarily caused by environmental factors and host susceptibility, resulting in significant economic losses to the poultry industry (Kaper et al., 2004; Newman et al., 2021). The APEC could cause both systemic and local infections in chickens, resulting in a variety of health issues such as perihepatitis, pericarditis, yolk peritonitis, salpingitis, cellulitis, osteomyelitis, arthritis, and other inflammatory diseases (Dho-Moulin and Fairbrother, 1999; Dias da Silveira et al., 2002). Furthermore, the APEC could adversely affect the growth performance, body weight gain, and feed conversion in broilers (Kemmett et al., 2013; Tarabees et al., 2019). Currently, there is no directly effective vaccine to protect chickens from APEC infection, mainly because the APEC has multiple serotypes and lacks cross-protection (Mehat et al., 2021). Antibiotic treatment has been proven effective in preventing and treating *E. coli* infections (Roth et al., 2019). However, both the overuse and misuse of antibiotics have led to rapid increase in antibiotic resistance among microorganisms, causing significant concerns in related areas (Nhung et al., 2017; Abdallah et al., 2019). Therefore, it is urgent to find effective and safe products to replace antibiotics for the prevention and treatment of APEC infection.



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The pomegranate peel is the waste residue generated during the processing of pomegranate juice and could be easily obtained at low prices. Notably, the pomegranate peel contains a large group of phenolic compounds at high concentrations, e.g., hydrolyzable tannins (i.e., punicalin, punicalagin, ellagic acid, and gallic acid), flavonoids (both anthocyanins and catechins), and nutrients (Akhtar et al., 2015; El-Hadary and Ramadan, 2019). These compounds exhibit strong antioxidant, antimicrobial, cardioprotective, apoptotic, and antigenotoxic potentials, with possible ameliorative effects on many critical diseases (Endo et al., 2010; Lee et al., 2010; Fawole et al., 2012). Furthermore, the pomegranate peel is an important source of organic acids, e.g., citric acid, malic acid, acetic acid, oxalic acid, tartaric acid, lactic acid, ascorbic acid, and fumaric acids, and many other nutrients (Poyrazoglu et al., 2002). The organic acids could acidify the digestive tract to generate the low pH level in the local environment, thereby improving the chicken's resistance to pathogens and creating an unfavorable environment for the proliferation of certain intestinal pathogens such as *E. coli* (Khan et al., 2022). Studies have shown that the addition of 4% pomegranate peel powder significantly reduced the quantity of *E. coli* compared to the control group ($P < 0.05$) (Ghasemi-Sadabadi et al., 2022). Moreover, studies have reported that the pomegranate peel extract has been shown to inhibit the growth of *E. coli*, i.e., at the highest concentration (2.7 mg/mL) of $H_2O$ extraction and ethanol peel extraction, the growth of *E. coli* was completely inhibited, resulting in 100% microbial growth inhibition rate (Kupnik et al., 2021). To date, plant microbial fermentation is widely used to develop new functional components in plants (Sugiharto and Ranjitkar, 2019). Microbial fermentation is frequently used to degrade indigestible substances into small, easily absorbed molecules and to increase the utilization of active ingredients (Cao, et al., 2012). For example, fermentation can increase the phenolic content of pomegranate wastes, thereby enhancing antioxidant effects (Verotta et al., 2018).

Although the pomegranate peel has been revealed with antibacterial and antioxidant effects, the studies on its effects as an inclusion in diets on the growth performance, health, and disease resistance of chickens are still lacking. Therefore, in order to determine the effect of the addition of pomegranate peel in broiler diets, the aim of this study was to evaluate the functions of fermented and unfermented pomegranate peels (**FP** and **UFP**) added to broiler diets in growth performance, taxonomic structure of intestinal microbiota, and disease resistance in broilers.

## MATERIALS AND METHODS

### *Preparation of Pomegranate Peel*

Both FP and UFP were obtained from the Hubei Huada Real Technology Co., Ltd., Jingzhou China. The FP was prepared as follows: the pomegranate peels were crushed, added with water to 50% moisture, heated to 80°C for 1 h, and then cooled to 37°C. Three species of probiotics (*Bacillus amyloliquefaciens* TL, *B. licheniformis* TN, and *Enterococcus faecalis*), were simultaneously added into the processed pomegranate peels mixture. After 72 h of fermentation, i.e., the level of tannin content stabilizes and does not increase any more, and the fermentation is complete. Then, the materials were dried, crushed, filtered through a 60-mesh sieve, and stored with ventilation. The FP contained tannic acid of ~17.21% with the moisture content of 8.20%. To make the UFP, the pomegranate peels were dried, crushed, filtered through a 60-mesh sieve, and stored with ventilation. The UFP contained tannic acid of ~15.61% with the moisture content of 14.67%.

### *Experimental Design*

All animal experiments was completed by strictly following the Guide for the Care and Use of Laboratory Animals Monitoring Committee of Hubei Province, China, with the experimental protocols approved by the Committee on the Ethics of Animal Experiments of the College of Veterinary Medicine, Huazhong Agricultural University (approval No. HZAUGE−2020−0001). A total of 240 one-day-old 817 broiler chicks (120 males and 120 females) were randomly and evenly divided into 4 groups, i.e., a blank control group (negative control [**NC**]), blank attacked group (positive control [**PC**]), and 2 experimental groups treated with FP and UFP, respectively, with 6 pens (10 chickens per pen) in each group. Chickens in both NC and PC groups were fed with a basal diet (no medicines or additives added), whereas the 2 experimental groups were fed with the basal diet with the addition of 0.2% FP and UFP, respectively. The nutritional components and levels of the basal diet were shown in Table 1.

The animal experiments were performed at the Hubei Huada Real Technology Co., Ltd. (Jingzhou, China). Prior to the trials, both potassium permanganate and formalin were used to disinfect the chicken coops, which were cleared of manure every day and maintained at ~33°C until the chickens were 7 d old. Then, the temperature was gradually decreased and finally maintained at 23°C. The chickens were given constant access to their group-specific feed throughout the day and unlimited water via nipple drinkers. The amount of feed consumption and the residual feed in each group were recorded daily. The body weight of each animal was measured once a week, i.e., on days 7, 14, 21, and 28, respectively.

### *Experimental Infection With* Escherichia coli

To activate the *E. coli*, the stored *E. coli* of lyophilized bacterial powder with serotypes O78 (obtained from the Laboratory of Veterinary Microbiology, Huazhong Agricultural University, Wuhan China) were cultured in Luria-Bertani liquid medium at 37°C and 200 r/min for 12 h.

**Table 1.** Nutrient composition and level of basal diet used in this study.

| Ingredient | Composition (%) |
|---|---|
| Calculated nutrient | |
| Corn | 60.78 |
| Soybean meal | 25.00 |
| Soybean oil | 1.00 |
| Flour | 5.00 |
| Fish meal | 5.00 |
| $CaHPO_4$ | 1.10 |
| NaCl | 0.35 |
| Limestone | 1.45 |
| Premix1 | 0.32 |
| Total | 100.00 |
| Calculated nutrient level | |
| AME (kcal/kg) | 2,850 |
| Crude protein | 20.17 |
| Calcium | 1.18 |
| Phosphorus | 0.56 |
| Nonphytate phosphorus | 0.40 |
| Lysine | 1.16 |
| Methionine | 0.32 |
| Threonine | 0.76 |
| Tryptophan | 0.21 |

The premix of diet provides the following nutrients per kg of diet: vitamin A, 9,600 IU; vitamin D3, 32,700 IU; vitamin B1, 1.5 mg; vitamin B2, 9.0 mg; vitamin B6, 3.0 mg; vitamin B12, 0.02 mg; vitamin E, 19 IU; vitamin K3, 1.40 mg; biotin, 0.95 mg; folic acid, 0.93 mg; D-pantothenic acid, 9.3 mg; Cu, 15 mg; Fe, 60 mg; Mn, 100 mg, Zn, 70 mg; I, 0.45 mg; Se, 0.59 mg.

During the passaging of *E. coli*, a small amount of bacterial solution was dipped by a sterile inoculation loop, streaked on a MacConkey plate, and then cultured in a 37°C incubator for 12−18 h. The single, pink, and smooth colonies of moderate size on the MacConkey plate were identified and collected using the inoculation loop to transfer to LB liquid medium, and cultured in a shaker at 37°C and 180 r/min for 12 h. The bacterial liquid passaging process were repeated 2−3 times.

From days 14 to 21, each chick in the PC, FP, and UFP groups was orally administered with 1 mL of a bacterial solution containing *E. coli* ($5 \times 10^8$ CFU/mL) daily, while the chicks in the NC group were orally administered with an equal amount of sterile saline solution. On day 7 postinfection (21 d old broilers), 6 chickens were randomly slaughtered from the APEC-infected groups (PC, FP, and UFP), and their livers (n = 6) and hearts (n = 6) were aseptically collected and stored at −20°C for determination of the bacterial load.

## Growth Performance

During the experiments, the BW and feed intake of chicks in each group were determined with an empty stomach, and the clinical signs and mortality were monitored daily. Four growth performance indicators, i.e., the average daily gain (**ADG**), average daily feed (**ADFI**), feed conversion radio (**FCR**), and mortality rate, were calculated using the formulae ADG = (final BW—initial BW)/days of experiment, ADFI = (feed amount provided during the test period—the remaining amount of feed during the test period)/days of experiment, FCR (%) = (ADFI intake/ADG of weight) × 100%, and mortality rate (%) = (the number of deaths in a group during the test period/the total number of animals in a group during the test period) × 100%, respectively.

## Sample Collection

A total of 6 broilers were randomly sampled from each of the 4 groups on day 28 after 12 h of fasting with the pen number and weight of each individual chicken recorded. Then, these chickens were slaughtered by cervical dislocation. A total of ∼3 mL blood was collected from the subwing vein with a vacuum coagulation tubelet, kept still at 25°C for 2 h, and then placed on ice. After the blood coagulated, the serum was separated and centrifuged at 3,000 r/min for 10 min to remove impurities. The supernatant was transferred into a clean centrifuge tube and stored at −20°C. A portion of cecum contents samples were collected, quickly frozen in liquid nitrogen, and stored in a refrigerator at −80°C for subsequent analyses.

## Serum Analysis

The levels of the enzymatic activities of 2 biochemical factors, i.e., alanine aminotransferase (**ALT**) and aspartate aminotransferase (**AST**), were measured by an automatic biochemical analyzer (BK-280, Shandong Blobase Biotechnology Co., Ltd., Shandong, China). The contents of malondialdehyde (**MDA**), superoxide dismutase (**SOD**), catalase (**CAT**), glutathione (**GSH**), and total antioxidant capacity (**T-AOC**) were measured using the enzyme-linked immunosorbent assay (**ELISA**) kits (Wuhan Meimian Biotechnology Co., Ltd., Wuhan, China). All experiments were strictly performed in accordance with the manufacturers' protocols and instructions.

## Histological Analysis

The liver, heart, duodenum, jejunum, and ileum tissue samples were fixed in 4% formalin solution (Biosharp Co., Ltd., Hefei, China), routinely embedded in paraffin, cut into 4 $\mu$m thick sections, and stained with hematoxylin and eosin (**HE**). Sections of the tissues from the chicks were examined under a microscope and photographed (Nikon Eclipse CI, Nikon Instruments, Tokyo, Japan). The measurements of villus height and crypt depth of duodenum, jejunum, and ileum were completed using CaseViewer software (version 2.0, Budapest, Hungary). Measurements of different intact villi were measured for each slice (for a total of 6 measurements in 3 successive fields of view).

## Determination of the Bacterial Load in Liver and Heart Tissues

***Establishment of the APEC Standard Curves*** To calculate the total number of bacteria using the plate

counting method, the bacterial solution was diluted to the series of concentrations of $10^9$−$10^4$ CFU/mL. The bacterial DNA was extracted with a Universal Genomic DNA Kit (CW2298M, CoWin Biosciences, Beijing, China) following the manufacturer's instructions. The cycle threshold (**CT**) value was measured by the fluorescence quantitative PCR based on the template of each bacterial concentration. The standard curve was established with the logarithm of the number of bacteria in the sample as the abscissa and the CT value as the ordinate.

***DNA Extraction of* Escherichia coli *in Liver and Heart Tissues*** DNA extraction of *E. coli* from liver and heart tissues was performed using the Universal Genomic DNA Kit (CW2298M, CoWin Biosciences) by following the procedures recommended by the manufacturer. Then, DNA was diluted 10-fold and used for quantitative real-time PCR (**qRT-PCR**) analysis using the Bio-Rad CFX96TM System and signal detection protocols by following the manufacturer's instructions (TaKaRa, Dalian, China). Each qRT-PCR experiment was repeated with 3 technical replicates using the gene *FimH* of *E. coli* as the internal reference and the forward primer F (5′-CTTATGGCGGCGTGTTATCT-3′) and the reverse primer R (5′-CTGCTCACAGGCGTCAAATA-3). Data were analyzed using GraphPad Prism v 8.3.0 (GraphPad, Inc., La Jolla, CA).

## *Intestinal Microorganism Analysis*

***Sample Collection, DNA Extraction, and Sequencing*** The PowerSoil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA) was used to extract the total genomic DNA of the microbial community from the cecal contents by following the manufacturer's instructions. DNA quality was determined on 1% agarose gel. DNA concentration and purity were evaluated using the NanoDrop 2000 UV-vis spectrophotometer (Thermo Scientific, Wilmington, DE). The hypervariable region V3-V4 of the bacterial 16S rRNA gene was amplified on the PCR thermocycler (ABI GeneAmp 9700, CA) based on the forward primer 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and the reverse primer 806R (5'-GGACTACHVGGGTWTCTAAT-3') by the following procedures: denaturation of 3 min (95°C), followed by a total of 27 cycles of denaturation of 30 s (95°C), annealing of 30 s (55°C), and extension of 45 s (72°C), and the final extension of 10 min (72°C); the reaction was kept at 4°C. The PCR (20 $\mu$L) contained the following chemical components: 4 $\mu$L 5× TransStart FastPfu buffer, 2 $\mu$L 2.5 mM dNTPs, 0.8 $\mu$L forward primer (5 $\mu$M) and reverse primer (5 $\mu$M), 0.4 $\mu$L TransStart FastPfu DNA polymerase, and 10 ng template DNA, with the final volume adjusted using $ddH_2O$. To ensure the reproducibility, each PCR was repeated with 3 biological replicates. The 2% agarose gel was used to collect the PCR products, which were then purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA) by following the manufacturer's protocols. The Quantus Fluorometer (Promega, Madison) was used to determine the concentrations of the purified PCR products, which were pooled in equimolar for the paired-end sequencing (2 × 300 bp) using an Illumina MiSeq platform (Illumina, San Diego, CA) by following the standard protocols of the Allwegene Technology Co., Ltd. (Beijing, China). Illumina Analysis Pipeline Version 2.6 was used to perform the image analysis, base calling, and error estimation of the sequencing data. The raw reads generated in this study were deposited into the Sequence Read Archive (**SRA**) database of the National Center for Biotechnology Information (**NCBI**) with an accession ID PRJNA982517.

***Sequencing Data Analysis*** The sequences shorter than 230 bp or of low quality (i.e., quality score $\leq$ 20, containing ambiguous bases, and not exactly matching the primer sequences and barcode tags) were removed from the raw sequencing data. Then, the qualified (clean) reads were separated using the sample-specific barcode sequences and clustered into the operational taxonomic units (**OTUs**) based on the similarity level of 97% (Edgar, 2013) use Uparse algorithm of Vsearch (v2.7.1). All sequences were classified into different taxonomic groups based on SILVA128 database using the Ribosomal Database Project (**RDP**) Classifier tool (Cole et al., 2009). Rarefaction analysis was performed using Mothur v.1.30.2 to calculate 3 alpha diversity indices (i.e., Chao1, Shannon, and Simpson). The variations in the species diversity among samples were also evaluated by the beta diversity analysis. The similarity between different samples was determined by both the clustering analysis and partial least squares discriminant analysis (**PLS-DA**) using R software (v3.6.0) based on the OTUs from each sample (Wang et al., 2012). The relative abundances of the microbes at the phylum and genus levels were determined using the R software (version 3.3.1).

## *Statistical Analysis*

Both the one-way analysis of variance (ANOVA) and Tukey's Honestly Significant Difference (Tukey's) multicomparison were performed using the SPSS version 26.0 (SPSS, Inc., Chicago, IL) to determine the significant differences between groups, with graphs generated using GraphPad Prism 8.3. (GraphPad, Inc.). Data were presented as the mean ± standard error of the mean (**SEM**) with the significance levels established at $P < 0.05$ (*) and $P < 0.01$ (**), respectively.

# RESULTS

## *Clinical Symptoms and Mortality in Broilers*

The observations showed that chicks challenged with APEC exhibited symptoms of lassitude, i.e., an inclination to huddle together with droopy wings and somnolescence observed. The acute cases generally did not produce clinical symptoms and died within 12 h. The anatomic examination revealed pericarditis and

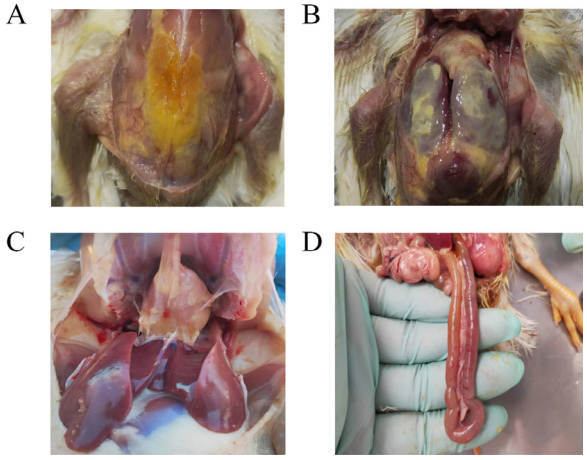


**Figure 1.** Pathological anatomy of diseased chickens after APEC challenge. (A) Yellow cheese-like substance oozing from the abdomen. (B) Greyish-white fibrous exudate and yellow caseous nutritive material covering the surface of the liver. (C) Clouding and thickening of the pericardium with purulent secretion in the pericardial cavity. (D) Bleeding of the duodenal mucosa, congestion, and hemorrhage of the bowel.

pericarditis, cloudy pericardium, greyish-white fibrous exudate covering the surface of the heart and liver, easily peeled off, yellow cheese-like material attached to the pectoral muscle, cloudy air sacs with yellow exudate, severe intestinal lesions, bleeding of the mucosa of the duodenum, jejunum, ileum, and other intestinal tissues, and congestion and bleeding of the intestinal canal (Figure 1). From days 1 to 28, the mortality rates reached 22.50%, 20.00%, and 21.25% in the PC, FP, and UFP groups, respectively. During the entire experiment, normal behaviors and no adverse reactions were observed in the chickens of the NC group.

## *Effect of Pomegranate Peel on the Growth Performance of Broilers Challenged With APEC*

The results of growth performance in the chickens revealed insignificant differences ($P > 0.05$) in the initial BWs among the 4 groups of chickens (Table 2). In week 3, the BWs were significantly lower in the PC group compared with the NC group, whereas the BWs were not significantly changed in both UFP and FP groups compared with NC group. The ADGs of chickens in both FP and UFP groups were significantly increased by 17.32% and 15.84% ($P < 0.01$) compared with the PC group, respectively, but were not significantly different from that of the NC group. The FCRs were lower in the FP and UFP groups compared to the PC group, but the difference is not significant ($P = 0.129$).

## *Effect of Pomegranate Peel on the Serum Biochemical Indices of Broilers Challenged With APEC*

The results of the effects of pomegranate peel on the serum biochemical indices of chickens were shown in Table 3. No significant differences were observed in the contents of CAT among the 4 groups of chickens ($P > 0.05$). Compared with the NC group, the contents of ALT, AST, and MDA were significantly increased in PC group ($P > 0.05$), whereas the activity of T-AOC, SOD were significantly decreased ($P < 0.01$). Compared with the PC group, the contents of ALT, AST, and MDA in the FP group were significantly reduced ($P < 0.01$), while the UFP group was revealed with significantly decreased contents of AST and MDA ($P < 0.01$).

## *Effect of Pomegranate Peel on the Intestinal Morphology of Broilers Challenged With APEC*

The results of the effects of pomegranate peel on the morphology of the small intestines of chickens were shown in Table 4. In the duodenum, the CDs in both FP and UFP groups were significantly decreased compared with the PC group ($P < 0.05$), while the villus height/crypt depth ratios in FP and UFP groups were significantly increased compared with the PC group ($P < 0.05$). In the jejunum, no significant difference was observed in VHs among the 4 groups of chickens ($P > 0.05$), while the villus height/crypt depth ratios in FP group were significantly higher than that in the PC group ($P < 0.05$). For the ileum, the VH in UFP group was significantly higher than that of the PC group ($P < 0.01$), while the CDs in the FP group were significantly decreased in comparison with the PC group ($P < 0.05$).

## *Effect of Pomegranate Peel on the Liver, Heart, and Intestinal Morphology of Broilers Challenged With APEC*

The pathological variations were observed in the histological sections of the liver, heart, and intestinal tissues of chickens in PC, FP, and UFP groups compared to the NC group (Figure 2). In the NC group, the liver cells were normally arranged and the tissue was densely stained without evident lesions, whereas the parenchyma destruction and a large number of inflammatory cells were observed in livers of chickens in the PC group (Figure 2). In the heart tissues of the PC group, the myocardial tissue was slightly abnormal in structure, i.e., the myocardial cells were loosely arranged with a few inflammatory cells infiltrating the tissue observed (Figure 2), compared to the NC group. For the duodenum, a small number of inflammatory cell infiltrations on the intestinal mucosal layer were observed in 3 groups of chicks (i.e., PC, FP, and UFP) challenged with APEC (Figure 2). In the jejunum, the mucosal epithelial cell degeneration (Figure 2) was revealed in the PC group. For the ileum, the number of goblet cells were increased in the PC group compared to the NC group (Figure 2).

**Table 2.** Growth performance in 4 groups of broilers (i.e., NC, PC, FP, and UFP).

| Item | Time | NC | PC | FP | UFP | *P*-value |
|---|---|---|---|---|---|---|
| BW (g) | Initial | 37.72 ± 0.31 | 39.16 ± 0.38 | 38.01 ± 0.73 | 37.86 ± 0.16 | 0.173 |
| | Week 1 | 104.37 ± 2.65 | 103.26 ± 2.69 | 116.45 ± 2.39 | 120.00 ± 10.71 | 0.176 |
| | Week 2 | 185.09 ± 4.48 | 187.78 ± 4.89 | 194.93 ± 6.49 | 197.98 ± 5.06 | 0.067 |
| | Week 3 | 380.85 ± 1.28$^{a}$ | 333.94 ± 2.14$^{b}$ | 380.19 ± 5.34$^{a}$ | 378.50 ± 8.45$^{a}$ | < 0.001 |
| | Week 4 | 612.63 ± 1.46$^{a}$ | 516.28 ± 16.17$^{b}$ | 591.45 ± 10.43$^{a}$ | 597.78 ± 17.42$^{a}$ | 0.003 |
| ADG (g/d) | 1 to 28 d | 21.30 ± 0.05$^{a}$ | 17.67 ± 0.61$^{b}$ | 20.73 ± 0.67$^{a}$ | 20.47 ± 0.40$^{a}$ | 0.004 |
| ADFI (g/d) | 1 to 28 d | 40.69 ± 0.21 | 37.25 ± 2.00 | 39.79 ± 1.31 | 38.01 ± 1.00 | 0.294 |
| FCR | 1 to 28 d | 1.91 ± 0.00 | 2.11 ± 0.06 | 1.92 ± 0.09 | 1.86 ± 0.08 | 0.129 |
| Mortality rate (%) | 1 to 28 d | 3.80 | 22.50 | 20.00 | 20.30 | |

Abbreviations: ADFI, average daily feed intake; ADG, average daily gain;BW, body weight; FCR, feed conversion rate; FP, group treated with fermented pomegranate peel; NC, negative control group; PC, positive control group; UFP, group treated with unfermented pomegranate peel.

Data are expressed as mean ± standard error of the mean (SEM). The superscripts a and b within the same row indicate the significant differences.

## *Effect of Pomegranate Peel on the Liver and Heart Bacterial Loads of Broilers Challenged With APEC*

The bacterial loads of the liver and heart tissues in chicks challenged with APEC were shown in Figure 3. The results indicated that the bacterial loads in the heart and liver tissues were significantly lower in the experimental groups (i.e., FP and UFP groups) than that of the PC group ($P < 0.01$), and the treatment of FP showed a higher antimicrobial effect than that of the UFP.

## *Effect of Pomegranate Peel on the Bacterial Diversity of the Intestinal Microbiota in Broilers*

To explore the effects of pomegranate peel on the taxonomic compositions of the intestinal microbiota of broilers, the V3-V4 regions of the 16S rRNA gene were sequenced based on the genomic DNA extracted from the contents of the cecum. The rarefaction curves of the samples generally tended to be flat, suggesting that the level of RNA-Seq analysis was sufficient to cover all taxa in the samples (Supplementary Figure 1). A total of 2,490,718 high-quality reads were obtained from 24 cecal contents samples (with an average of 103,779 reads per sample) (Supplementary Table 1). The alpha diversity indices were determined based on the OTUs, i.e., the observed-species (Figure 4A) presented the observed OTUs, the chao1 indices (Figure 4B) were measured to evaluate the community richness, and the Shannon's diversity indices (Figure 4C) were determined to evaluate the community diversity. Beta diversity analysis was performed to assess the differences in species complexity among different samples. The PLS-DA was used to evaluate the principal coordinates and visualize the multidimensional data (Supplementary Figure 2). The results revealed no significant difference in Shannon diversity among the 4 groups of chicks ($P > 0.05$), while both the Chao1 indices ($P < 0.05$) and observed-species ($P < 0.05$) were significantly increased in the UFP group compared to the PC group.

In order to further explore the effects of pomegranate peel on the cecal microbiota of chickens, the classification of OTUs was investigated at the phylum level (Figure 5A). Both *Bacteroides* and *Firmicutes* were identified as the top 2 bacterial phyla with the highest relative abundances in the cecal microbes of chickens (Figure 5B). The infection of APEC significantly decreased the relative abundance of *Bacteroidetes* and increased the relative abundance of *Firmicutes* compared to the NC group ($P < 0.01$). However, compared with the PC group, the relative abundances of *Bacteroidetes* and *Firmicutes* were not significantly different in either FP or UFP groups. No significant difference was observed in the relative abundances of *Proteobacteria* and *campilobacterota* in all 4 groups of chickens ($P > 0.05$).

The effects of pomegranate peel on the fecal microbiota in the 4 groups of chickens were further evaluated based on the top 20 genera with the highest relative abundances (Figure 6A). The results showed that at the genus level, the gut microbiota was relatively dominated by *Bacteroides, Faecalibacterium, Alistipes, Streptococcus, Ruminococcus_torques_group*, and *Lachnoclostridium* in

**Table 3.** Serum biochemical indices in 4 groups of broilers (i.e., NC, PC, FP, and UFP).

| Item | NC | PC | FP | UFP | *P*-value |
|---|---|---|---|---|---|
| ALT (U/L) | 1.97 ± 0.15$^{b}$ | 2.90 ± 0.22$^{a}$ | 2.02 ± 0.12$^{b}$ | 2.93 ± 0.17$^{a}$ | <0.001 |
| AST (U/L) | 197.30 ± 5.47$^{b}$ | 264.42 ± 5.20$^{a}$ | 189.67 ± 6.72$^{b}$ | 196.07 ± 2.70$^{b}$ | <0.001 |
| T-AOC (U/mL) | 6.49 ± 0.48$^{a}$ | 2.62 ± 0.55$^{b}$ | 2.47 ± 0.55$^{b}$ | 3.76 ± 0.99$^{b}$ | 0.001 |
| GSH (umol/L) | 39.83 ± 2.12$^{a}$ | 33.33 ± 2.63$^{ab}$ | 27.25 ± 1.87$^{b}$ | 27.06 ± 2.65$^{b}$ | 0.003 |
| SOD (U/mL) | 22.99 ± 0.52$^{a}$ | 20.36 ± 0.52$^{b}$ | 22.46 ± 0.76$^{ab}$ | 20.74 ± 0.39$^{b}$ | 0.008 |
| MDA (umol/mL) | 6.14 ± 0.52$^{b}$ | 11.84 ± 1.21$^{a}$ | 5.98 ± 0.29$^{b}$ | 7.52 ± 0.62$^{b}$ | <0.001 |
| CAT (U/mL) | 13.35 ± 1.71 | 14.67 ± 1.99 | 16.38 ±3.95 | 14.24 ±1.23 | 0.621 |

Abbreviations: FP, group treated with fermented pomegranate peel;NC, negative control group; PC, positive control group; UFP, group treated with unfermented pomegranate peel.

Data are expressed as mean ± standard error of the mean (SEM). The superscripts a and b within the same row indicate the significant difference.

**Table 4.** The intestinal morphology in 4 groups of broilers (NC, PC, FP, and UFP).

| Item | | NC | PC | FP | UFP | *P*-value |
|---|---|---|---|---|---|---|
| Duodenum ($\mu$m) | VH | 1,348.55 ± 12.42 | 1,219.77 ± 26.59 | 1,311.12 ± 51.57 | 1,309.43 ± 62.28 | 0.217 |
| | CD | 161.52 ± 3.27$^{b}$ | 180.07± 3.56$^{a}$ | 167.90 ± 4.81$^{b}$ | 164.47 ± 5.56$^{b}$ | 0.037 |
| | VCR | 8.37 ± 0.19$^{a}$ | 6.78 ± 0.24$^{b}$ | 7.80 ± 0.12$^{a}$ | 7.95 ± 0.14$^{a}$ | <0.001 |
| Jejunum ($\mu$m) | VH | 1,024.48 ± 43.98 | 933.97 ± 41.85 | 1,019.38 ± 23.51 | 1,008.40 ± 17.24 | 0.227 |
| | CD | 128.90 ± 5.39 | 144.58 ± 8.27 | 135.05 ± 7.04 | 137.38 ± 7.08 | 0.482 |
| | VCR | 7.96 ± 0.15$^{a}$ | 6.50 ± 0.19 $^{b}$ | 7.65 ± 0.45$^{a}$ | 7.14 ± 0.30$^{ab}$ | 0.011 |
| Ileum ($\mu$m) | VH | 810.40 ± 27.99$^{a}$ | 676.05 ± 11.14$^{c}$ | 708.92 ± 20.23$^{bc}$ | 786.52 ± 7.53$^{a}$ | <0.001 |
| | CD | 125.43 ± 5.21$^{ab}$ | 129.13 ± 3.02$^{a}$ | 111.16 ± 3.60 $^{b}$ | 116.60 ± 5.05$^{ab}$ | 0.032 |
| | VCR | 6.49 ± 0.24$^{a}$ | 5.24 ± 0.14$^{b}$ | 6.39 ± 0.19$^{a}$ | 6.81 ± 0.1$^{a}$ | 0.001 |

Abbreviations: CD, crypt depth; FP, group treated with fermented pomegranate peel; NC, negative control group; PC, positive control group; UFP, group treated with unfermented pomegranate peel; VCR, villus height/crypt depth ratio; VH, villus height.

Data are expressed as mean ± standard error of the mean (SEM). The superscripts a and b within the same row indicate the significant difference.

chickens (Figure 6B). The infection of *E. coli* caused the decreased relative abundance in *Bacteroides* of the PC group, while these changes were reversed in both UFP and FP groups, though the changes were not statistically significant ($P > 0.05$). Compared to the PC group, the relative abundance of *Ruminococcus_torques_group* in the UFP group was significantly increased ($P < 0.05$). The relative abundances of *Alistipes* in the UFP and FP groups were significantly lower than those in both NC and PC groups.

The effect size analysis and linear discriminant analysis were performed to compare the bacterial composition from phylum to species levels among the 4 groups of chicks (Figure 7). The results revealed that at the genus level, *Alistipes, Turicibacter*, and *Lachnospira* showed the highest compositions in the PC group, genera *Ruminococcus_torques_group, Ruminiclostridium, Roseburia*, and *Fusicatenibacter* were revealed with the highest compositions in the UFP group, and 3 genera (i.e., *Eubacterium, Ruminococcaceae*, and Enterococcus) presented the highest compositions in the FP group.

## DISCUSSION

The *avian pathogenic E. coli* is well known as a type of *E. coli* to cause colibacillosis, which is a common bacterial disease in poultry (Ewers et al., 2003). Compared to other poultry, chickens are particularly susceptible to this disease, and each onset causes significant losses to the local poultry industry (Antão et al., 2008). Currently, the use of antibiotics is the primary method for treatment of *E. coli* (Fancher et al., 2021). However, the

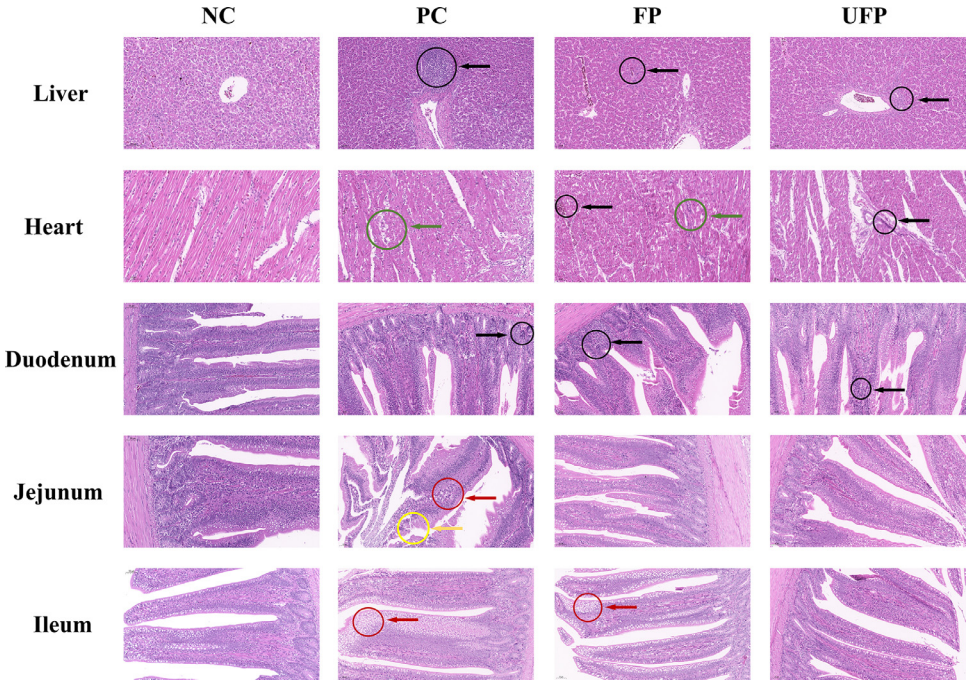


**Figure 2.** Effect of pomegranate peel on the liver, heart, and intestinal morphology of chickens challenged with APEC based on hematoxylin and eosin (HE) staining observed under 20× magnifications. NC, negative control group; PC, positive control group; FP, group treated with fermented pomegranate peel; UFP, group treated with unfermented pomegranate peel. Black arrows and circles indicate the inflammatory cell infiltration; green arrows and circles present the degeneration and detachment of the epithelial cells in the mucosal layer; red arrows and circles indicate marked increase in the number of cupped cells; yellow arrow and circle indicate the abnormal myocardial tissue structure and disorganization.

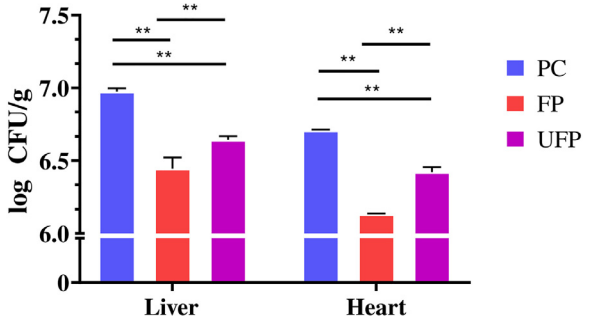


**Figure 3.** Effect of pomegranate peel on the bacterial loads of liver and heart tissues in chickens. Abbreviations: PC, positive control group; FP, group treated with fermented pomegranate peel; UFP, group treated with unfermented pomegranate peel. Symbol "**" indicates the significant difference based on $P < 0.01$.

emergence of issues such as drug resistance and the extension of drug rest periods have led to an increased interest in the treatment of *E. coli* using the herbal feed additives (Wang et al., 2010; Azam et al., 2019). These additives have shown several advantages in promoting growth, regulating the intestinal tract, and preventing and treating diseases (Abdallah et al., 2019). Studies have shown that extracts of pomegranate peel are effective inhibitors of several bacterial taxa such as *Listeria monocytogenes, Staphylococcus aureus*, and *E. coli* (Hanafy et al., 2021). Therefore, pomegranate peel is used in traditional herbal medicine and as an intestinal astringent to relieve diarrhea and enteritis (Alzoreky and Nakahara, 2003; Voravuthikunchai et al., 2005; Al-Zoreky, 2009). Our study investigated the potential effects of pomegranate peel, which is rich in tannins, in the forms of either fermented or unfermented, on the enhancement of growth performance, improvement of immunity and antioxidant capacity, and decrease of the prevalence of *E. coli* diseases in broilers. The study also explored the potential advantages of using pomegranate peel in the poultry industry.

Studies have shown that colibacillosis is frequently associated with diseases in older broilers (>2 wk old) (Kemmett et al., 2014). In this study, the 14-day-old broilers were orally (by gavage) administrated with APEC, which most closely resembled the natural infection state by *E. coli*. All the chickens in each group were healthy before the APEC infection, whereas the mortality rate after the infection with APEC was increased to 22.5%. The clinical symptoms of chicken death after infection were consistent with those previously reported by the study of Forgetta et al. (2012), suggesting that the chicken model of pullorosis was successfully established in our study (Figure 1). It was worth noting that although the mortality rate was not significantly changed by the treatment of pomegranate peel, the tissues in liver, heart, and small intestine (duodenum, jejunum, and ileum) of the chickens in the FP and UFP groups were dense and intact, showing improved morphological features compared to the PC group (Figure 2). These results were consistent with those previously

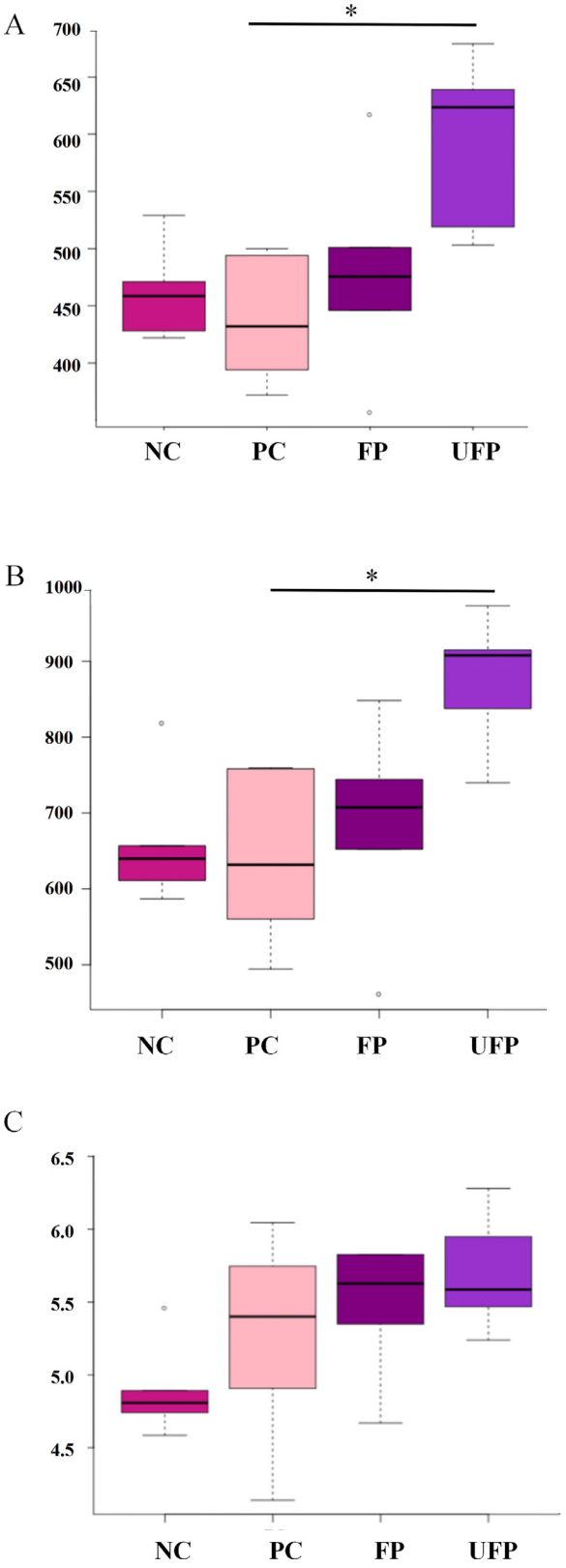


**Figure 4.** Effects of pomegranate peel on the intestinal microbiota diversity in broilers based on alpha diversity indices of observed species (A), Chao index (B), and Shannon index (C). Symbol "*" indicates significant difference based on $P < 0.05$. Abbreviations: NC, negative control group; PC, positive control group; FP, group treated with fermented pomegranate peel; UFP, treated with unfermented pomegranate peel.

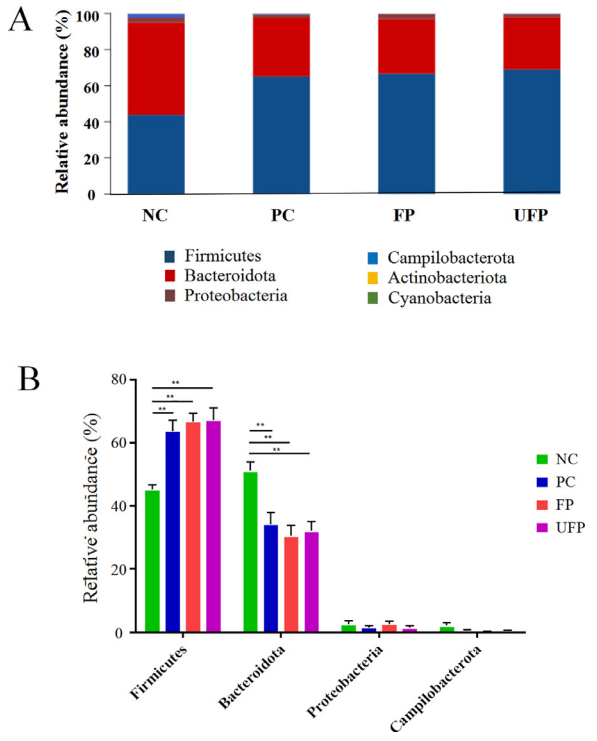


**Figure 5.** Effects of pomegranate peel on the compositions of intestinal microbiota in chickens characterized by the taxonomic distributions of the microbial communities in fecal samples at phylum level (A) and the top 4 relatively abundant bacterial phyla (B). Symbol "**" indicates the significant difference at $P < 0.01$. Abbreviations: NC, negative control group; PC, positive control group; FP, group treated with fermented pomegranate peel; UFP, group treated with unfermented pomegranate peel.

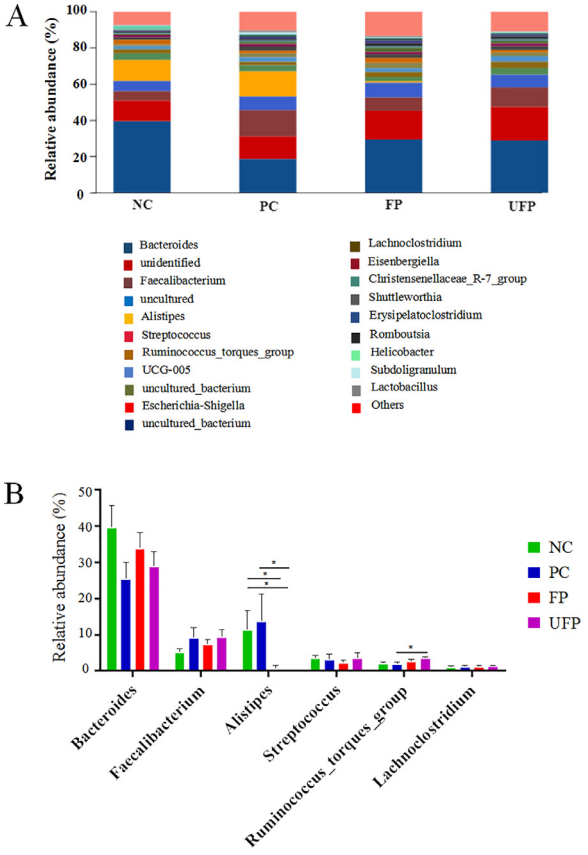


**Figure 6.** Comparison of the gut microbiota community at the genus level in 4 groups of chickens showing the relative abundances of the top 20 (A) and the top 6 (B) bacterial taxa in the microbiota of the chickens with the statistical significance. Values are represented as the mean ± stand error of the mean (SEM) (n = 6 chickens per group). The significant difference is determined based on $P < 0.05$ (*). Abbreviations: NC, negative control group; PC, positive control group; FP, group treated with fermented pomegranate peel; UFP, group treated with unfermented pomegranate peel.

reported, i.e., male broilers of 21-day-old challenged with APEC and subsequently administered orally with extracts of *Punica granatum* were revealed with decreased morbidity and inflammation induced by APEC (Zhong et al., 2014). These findings suggested that feeding pomegranate peel could effectively prevent clinical symptoms of *E. coli* infection in chickens and promote growth by reducing intestinal lesions. Furthermore, our results showed that challenging chickens with *E. coli* O78 ($5 \times 10^8$ CFU/chicken) resulted in the average APEC loads of 6.97 log (CFU/g) in the liver and 6.71 log (CFU/g) in the heart (Figure 3), whereas the groups fed with either unfermented or fermented pomegranate peels showed significant reduction in bacterial burdens in the liver and heart tissues. Previous studies showed that a 50% concentration of tannin extract (from pomegranate peel) was revealed with a maximum circle of inhibition against *E. coli* ranging from 12 ± 0.5 to 30.3 ± 0.2, indicating that tannin is an effective natural substance against *E. coli* infection (Hamdi Abdulkareem et al., 2022). Furthermore, the pomegranate peel polyphenols, a group of secondary metabolites extracted from pomegranate peel, were introduced and embedded into chitosan to form stable nanoparticles, which could inhibit *E. coli* O157:H7, at high inhibition rates > 95% (Cai et al., 2021). Therefore, it could be concluded that pomegranate peel could provide a protective effect on broilers which were orally challenged with APEC.

## *Effects of Pomegranate Peel on the Growth Performance in Broilers Challenged With APEC*

Studies showed that the growth performance of broilers could be negatively impacted by *E. coli* infection, leading to symptoms such as depression, poor appetite, and decreased feed intake (Kabir, 2010; da Rosa et al., 2020). It is well known that pomegranate peel (either extract or powder) could improve body weight, feed intake, feed efficiency, carcase, and organ parameters in broilers (Kishawy et al., 2019; Sharifian et al., 2019; Abdel Baset et al., 2020). Our study found that broilers challenged with APEC infection experienced a decrease in ADG and ADFI, and an increase in FCR (Table 2). However, compared to the PC group, the FP and UFP groups showed higher growth performance prior to challenge, though the difference was not significant (Table 2). These results suggested that pomegranate peel could effectively help broilers quickly recover from the *E. coli* infection. This was probably because that either FP or UFP could inhibit the malignant effects of chronic colibacillosis in broiler chickens to prevent the possibility of becoming "zombie chickens" due to the

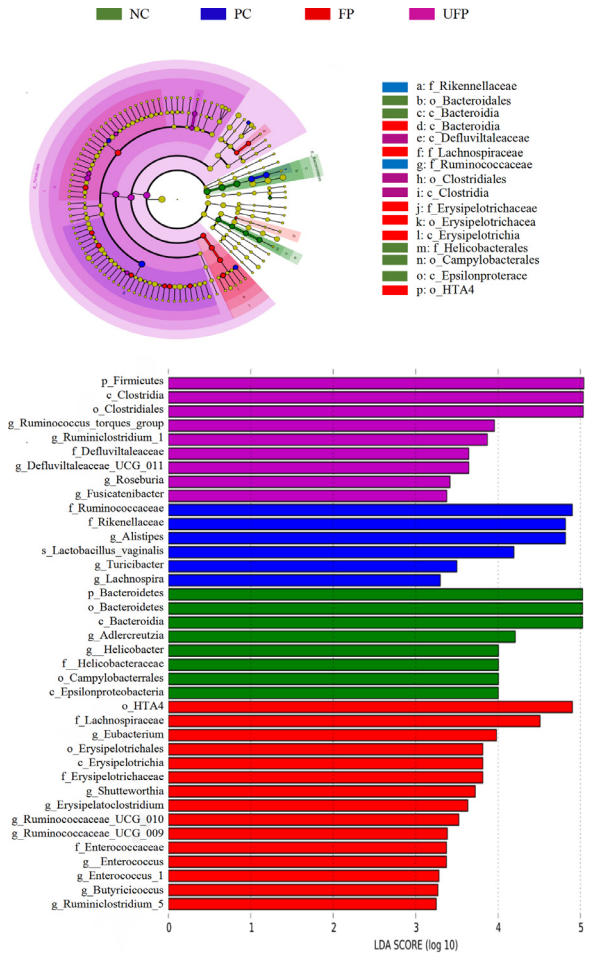


**Figure 7.** Bacterial taxa with significant difference in their relative abundances between different groups of broilers identified by LefSe analysis using default parameters. (A) Cladogram. (B) Histograms. NC, negative control group; PC, positive control group; FP, group treated with fermented pomegranate peel; UFP, group treated with unfermented pomegranate peel. The taxonomic ranks of phylum, class, order, family, genus, and species of microbial taxa are abbreviated as lowercase letters p, c, o, f, g, and s, respectively, given at the beginning of the name of each taxon.

disease. Previous studies have shown the beneficial effects of polyphenols and probiotics in combating inflammatory diseases. For example, addition of probiotics improved growth performance in broilers infected with APCE O78 (Tarabees, et al., 2019), and pomegranate polyphenol extract was effective in reducing the inflammatory response following attack by APEC strains (Zhong, et al., 2014). These results were consistent with our findings. These studies suggested that the naturally occurring polyphenols in pomegranate peel could be a potential alternative medicine for the prevention or treatment of avian *E. coli* disease.

## *Effects of Pomegranate Peel on Serum Biochemical Indices in Broilers Challenged With APEC*

Serum biochemical factors are generally considered accurate indicators of the nutritional, physiological, and pathological status of the broilers (Saeed et al., 2018). Serum AST and ALT are 2 important intracellular enzymes commonly used to assess the hepatocyte damage (Zhang et al., 2022). For example, studies showed that acrylamide significantly increased the serum levels of AST and ALT in rats, while rats co-treated with both pomegranate peel extract and acrylamide showed significant normalization of AST and ALT levels (Sayed et al., 2022). Moreover, studies showed that the concentration of serum AST was decreased when 4% pomegranate peel was added in the diet (Ghasemi-Sadabadi et al., 2021). These results were consistent with the findings revealed in our study, showing that compared to the NC group, the levels of ALT and AST were significantly increased in the serum of APEC-infected broilers, indicating that *E. coli* caused liver damage. However, AST levels were significantly lower in the UFP and FP groups and ALT levels were significantly lower in the FP group compared to the PC group (Table 3), suggesting that pomegranate peels could attenuate the liver injury caused by APEC and were more effective after fermentation, which could be attributed to the introduction of probiotics during the fermentation process. As indicated in previous studies, the addition of probiotics inhibited the colonization of harmful bacteria and increased the resistance of chicks to pathogens (Wang, et al., 2021). The T-AOC antioxidant index generally reflects the antioxidant capacity of the active substances in the organism. Studies have shown that the hepatic antioxidant enzymatic activities of T-AOC were markedly increased when the mice were administrated with high dosage of pomegranate peel polysaccharides (Wu et al., 2019). The high antioxidant activity of pomegranate peel is due to the presence of a variety of phenolic components, which directly remove the free radical species and improve the defense systems of cells, thereby activating antioxidant enzymes in the body (Chidambara Murthy et al., 2002). This inconsistent finding in our research on the T-AOC antioxidant index of broilers was probably due to the body produced a large amount of reactive oxygen species (**ROS**) during the infection process (Gao, et al., 2019), which increased the difficulty in against ROS, and thus the T-AOC values in the FP and UFP groups were not improved compared with those in the PC group (Table 3). MDA is an indicator of lipid peroxidation and oxidative damage caused by ROS, indirectly reflecting the extent of cellular damage (Zuo et al., 2014). Our results showed that the serum MDA level of broilers was significantly increased by the APEC infection (Table 3), while the MDA levels in the groups treated with pomegranate peel were similar to those in the group without APEC infection, indicating that feeding pomegranate peel decreased the organism damage and facilitated the recovery from APEC infection. Similarly, studies have shown that the concentration of MDA was decreased by the treatment of 1% pomegranate peel powder ($P < 0.05$) (Ahmadipour et al., 2021).

### Effects of Pomegranate Peel on Intestinal Morphology in Broilers Challenged With APEC

The small intestine is the main organ for digestion and absorption, which are vital for the growth of broiler chickens (Weurding et al., 2001). The intestinal villous epithelial cells are constantly replaced, with the crypt constantly producing epithelial cells, which migrate and differentiate towards the tip of the intestinal villi to replenish the physiological loss of intestinal villous epithelial cells and maintain a dynamic balance (de Santa Barbara et al., 2003; Wang et al., 2020). The VCR is an important parameter to assess the absorption capacity of the small intestine, i.e., the increase in VCR indicates a rapid renewal rate of intestinal epithelial cells, high intestinal digestion and absorption, and high feed utilization (Yaqoob et al., 2022). In our study, broilers challenged with APEC showed a significantly lower VCR than that of the NC group, whereas the addition of pomegranate peels to the diet resulted in the restored VCR close to normal levels (Table 4). It was reported that the use of 8% pomegranate peel powder in the diet showed a significant effect on villus height, crypt depth, and villus/crypt ratio of broilers ($P < 0.05$) (Ghasemi-Sadabadi et al., 2022). Furthermore, it has been shown that the jejuna of mice infected with *Eimeria papillata* are characterized by inflammation, which is alleviated by the treatment with pomegranate (Amer et al., 2015). These results were consistent with our findings, showing that the groups fed with either fermented or unfermented pomegranate peels were less affected by the APEC infection, recovered quickly, and showed high maintenance of intestinal morphology, which was probably the cause of unaffected BW gain.

### Effects of Pomegranate Peel on Microbial Diversity of the Intestinal Microbiota in Broilers Challenged with APEC

The microbiome of the broiler gastrointestinal tract (**GIT**) has been extensively studied and well documented to play an important role in the health of the host (Clavijo and Flórez, 2018; Stamilla et al., 2021; Zaytsoff et al., 2022). In our study, the broiler intestinal microbiota was analyzed using 16S rRNA sequencing technology to investigate the effect of pomegranate peel on the taxonomic compositions of the broiler intestinal microbial community under the APEC challenge. The results revealed no significant difference in the alpha diversities between the PC and NC groups (Figure 4), suggesting that species richness and diversity of bacterial communities were not significantly affected by the APEC challenge. However, the addition of unfermented pomegranate increased the alpha diversity indices (i.e., observed-species and chao1) of the microbial community of broilers challenged by APEC. Studies have reported that the pomegranate by-products enhance the growth of total bacteria of both bifidobacteria and lactobacilli (Bialonska et al., 2010). Pomegranate peel is a rich source of phenolics, including tannins and flavonoids (Fahmy and Farag, 2021). The interaction between tannins and gut bacteria is a complex process that is influenced by the abundances and types of bacterial taxa as well as the numbers and types of phenolics consumed by the host (Russo et al., 2018; Andishmand et al., 2023). Furthermore, the gut bacteria are capable of metabolizing polyphenols, while a metabolite released by a bacterium could affect the growth of the bacteria producing the metabolite as well as the adjacent microbiota (Bialonska et al., 2009). Our results showed that *Bacteroidota* and *Firmicutes* were the most important representatives of intestinal bacteria. These results were consistent with those previously reported (Chica Cardenas et al., 2021). Moreover, our study revealed a change in the taxonomic structure of the intestinal microbiota of *E. coli* infected broilers, i.e., decreased proportion of *Bacteroides* and an increased relative abundance of *Firmicutes* (Figure 5). Studies showed that *E. coli* infection could cause an imbalance between *Bacteroidetes* and *Firmicutes*, and the B/F ratio could be used as an indicator to evaluate the imbalance of intestinal microbiota caused by various diseases (Hold et al., 2014; Liu et al., 2020). The genus *Alistipes* is recently established with species mostly isolated from patients suffering from certain intestine-related conditions (Parker et al., 2020). Although it is unclear whether this taxon plays a dominant role in the observed clinical phenotype, it is certain that the relative abundance of *Alistipes* is strongly associated with intestinal dysbiosis (Cai et al., 2020). In the present study, the relative abundance of *Alistipes* was significantly decreased by the pomegranate peel added to the diet compared with the control group (Figure 6). It was reported that supplementation with 5% chitin-glucan and 0.5% pomegranate peel extracts could reduce the relative abundance of *Alistipes* in cecal contents of mice fed with a high-fat diet (Neyrinck et al., 2019). Furthermore, some producers of short-chain fatty acids in *Ruminococcaceae* are involved in the maintenance of intestinal health (Biddle et al., 2013). Previous reports have shown that pomegranate peel polyphenols could significantly elevate the relative proportion of *Ruminococcaceae* in rats (Shi et al., 2022). Similar results were revealed in our study, showing that the relative abundance of *Ruminococcus_torques_group* in the UFP group was significantly increased compared with the PC group (Figure 6). Studies have revealed significant increases in acetate, propionate, and butyrate production due to tannin supplementation (Molino et al., 2022). Notably, the tannin treatment consistently and strongly increased the levels of unclassified members of *Ruminococcaceae* and other genera of the same bacterial family (Díaz Carrasco et al., 2018). Finally, the tannin supplements could induce the beneficial changes in the gut microbiota (Molino et al., 2021), while the pomegranate rind is rich in tannins, suggesting that pomegranate rind improves the disease resistance in broilers by altering the bacterial structure of the gut microbiota.

Our study revealed that the addition of pomegranate peel to the diet showed a protective effect on chickens

challenged by APEC, maintaining growth performance in broilers. Furthermore, the supplementation with pomegranate peel altered the gut microbiota of broiler chickens, mainly by increasing the relative abundances of beneficial bacteria, such as *Ruminococcus_torques_group*. More importantly, these beneficial bacteria helped maintain the gut microbiota in a host-friendly manner, effectively decreasing the abnormal changes in the taxonomic structure of gut microbiota in broilers with APEC infection and creating favorable microbial communities for subsequent restoration of balance in the gut microbiota. Because the use of antibiotics in the poultry industry is restricted, pomegranate peel has become an effective feed additive due to its high availability (as a by-product of pomegranate), cost-effective, and high antibacterial properties. Our study provides strong experimental evidence to support the application of pomegranate peel as feed additives in poultry breeding.

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## DISCLOSURES

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.psj.2023.103304.

## REFERENCES

Abdallah, A., P. Zhang, Q. Z. Zhong, and Z. W. Sun. 2019. Application of traditional Chinese herbal medicine by-products as dietary feed supplements and antibiotic replacements in animal production. Curr. Drug Metab. 20:54–64.

Abdel Baset, S., E. A. Ashour, M. E. Abd El-Hack, and M. M. El-Mekkawy. 2020. Effect of different levels of pomegranate peel powder and probiotic supplementation on growth, carcass traits, blood serum metabolites, antioxidant status and meat quality of broilers. Anim. Biotechnol. 33:690–700.

Ahmadipour, B., S. Pat, S. Abaszadeh, H. Hassanpour, and F. Khajali. 2021. Pomegranate peel as a phytogenic in broiler chickens: Influence upon antioxidant, lipogenesis and hypotensive response. Vet. Med. Sci. 7:1907–1913.

Akhtar, S., T. Ismail, D. Fraternale, and P. Sestili. 2015. Pomegranate peel and peel extracts: chemistry and food features. Food Chem. 174:417–425.

Al-Zoreky, N. S. 2009. Antimicrobial activity of pomegranate (*Punica granatum* L.) fruit peels. Int. J. Food Microbiol. 134:244–248.

Alzoreky, N. S., and K. Nakahara. 2003. Antibacterial activity of extracts from some edible plants commonly consumed in Asia. Int. J. Food Microbiol. 80:223–230.

Amer, O. S. O., M. A. Dkhil, W. M. Hikal, and S. Al-Quraishy. 2015. Antioxidant and anti-inflammatory activities of pomegranate (*Punica granatum*) on Eimeria papillata-induced infection in mice. Biomed. Res. Int. 2015:219670.

Andishmand, H., S. Azadmard-damirchi, H. Hamishekar, M. Torbati, M. S. Kharazmi, G. P. Savage, C. Tan, and S. M. Jafari. 2023. Nano-delivery systems for encapsulation of phenolic compounds from pomegranate peel. Adv. Colloid Interface Sci. 311:102833.

Antão, E.-M., S. Glodde, G. Li, R. Sharifi, T. Homeier, C. Laturnus, I. Diehl, A. Bethe, H.-C. Philipp, R. Preisinger, L. H. Wieler, and C. Ewers. 2008. The chicken as a natural model for extraintestinal infections caused by avian pathogenic *Escherichia coli* (APEC). Microb. Pathog. 45:361–369.

Azam, M., M. Mohsin, R. Sajjad ur, and M. K. Saleemi. 2019. Virulence-associated genes and antimicrobial resistance among avian pathogenic *Escherichia coli* from colibacillosis affected broilers in Pakistan. Trop. Anim. Health Prod. 51:1259–1265.

Bialonska, D., S. G. Kasimsetty, K. K. Schrader, and D. Ferreira. 2009. The effect of pomegranate (*Punica granatum* L.) byproducts and ellagitannins on the growth of human gut bacteria. J. Agric. Food Chem. 57:8344–8349.

Bialonska, D., P. Ramnani, S. G. Kasimsetty, K. R. Muntha, G. R. Gibson, and D. Ferreira. 2010. The influence of pomegranate by-product and punicalagins on selected groups of human intestinal microbiota. Int. J. Food Microbiol. 140:175–182.

Biddle, A., L. Stewart, J. Blanchard, and S. Leschine. 2013. Untangling the genetic basis of Fibrolytic specialization by Lachnospiraceae and Ruminococcaceae in diverse gut communities. Diversity 5:627–640.

Cai, M., G. Zhang, C. Li, X. Chen, H. Cui, and L. Lin. 2021. *Pleurotus eryngii* polysaccharide nanofiber containing pomegranate peel polyphenol/chitosan nanoparticles for control of *E. coli* O157:H7. Int. J. Biol. Macromol. 192:939–949.

Cai, T. T., X. L. Ye, R. R. Li, H. Chen, Y. Y. Wang, H. J. Yong, M. L. Pan, W. Lu, Y. Tang, H. Miao, A. M. Snijders, J. H. Mao, X. Y. Liu, Y. B. Lu, and D. F. Ding. 2020. Resveratrol modulates the gut microbiota and inflammation to protect against diabetic nephropathy in mice. Front. Pharmacol. 11:1249.

Cao, F. L., X. H. Zhang, W. W. Yu, L. G. Zhao, and T. Wang. 2012. Effect of feeding fermented Ginkgo biloba leaves on growth performance, meat quality, and lipid metabolism in broilers. Poult. Sci. 91:1210–1221.

Chica Cardenas, L. A., V. Clavijo, M. Vives, and A. Reyes. 2021. Bacterial meta-analysis of chicken cecal microbiota. Peer J. 9: e10571.

Chidambara Murthy, K. N., G. K. Jayaprakasha, and R. P. Singh. 2002. Studies on antioxidant activity of pomegranate (*Punica granatum*) peel extract using in vivo models. J. Agric. Food Chem. 50:4791–4795.

Clavijo, V., and M. J. V. Flórez. 2018. The gastrointestinal microbiome and its association with the control of pathogens in broiler chicken production: a review. Poult. Sci. 97:1006–1021.

Cole, J. R., Q. Wang, E. Cardenas, J. Fish, B. Chai, R. J. Farris, A. S. Kulam-Syed-Mohideen, D. M. McGarrell, T. Marsh, G. M. Garrity, and J. M. Tiedje. 2009. The ribosomal database project: improved alignments and new tools for rRNA analysis. Nucleic Acids Res. 37:D141–D145.

da Rosa, G., D. F. Alba, A. D. Silva, A. Gris, R. E. Mendes, V. B. Mostardeiro, T. F. Lopes, M. R. C. Schetinger, L. M. Stefani, M. T. Lopes, M. M. Boiago, and A. S. da Silva. 2020. Impact of *Escherichia coli* infection in broiler breeder chicks: the effect of oxidative stress on weight gain. Microb. Pathog. 139:103861.

de Santa Barbara, P., G. R. van den Brink, and D. J. Roberts. 2003. Development and differentiation of the intestinal epithelium. Cell. Mol. Life Sci. 60:1322–1332.

Dho-Moulin, M., and J. M. Fairbrother. 1999. Avian pathogenic *Escherichia coli* (APEC). Vet. Res. 30:299–316.

Dias da Silveira, W., A. Ferreira, M. Brocchi, L. Maria de Hollanda, A. F. Pestana de Castro, A. Tatsumi Yamada, and M. Lancellotti. 2002. Biological characteristics and pathogenicity of avian *Escherichia coli* strains. Vet. Microbiol. 85:47–53.

Díaz Carrasco, J. M., E. A. Redondo, N. D. Pin Viso, L. M. Redondo, M. D. Farber, and M. E. Fernández Miyakawa. 2018. Tannins and bacitracin differentially modulate gut microbiota of broiler chickens. Biomed. Res. Int. 2018:1879168.

Edgar, R. C. 2013. UPARSE: highly accurate OTU sequences from microbial amplicon reads. Nat. Methods 10:996–998.

El-Hadary, A. E., and M. F. Ramadan. 2019. Phenolic profiles, antihyperglycemic, antihyperlipidemic, and antioxidant properties of pomegranate (*Punica granatum*) peel extract. J. Food Biochem. 43:e12803.

Endo, E. H., D. A. G. Cortez, T. Ueda-Nakamura, C. V. Nakamura, and B. P. Dias. 2010. Potent antifungal activity of extracts and pure compound isolated from pomegranate peels and synergism with fluconazole against *Candida albicans*. Res. Microbiol. 161:534–540.

Ewers, C., T. Janssen, and L. H. Wieler. 2003. Avian pathogenic *Escherichia coli* (APEC). Berl. Munch Tierarztl Wochenschr. 116:381–395.

Fahmy, H. A., and M. A. Farag. 2021. Ongoing and potential novel trends of pomegranate fruit peel; a comprehensive review of its health benefits and future perspectives as nutraceutical. J. Food Biochem. 46:e14024.

Fancher, C. A., H. T. Thames, M. G. Colvin, M. Smith, A. Easterling, N. Nuthalapati, L. Zhang, A. Kiess, T. T. N. Dinh, A. Theradiyil Sukumaran, and S. L. Chen. 2021. Prevalence and molecular characteristics of avian pathogenic *Escherichia coli* in "no antibiotics ever" broiler farms. Microbiol. Spectr. 9:e0083421.

Fawole, O. A., N. P. Makunga, and U. L. Opara. 2012. Antibacterial, antioxidant and tyrosinase-inhibition activities of pomegranate fruit peel methanolic extract. BMC Complement Altern. Med. 12:200.

Forgetta, V., H. Rempel, F. Malouin, R. Vaillancourt Jr., E. Topp, K. Dewar, and M. S. Diarra. 2012. Pathogenic and multidrug-resistant *Escherichia fergusonii* from broiler chicken. Poult. Sci. 91:512–525.

Gao, Q. Q., L. Xia, X. B. Wang, Z. Q. Ye, J. B. Liu, and S. Gao. 2019. SodA contributes to the virulence of avian pathogenic $O_2$ Strain E058 in experimentally infected chickens. J. Bacteriol. 201 e00625-18.

Ghasemi-Sadabadi, M., Y. Ebrahimnezhad, N. Maheri-Sis, J. G. Ghalehkandi, and A. Shaddel-Teli. 2021. Immune response and antioxidant status of broilers as influenced by oxidized vegetable oil and pomegranate peel. J. Anim. Sci. Technol. 63:1034–1063.

Ghasemi-Sadabadi, M., Y. Ebrahimnezhad, N. Maheri-Sis, A. Shaddel-Teli, J. G. Ghalehkandi, and T. Veldkamp. 2022. Effects of supplementation of pomegranate processing by-products and waste cooking oils as alternative feed resources in broiler nutrition. Sci. Rep. 12:21216.

Hamdi Abdulkareem, M., I. Abbas Abood, and M. Munis Dakheel. 2022. Antimicrobial resistance of tannin extract against *E. coli* isolates from sheep. Arch. Razi. Inst. 77:697–701.

Hanafy, S. M., Y. M. Abd El-Shafea, W. D. Saleh, and H. M. Fathy. 2021. Chemical profiling, in vitro antimicrobial and antioxidant activities of pomegranate, orange and banana peel-extracts against pathogenic microorganisms. J. Genet. Eng. Biotechnol. 19:80.

Hold, G. L., Q. Zhu, Z. Jin, W. Wu, R. Gao, B. Guo, Z. Gao, Y. Yang, and H. Qin. 2014. Analysis of the intestinal lumen microbiota in an animal model of colorectal cancer. PLoS One 9:e90849.

Kabir, S. M. L. 2010. Avian colibacillosis and salmonellosis: a closer look at epidemiology, pathogenesis, diagnosis, control and public health concerns. Int. J. Environ. Res. Public Health 7:89–114.

Kaper, J. B., J. P. Nataro, and H. L. T. Mobley. 2004. Pathogenic *Escherichia coli*. Nat. Rev. Microbiol. 2:123–140.

Kemmett, K., T. Humphrey, S. Rushton, A. Close, and P. Wigley. 2013. A Longitudinal Study Simultaneously Exploring the Carriage of APEC Virulence Associated Genes and the Molecular Epidemiology of Faecal and Systemic in Commercial Broiler Chickens. Plos One. 8:e67749.

Kemmett, K., N. J. Williams, G. Chaloner, S. Humphrey, P. Wigley, and T. Humphrey. 2014. The contribution of systemic *Escherichia coli* infection to the early mortalities of commercial broiler chickens. Avian Pathol. 43:37–42.

Khan, R. U., S. Naz, F. Raziq, Q. Qudratullah, N. A. Khan, V. Laudadio, V. Tufarelli, and M. Ragni. 2022. Prospects of organic acids as safe alternative to antibiotics in broiler chickens diet. Environ. Sci. Pollut. Res. Int. 29:32594–32604.

Kishawy, A. T. Y., S. A. Amer, M. E. Abd El-Hack, I. M. Saadeldin, and A. A. Swelum. 2019. The impact of dietary linseed oil and pomegranate peel extract on broiler growth, carcass traits, serum lipid profile, and meat fatty acid, phenol, and flavonoid contents. Asian-Australas J. Anim. Sci. 32:1161–1171.

Kupnik, K., M. Primožič, K. Vasić, Ž. Knez, and M. Leitgeb. 2021. A comprehensive study of the antibacterial activity of bioactive juice and extracts from pomegranate (*Punica granatum* L.). Plants (Basel) 10:1554.

Lee, C. J., L. G. Chen, W. L. Liang, and C. C. Wang. 2010. Anti-inflammatory effects of *Punica granatum* Linne in vitro and in vivo. Food Chem. 118:315–322.

Liu, X., S. He, Q. Li, X. Mu, G. Hu, and H. Dong. 2020. Comparison of the gut microbiota between pulsatilla decoction and levofloxacin hydrochloride therapy on *Escherichia coli* infection. Front. Cell Infect. Microbiol. 10:319.

Mehat, J. W., A. H. M. van Vliet, and R. M. La Ragione. 2021. The avian pathogenic *Escherichia coli* (APEC) pathotype is comprised of multiple distinct, independent genotypes. Avian Pathol. 50:402–416.

Molino, S., A. Lerma-Aguilera, N. Jiménez-Hernández, M. J. Gosalbes, J.Á. Rufián-Henares, and M. P. Francino. 2021. Enrichment of food with tannin extracts promotes healthy changes in the human gut microbiota. Front. Microbiol. 12:625782.

Molino, S., A. Lerma-Aguilera, N. Jiménez-Hernández, J. Á. Rufián Henares, and M. P. Francino. 2022. Evaluation of the effects of a short supplementation with tannins on the gut microbiota of healthy subjects. Front. Microbiol. 13:848611.

Newman, D. M., N. L. Barbieri, A. L. de Oliveira, D. Willis, L. K. Nolan, and C. M. Logue. 2021. Characterizing avian pathogenic *Escherichia coli* (APEC) from colibacillosis cases, 2018. Peer J. 9:e11025.

Neyrinck, A. M., E. Catry, B. Taminiau, P. D. Cani, L. B. Bindels, G. Daube, C. Dessy, and N. M. Delzenne. 2019. Chitin−glucan and pomegranate polyphenols improve endothelial dysfunction. Sci. Rep. 9:14150.

Nhung, N. T., N. Chansiripornchai, and J. J. Carrique-Mas. 2017. Antimicrobial resistance in bacterial poultry pathogens: a review. Front. Vet. Sci. 4:126.

Parker, B. J., P. A. Wearsch, A. C. M. Veloo, and A. Rodriguez-Palacios. 2020. The genus *Alistipes*: gut bacteria with emerging implications to inflammation, cancer, and mental health. Front. Immunol. 11:906.

Poyrazoglu, E., V. Gokmen, and N. Artik. 2002. Organic acids and phenolic compounds in pomegranates (*Punica granatum* L.) grown in Turkey. J. Food Compost. Anal. 15:567–575.

Roth, N., C. Hofacre, U. Zitz, G. F. Mathis, K. Moder, B. Doupovec, R. Berghouse, and K. J. Domig. 2019. Prevalence of antibiotic-resistant *E. coli* in broilers challenged with a multi-resistant E. coli strain and received ampicillin, an organic acid-based feed additive or a synbiotic preparation. Poult. Sci. 98:2598–2607.

Russo, M., C. Fanali, G. Tripodo, P. Dugo, R. Muleo, L. Dugo, L. De Gara, and L. Mondello. 2018. Analysis of phenolic compounds in different parts of pomegranate (*Punica granatum*) fruit by HPLC-PDA-ESI/MS and evaluation of their antioxidant activity: application to different Italian varieties. Anal. Bioanal. Chem. 410:3507–3520.

Saeed, M., X. Yatao, F.-u. Hassan, M. Arain, M. Abd El-Hack, A. Noreldin, and C. Sun. 2018. Influence of graded levels of l-theanine dietary supplementation on growth performance, carcass traits, meat quality, organs histomorphometry, blood chemistry and immune response of broiler chickens. Int. J. Mol. Sci. 19:462.

Sayed, S., S. S. Alotaibi, A. M. El-Shehawi, M. M. Hassan, M. Shukry, M. Alkafafy, and M. M. Soliman. 2022. The anti-inflammatory, anti-apoptotic, and antioxidant effects of a pomegranate-peel extract against acrylamide-induced hepatotoxicity in rats. Life (Basel) 12:224.

Sharifian, M., S. J. Hosseini-Vashan, M. H. F. Nasri, and A. H. Perai. 2019. Pomegranate peel extract for broiler chickens under heat stress: Its influence on growth performance, carcass traits, blood metabolites, immunity, jejunal morphology, and meat quality. Livest. Sci. 227:22–28.

Shi, H., J. Yang, and J. Li. 2022. Pomegranate peel polyphenols interaction with intestinal flora and its metabolic transformation. Xenobiotica 52:442–452.

Stamilla, A., S. Ruiz-Ruiz, A. Artacho, J. Pons, A. Messina, C. Lucia Randazzo, C. Caggia, M. Lanza, and A. Moya. 2021. Analysis of the microbial intestinal tract in broiler chickens during the rearing period. Biology (Basel) 10:942.

Sugiharto, S., and S. Ranjitkar. 2019. Recent advances in fermented feeds towards improved broiler chicken performance, gastrointestinal tract microecology and immune responses: a review. Anim. Nutr. 5:1–10.

Tarabees, R., M. S. El-Sayed, A. A. Shehata, and M. S. Diab. 2019. Effects of the probiotic candidate *E. faecalis*-1, the Poulvac *E. coli* vaccine, and their combination on growth performance, caecal microbial composition, immune response, and protection against *E. coli* O78 challenge in broiler chickens. Probiotics Antimicrob. Proteins 12:860–872.

Verotta, L., L. Panzella, S. Antenucci, V. Calvenzani, F. Tomay, K. Petroni, E. Caneva, and A. Napolitano. 2018. Fermented pomegranate wastes as sustainable source of ellagic acid: antioxidant properties, anti-inflammatory action, and controlled release under simulated digestion conditions. Food Chem. 246:129–136.

Voravuthikunchai, S. P., T. Sririrak, S. Limsuwan, T. Supawita, T. Iida, and T. Honda. 2005. Inhibitory effects of active compounds from *Punica granatum* pericarp on verocytotoxin production by enterohemorrhagic *Escherichia coli* O157 : H7. J. Health Sci. 51:590–596.

Wang, M., C. Yang, Q. Y. Wang, J. Z. Li, Y. L. Li, X. Q. Ding, J. Yin, H. S. Yang, and Y. L. Yin. 2020. The growth performance, intestinal digestive and absorptive capabilities in piglets with different lengths of small intestines. Animal 14:1196–1203.

Wang, X.-M., X.-P. Liao, W.-J. Zhang, H.-X. Jiang, J. Sun, M.-J. Zhang, X.-F. He, D.-X. Lao, and Y.-H. Liu. 2010. Prevalence of serogroups, virulence genotypes, antimicrobial resistance, and phylogenetic background of avian pathogenic *Escherichia coli* in south of China. Foodborne Pathog. Dis. 7:1099–1106.

Wang, Y., H. F. Sheng, Y. He, J. Y. Wu, Y. X. Jiang, N. F. Y. Tam, and H. W. Zhou. 2012. Comparison of the levels of bacterial diversity in freshwater, intertidal wetland, and marine sediments by using millions of Illumina tags. Appl. Environ. Microbiol. 78:8264–8271.

Wang, Y. M., J. Y. Li, Y. H. Xie, H. X. Zhang, J. H. Jin, L. X. Xiong, and H. Liu. 2021. Effects of a probiotic-fermented herbal blend on the growth performance, intestinal flora and immune function of chicks infected with. Poult. Sci. 100:101196.

Weurding, R. E., A. Veldman, W. A. G. Veen, P. J. van der Aar, and M. W. A. Verstegen. 2001. Starch digestion rate in the small intestine of broiler chickens differs among feedstuffs. J. Nutr. 131:2329–2335.

Wu, Y., C.-p. Zhu, Y. Zhang, Y. Li, and J.-r. Sun. 2019. Immunomodulatory and antioxidant effects of pomegranate peel polysaccharides on immunosuppressed mice. Int. J. Biol. Macromol. 137:504–511.

Yaqoob, M. U., M. Yousaf, M. I. Khan, and M. Wang. 2022. Effect of $\beta$-mannanase supplementation on growth performance, ileal digestibility, carcass traits, intestinal morphology, and meat quality in broilers fed low-ME diets. Animals (Basel) 12:1126.

Zaytsoff, S. J. M., T. Montina, V. F. Boras, J. Brassard, P. E. Moote, R. R. E. Uwiera, and G. D. Inglis. 2022. Microbiota transplantation in day-old broiler chickens ameliorates necrotic enteritis via modulation of the intestinal microbiota and host immune responses. Pathogens 11:972.

Zhang, Z. F., Y. Xi, S. T. Wang, L. Y. Zheng, Y. Qi, S. S. Guo, and B. Y. Ding. 2022. Effects of Chinese gallnut tannic acid on growth performance, blood parameters, antioxidative status, intestinal histomorphology, and cecal microbial shedding in broilers challenged with aflatoxin B1. J. Anim. Sci. 100:skac099.

Zhong, X., Y. Shi, J. Chen, J. Xu, L. Wang, R. C. Beier, X. Hou, and F. Liu. 2014. Polyphenol extracts from *Punica granatum* and *Terminalia chebula* are anti-inflammatory and increase the survival rate of chickens challenged with *Escherichia coli*. Biol. Pharm. Bull. 37:1575–1582.

Zuo, L., B. A. Rose, W. J. Roberts, F. He, and A. K. Banes-Berceli. 2014. Molecular characterization of reactive oxygen species in systemic and pulmonary hypertension. Am. J. Hypertens. 27:643–650.