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Aerial parts of Angelica sinensis supplementation for improved broiler growth and intestinal health

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ABSTRACT This research examined the impact of incorporating *Angelica sinensis's* aerial components (**APA**), commonly referred to as "female ginseng", into broilers' diet. Two hundred eighty-eight 1-day-old Cobb 500 broilers were randomly assigned to the 4 experimental groups with 6 replications and 12 birds/replicate. The 4 groups were fed the diets included 4 concentrations of APA (0, 1, 2, and 3%, respectively). The study spanned 42 d, categorized as the starter phase (1−21 d) and the finisher phase (22−42 d). Notably, broilers fed with 3% APA demonstrated a pronounced surge in feed consumption and weight gain during the 22 to 42 d and over the full 42-d period ($P < 0.05$). Furthermore, when examining the broilers' intestinal structure, there was a notable increase in the villus height and villi ratio across the duodenum, jejunum, and ileum, with a decrease in crypt depth upon 3% APA inclusion ($P < 0.05$). On a molecular note, certain genes connected to the intestinal mechanical barrier, such as Zona Occludens 1 and Claudin-2, saw significant elevation in the jejunum ($P < 0.05$). The jejunum also displayed heightened levels of antimicrobial peptides like lysozyme, mucin 2, sIgA, IgG, and IgM, showcasing an enhanced chemical and immune barrier ($P < 0.05$). Delving into the 16SrDNA sequencing of intestinal content, a higher microbial diversity was evident with a surge in beneficial bacteria, particularly Firmicutes, advocating a resilient and balanced microecosystem. The findings imply that a 3% APA dietary addition bolsters growth metrics and fortifies the intestinal barrier's structural and functional integrity in broilers.

Key words: aerial parts of Angelica sinensis, broiler growth performance, intestinal barrier, intestinal homeostasis

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INTRODUCTION

With the growing economy and the rise in living standards, people's demand for animal products has increased. This strong market environment has gradually transformed animal husbandry into industrialization. Outdated production capacities will be gradually eliminated with the tightening of national policies. For instance, after the strengthening of environmental protection policies and government supervision, antibiotics now play a vital role in promoting animal growth and development, improving animal feed remuneration, and preventing infections (Castanon, 2007; Suresh et al., 2018). However, the large-scale use of antibiotics, raises serious concerns about drug resistance and a potential risk to human health (Van Boeckel et al., 2015). In recent years, many countries have restricted or even banned the use of antibiotics in animal feed (Brüssow, 2017). Particularly, China has barred the production of commercial feed containing feed additives such as growth-promoting drugs since July 1, 2020. Therefore, minimizing or replacing the use of antibiotics has become an urgent issue in animal husbandry, particularly during the breeding process. Many recent studies have shown that the active substances in plants can enhance the innate immunity of animals, promoting the growth and development of animals, reducing oxidative stress, maintaining intestinal integrity, promoting the growth of beneficial bacteria, and reducing intestinal infections (Hyun et al., 2018).

Chinese herbal medicines are natural substances with low toxicity and no residual effects. Worldwide, scientists are exploring various herbal medicines as alternatives to antibiotics in animal husbandry. However, this has also indirectly led to the phenomenon of "human and animal competition for medicine", due to the limited resources of such medicinal materials. Therefore,

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rationally utilizing Chinese herbal medicine resources is also an issue in promoting the development of animal husbandry in China. Angelica is a traditional Chinese medicine with a long history of application that can be traced back to the Han Dynasty. According to "Shen Nong's Materia Medica", Angelica, also known as "female ginseng", has the functions of nourishing blood, promoting blood circulation, relieving pain, and moistening the intestines (Shang, 1999; Shan et al., 2014). In the current Chinese Pharmacopoeia (Chinese Pharmacopoeia Commission, 2010), Angelica sinensis is used in more than 80 kinds of Chinese medicinal materials for its wide range of pharmacological properties. Furthermore, modern pharmacological studies have also shown that Angelica sinensis is not only safe and nontoxic but also has anticancer, antioxidation, immune regulation, and neuroprotection properties (Chen et al., 2013). Additionally, both *in vitro* and *in vivo* studies have shown that the active substances in Angelica can protect the heart from arrhythmia and atherosclerosis (Hu et al., 1991; Liu et al., 2001; Yao et al., 2015; Zhou et al., 2015). The roots of Angelica sinensis are the main medicinal parts, while the stems, leaves, and other aboveground parts are usually discarded. Since the stems and leaves of Angelica are much larger resources, discarding them could be a serious waste of natural medicinal resources. Studies have shown that aerial parts of Angelica sinensis (**APA**) have bioactive substances similar to those in roots (Zhou et al., 2012). Therefore, APA has potential medicinal value. To date, there are no reports on the health properties of APA as an animal feed additive. Therefore, in this study, we evaluated the influence of dietary supplementation with APA on the growth performance and intestinal health of broilers.

MATERIALS AND METHODS

Ethics Statement

All experimental animal procedures in the current study were carried out with the approval of the Faculty Animal Policy and Welfare Committee of Gansu Agricultural University (No. GSAU-Eth-AST-2021-001).

Test Materials

The APA used in this study was collected from Kangle County, Linxia Prefecture, Gansu Province, China, in October 2021. The plant parts 10-13 cm above the ground were cut as the original samples and naturally dried in a sunroom. Dried APA (122.16 mg/g flavone, 24.20 mg/g polyphenol, 18.82 mg/g polysaccharide) was crushed and stored for later use. The contents of nutrients in APA were determined using conventional methods. The crude protein content was determined by the Kjeldahl method (Standardization Administration of China, 2018), the crude fat content was determined by the Soxhlet extraction method (Standardization Administration of China, 2006), the crude ash content was determined by the high-temperature burning method (Standardization Administration of China, 2007), the calcium content was determined by the potassium permanganate titration method (Standardization Administration of China, 2018), and the phosphorus content was determined by the spectrophotometric method (Standardization Administration of China, 2018a). The results of the measurements are presented in Table 1.

Table 1. Nutrition content of Aerial parts of angelica sinensis.

Items	DM (%)	CP (%)	EE (%)	Ash (%)	Ca (%)	P (%)
APA	90.22	13.22	1.22	14.80	2.44	0.24

Note: CP: crude protein, EE: crude fat, NFE: nitrogen-free extract, Ash: crude ash, Ca: calcium, P: phosphorus.

Experimental Design and Broilers Management

We sourced 288 healthy, day-old Cobb500 white-feathered broilers from Shaanxi Baoji Hualong Animal Husbandry Co., Ltd. These birds were then randomly allocated into 4 distinct treatments diets included 0, 1, 2, and 3% APA. Each treatment consisted of 6 replicates, with each replicate housing 12 birds. Experiment diets with isocaloric and isonitrogenous were formulated according to the commercial nutrient specifications used in China (Ministry of Agriculture of the People's Republic of China PRC, 2004) as shown in Table 2. Before mixing the diets, APA was ground to pass through a 3.0 mm screen and sample was determined for the content of dry matter (**DM**), crude protein (**CP**), crude ash, crude fat (**EE**), crude ash (**Ash**), Calcium (**Ca**) and phosphorus (**P**) of APA was calculated value. The study spanned 42 d and was categorized into 2 distinct phases: the initial or "starter" phase (1-21 d) followed by the "finisher" phase (22-42 d). Housing for these broilers involved a 3-tiered cage setup, where each tier accommodated 12 birds, effectively making each tier a separate experimental unit (length: 160 cm, width: 70 cm, and height: 40 cm). Each cage was fitted with a nipple drinker and tube feeder. Birds had ad libitum access to feed and water throughout the experimental period. The birds were raised in a temperature-controlled room maintained at 36°C at the arrival of the broilers and gradually decreased following the breeder's guidelines (Cobb, 2019). Lighting was provided for 24 h during the first 5 d of the experiment and reduced to 23 h/d from d 6 to 42. Birds were vaccinated against Newcastle disease (7 and 21 d posthatching) and infectious bursa disease (14 and 28 d posthatching).

Sample Collection

For intestinal tissue procurement: The experimental animals were euthanized by cervical dislocation, sections approximately 2 cm long from the central portions of the duodenum, jejunum, and ileum were excised utilizing a sterilized scalpel. These segments were subsequently cleansed of any intestinal contents using phosphate buffer

Table 2. Ingredient composition and nutrient content of the basal diets.

Ingredients	Starter				Finisher			
APA	0	1	2	3	0	1	2	3
Maize	60.63	59.6	59.17	58.68	65.05	63.72	62.72	61.72
Vegetable oil	1.3	1.82	1.88	1.94	3.1	3.62	4.22	4.82
Soya bean meal	22	22.5	22.9	23.3	15	16.8	17.2	17.6
Cottonseed meal	2	2	2	2	3	3	3	3
Corn gluten meal	3	3	3	3	3	3	3	3
Distillers dried grains with solubles	2	2	2	2	3	2	2	2
Wheat bran	4	3	2	1	4	3	2	1
Limestone	1.3	1.3	1.3	1.3	0.8	0.8	0.8	0.8
Calcium phosphate dibasic	1.8	1.8	1.8	1.8	1.2	1.2	1.2	1.2
Premix[1]	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
NaCl	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4
DL-methionine	0.25	0.25	0.25	0.25	0.18	0.18	0.18	0.18
L-lysine	0.62	0.63	0.63	0.63	0.57	0.58	0.58	0.58
Chloride choline	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Sum	100	100	100	100	100	100	100	100
Calculated Nutrient Content[2]								
Metabolic energy, MJ/kg	12.36	12.37	12.35	12.39	12.98	13.01	12.96	13.02
Crude protein, %	20.00	19.96	20.12	20.34	18.00	17.98	18.15	18.32
Ca, %	1.00	1.03	1.02	1.01	0.67	0.69	0.66	0.68
Available P, %	0.45	0.46	0.44	0.49	0.34	0.35	0.37	0.38
Digestible Met, %	0.50	0.51	0.49	0.52	0.40	0.42	0.40	0.43
Digestible Lys, %	1.25	1.26	1.35	1.41	1.08	1.09	1.15	1.22

[1]The premix provides per kilogram of feed- vitamin A: 11600 IU; Vitamin D3: 2600 IU; Vitamin E: 20 IU; Vitamin K3: 2.0 mg; Vitamin B1: 2.4 mg; Vitamin B2: 8.0 mg; Vitamin B6: 4.0 mg; Vitamin B12: 0.02 mg; Niacinamide: 36.0 mg; Pantothenic Acid: 12.8 mg; Folic Acid: 1.2 mg; Biotin: 0.048 mg; Antioxidants: 200 mg; Iron: 114 mg; Copper: 13.0 mg; Manganese 137.0 mg; Potassium: 4.35 mg; Selenium: 0.36 mg. The late premix provides per kilogram of diet- vitamin A: 4350 IU; vitamin D3: 975 IU; vitamin E: 7.5 IU; vitamin K3: 0.75 mg; vitamin B1: 0.9 mg; Vitamin B2: 3.0 mg; Vitamin B6: 1.5 mg; Vitamin B12: 0.00075 mg; Niacinamide: 13.5 mg; Pantothenic acid: 4.8 mg; Folate: 0.45 mg; Biotin: 0.018 mg; Antioxidants: 200 mg; Iron: 91.55 mg; Copper: 13.25 mg; Manganese: 113.82 mg; Potassium: 4.35 mg; Selenium: 0.36 mg. The ME, CP, Ca, Available P, and Met Lys of basal diets were calculated.

[2]Metabolic energy and amino acid contents were calculated using values from the NRC requirements, but other nutrient contents were measured value.

saline (**PBS**). Once cleaned, these samples were immersed in a solution containing 4% paraformaldehyde. The residual part of the jejunum underwent a longitudinal incision and was washed using PBS to clear away any lingering chyme. The mucosal layer of the jejunum was then gently scraped off with a sanitized glass slide, gathered into a sterile 2.0 mL cryotube, sealed tightly, promptly frozen using liquid nitrogen, and preserved at a temperature of −80°C.

For the cecum specimen collection: Upon the broiler's euthanization, the entire cecum was carefully extracted using a sanitized scalpel. A normal saline solution was employed for its rinsing. A portion of the cecum, approximately 2 g in weight, was excised and transferred into a sanitized cryopreservation tube. Immediately after, these sample tubes were flash-frozen in liquid nitrogen and maintained at −80°C.

Growth Performance

Daily, any incidences of mortality among the animals were diligently noted for every individual cage. On the 21st and 42nd mornings, after ensuring a 12-hour period without feed, both the average consumption of feed (denoted as AFI) and the mean increase in weight (termed AWG) were systematically assessed. To ensure data accuracy, parameters such as the ratio of feed to weight gain ratio (represented as F/G) and in each feeding phase; mortalities were removed and recorded to adjust the feed intake as outlined by Olanrewaju et al (2018).

Intestinal Morphology

After collection, specimens from the duodenum, jejunum, and ileum underwent an immersion process in a solution containing 4% paraformaldehyde. This was followed by a methodical preparation process that included dehydration, clearing, wax immersion, and embedding. Once prepared, the samples were sectioned appropriately to undergo staining with hematoxylin and eosin (**HE**). Capturing the detailed histological features, images were taken using a BA210 digital trinocular camera microscope system, a product of Macaudi Industrial Group Co., Ltd. Initially, a panoramic view of each section was obtained at a lower magnification. Then, specific regions of interest were zeroed in on and imaged at a 40X magnification. Measurements were extracted for both the villus height (VH) and crypt depth (CD) from ten distinct and well-preserved villi and crypt structures on every section. With these measurements in hand, the ratio between villus height and crypt depth (VH/CD) was methodically derived.

Intestinal Tight Junction Protein

We determined the expression level of 3 intestinal tight junction-related genes (*Zona Occludens 1, Occludin*, and *Claudin-2*) in the jejunum by qRT-PCR. (Initial denaturation at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 15 s, and annealing and extension at 60°C for 30 s) β-actin was used as the internal reference gene, and each sample set up had 3

Table 3. Primers information.

Gene name	Primer sequence
ZO-1	F: GAAGAGAGCACAGAACGCAG
	R: CACTTGTGGCAAGCTGAAGT
Occludin	F: TCCTCATCGTCATCCTGCTC
	R: TTCTTCACCCACTCCTCCAC
Claudin-2	F: TGCTGCGAGATTTCCACAAC
	R: CAGGAGGCACAGAGGATGAA
β-actin	F: AGTACCCCATTGAACACGGT
	R: CTCTGTTGGCTTTGGGGTTC

replicates. Relative gene expression levels were calculated using the $2^{-\Delta\Delta CT}$ method. The primer sequences used are listed in Table 3.

Intestinal Secretory Factors and Mucins

The contents of antibacterial peptide (Attccin, pg/L), lysozyme (Lysozyme, pg/L), and secretory mucin (MUC-2, pg/L) in jejunum were determined by the respective ELISA kit (Jiangsu Meimian Industrial Co. Ltd., Jiangsu, China) following the kit instructions.

Intestinal Immune Factor

To quantify the concentrations of specific immunoglobulins within the jejunal mucosa, we utilized ELISA kits supplied by Jiangsu Meimian Industrial Co. Ltd. Following the protocols detailed in the kit's guidance, measurements for secretory immunoglobulin A (sIgA, μg/mL), immunoglobulin G (IgG, μg/mL), and immunoglobulin M (IgM, μg/mL) were successfully determined.

Analysis of Cecal Microbial Diversity

Genomic DNA from the provided samples was isolated utilizing the DNA Extraction Kit (M5636-02, OMEGA Bio-Tek, USA) and then preserved at a temperature of −20°C. To evaluate both the amount and integrity of the extracted DNA, we employed a NanoDrop NC2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA) along with agarose gel electrophoresis. We targeted the V3-V4 region of the bacterial 16S rDNA gene using primers: forward 338F (5′-ACTCCTACGGGAGGCAGCA-3′) and reverse 806R (5′-GGACTACHVGGGTWTCTAAT-3′). To facilitate multiplexed sequencing, a distinct 7-bp barcode was integrated into each primer set. The amplification procedure via PCR was structured with an initial 98°C denaturation step for 5 min. This was followed by 25 repetitions consisting of 98°C for 30 s, 53°C for 30 s, and 72°C for 45 s. The process was concluded with a 72°C extension for a 5-min duration.

After PCR amplification, we purified the products with VAHTSTM DNA Clean Beads (Vazyme, China). Their concentration was then assessed through Invitrogen's quantitative-it PicoGreen dsDNA Assay Kit. Once quantified, these amplicons were combined in equimolar ratios. For sequencing, we employed the Illumina NovaSeq platform from Shanghai Personal Biotechnology Co., Ltd., China, executing a paired-end read with 2 × 250 bp using the accompanying NovaSeq 6000 SP kit that facilitates 500 cycles.

For the in-depth analysis of sequencing data, both QIIME2 and the R software package (version 3.2.0) were utilized. Through QIIME2, we derived various alpha diversity metrics, including the Chao1, Shannon, and Simpson measures. Using principal component analysis (**PCoA**), we probed the disparities in microbial community architectures. Additionally, when investigating taxonomic distinctions at both the phylum and genus strata, the LefSe tool was pivotal. This tool facilitated the computation of LDA (linear discriminant analysis) effect sizes and played a crucial role in discerning statistically significant biomarkers across different sample groups.

Statistical Analysis

The differences among the groups were statistically analyzed by one-way analysis of variance with SPSS 20.0, sourced from IBM Corporation. Shapiro-Wilk and Levene's tests were used to test the normal distribution of data as well as the homogeneity of variance. Tukey's multiple comparison test was used to determine the significance of mean differences. Polynomial orthogonal contrasts were used to determine the linear and quadratic responses of measured parameters to dietary APA concentrations. The results were expressed as mean and pooled standard errors of the means (SEM). A probability value of $P < 0.05$ was considered statistically significant. Lastly, any necessary visual enhancements and modifications of figures were proficiently handled with the GraphPad Prism 8 tool.

RESULTS

Growth Performance

During the initial phase spanning d 1 to 21, when assessed against the control group with 0% APA inclusion, it was observed that the APA dietary regimen did not influence the AFI and AWG metrics in broilers ($P > 0.05$), however, 3%APA supplementation significantly decreased the F/G ratio of broilers ($P < 0.05$), as evident from the results in Table 4. Nevertheless, as the birds transitioned into the finisher phase from d 22 to 42, there was a marked increase in both AFI and AWG for the broilers in the dietary groups receiving 2% and 3% APA, this enhancement was statistically significant when contrasted with the control group, with results compiled in Table 4 indicating a significance level of $P < 0.05$. This trend remained consistent when considering the entire duration of the study, from d 1 to d 42.

Table 4. Effects of dietary supplementation with APA on the growth performance of broilers.

Items	Groups				SEM	*P* value		
	Con	1% APA	2% APA	3% APA		ANOVA	Linear	Quadratic
Starter (1-21 d)								
AFI (g)	666.49	656.93	646.10	658.47	6.70	0.897	0.586	0.472
AWG (g)	421.19	432.99	425.21	447.66	5.00	0.259	0.115	0.589
F/G (g/g)	1.56^{a}	1.54^{ab}	1.53^{ab}	1.49^{b}	0.04	0.047	0.007	0.583
Finisher (22-42 d)								
AFI (g)	1745.84^{b}	1865.28^{ab}	1986.13^{a}	2037.22^{a}	33.63	0.003	<0.001	0.515
AWG (g)	919.81^{c}	973.99^{bc}	1076.26^{ab}	1097.17^{a}	20.83	0.001	<0.001	0.592
F/G (g/g)	1.91	1.92	1.86	1.86	0.02	0.294	0.108	0.782
All period (1-42 d)								
AFI (g)	2411.07^{b}	2531.77^{ab}	2636.01^{ab}	2704.71^{a}	36.14	0.013	<0.001	0.667
AWG (g)	1341.00^{b}	1406.99^{ab}	1494.62^{a}	1540.81^{a}	23.82	0.006	<0.001	0.795
F/G (g/g)	1.80	1.80	1.77	1.75	0.01	0.327	0.099	0.707

Abbreviations: AFI, average feed intake. AWG, average weight gain. F/G, feed to weight gain ratio. Note: Values with the same or no letter superscripts within the same row denote no significant difference ($P>0.05$), while with different letter superscripts indicate significant difference ($P<0.05$). The same applies to other Tables.

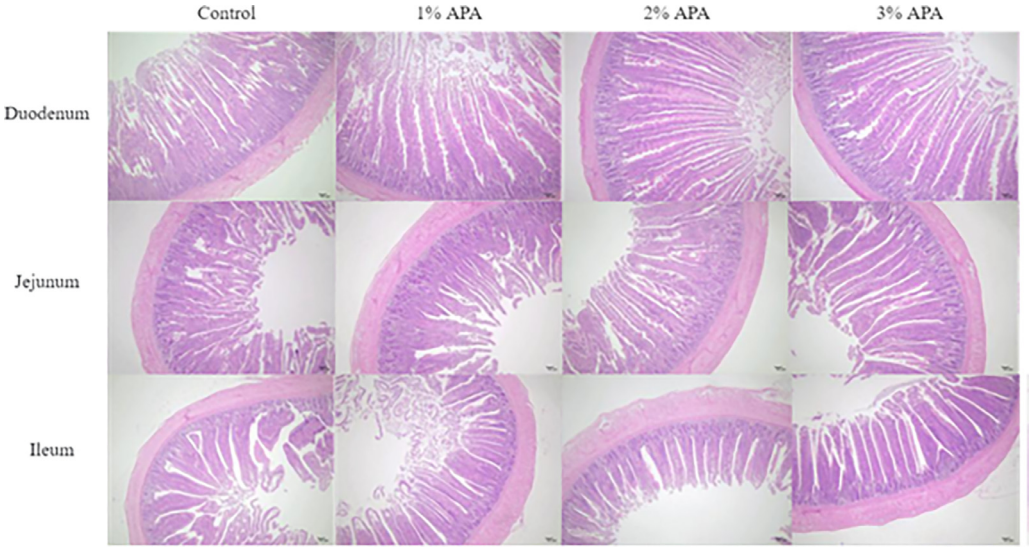

Figure 1. Tissue morphology of the duodenum, jejunum, and ileum.

Intestinal Mechanical Barrier

We explored the impact of APA dietary supplementation on the histological structures of broilers' duodenum, jejunum, and ileum, as visualized in Figure 1 and detailed in Table 5. In the duodenum, when matched against the 0% APA baseline, the 1% APA group showed a 24% up-regulation in VH and a 77% enhancement in the VH/CD ratio. In the 2% APA set, these figures jumped to 51% and 119%, while in the 3% APA ensemble, they up-regulation to 78% and 276%. Correspondingly, the CD recorded declines of 28, 27, and 52% across the 1, 2, and 3% APA groups. Turning our attention to the jejunum, the 1% APA segment noted an upswing of 19% in VH and 5% in CD. The 2% APA cohort registered upticks of 46% and 43% in VH

Table 5. Effect of dietary APA supplementation on intestinal tissue morphology of broilers.

Items	Groups				SEM	*P* value		
	Con	1% APA	2% APA	3% APA		ANOVA	Linear	Quadratic
Duodenum								
VH, um	1255.40^{d}	1,554.23^{c}	1,907.94^{b}	2,238.22^{a}	61.88	<0.001	<0.001	0.680
CD, um	267.63^{a}	192.07^{b}	192.89^{b}	127.86^{c}	66.83	<0.001	<0.001	0.719
VH/CD	4.83^{c}	8.55^{b}	10.61^{b}	18.15^{a}	5.70	<0.001	<0.001	0.050
Jejunum								
VH, um	1084.25^{d}	1,291.9^{c}	1,578.15^{b}	1,735.27^{a}	41.35	<0.001	<0.001	0.195
CD, um	137.66	153.22	134.79	130.59	30.70	0.388	0.367	0.315
VH/CD	8.46^{b}	8.87^{b}	12.14^{a}	13.76^{a}	0.86	<0.001	<0.001	0.456
Ileum								
VH, um	667.05^{d}	869.12^{c}	943.50^{b}	1111.35^{a}	27.25	<0.001	<0.001	0.392
CD, um	113.48	105.37	97.42	91.72	3.33	0.103	0.014	0.852
VH/CD	6.19^{c}	8.33^{bc}	10.23^{ab}	12.45^{a}	0.48	<0.001	<0.001	0.954

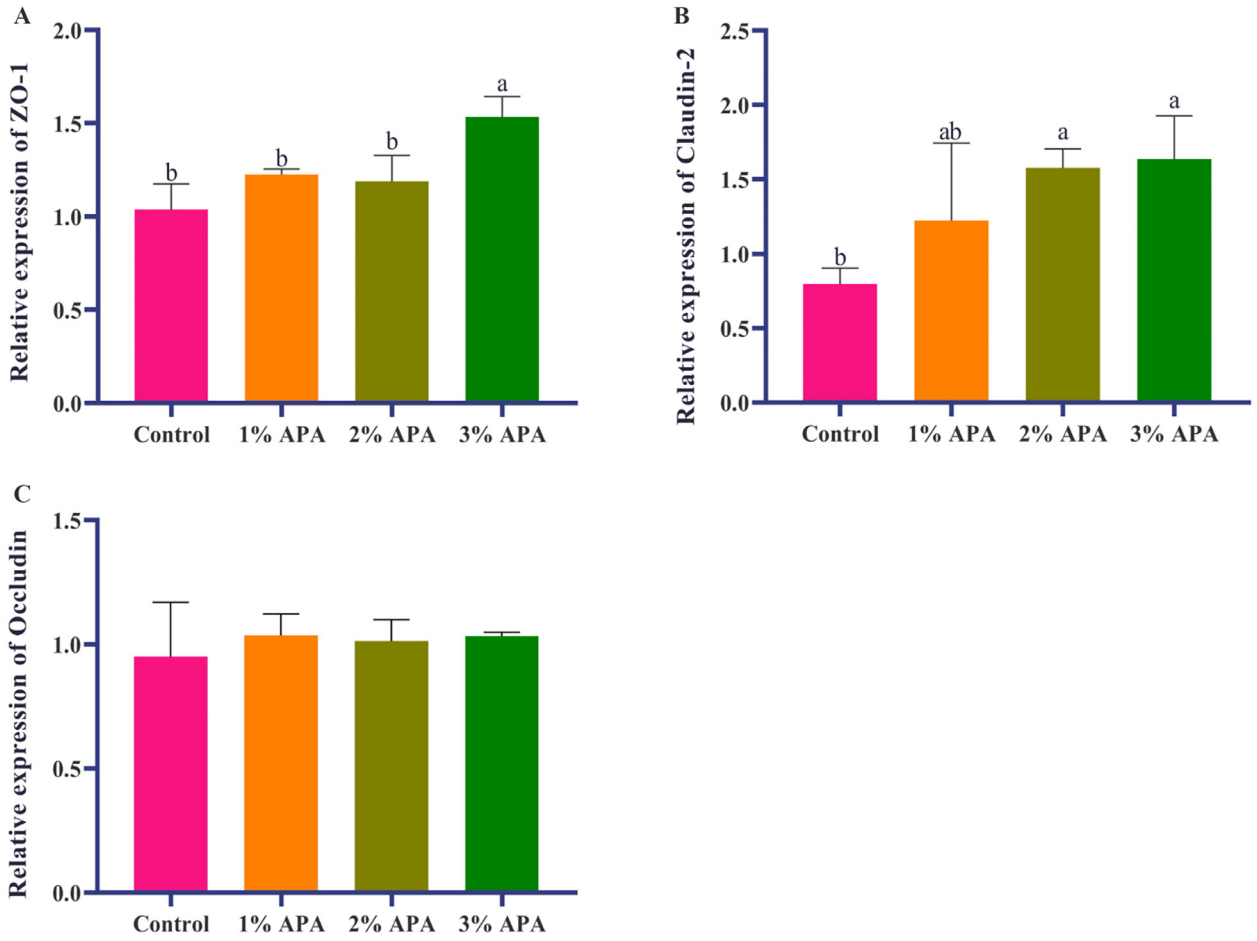

Figure 2. Expression of jejunal mucosal barrier-related genes. Expression of (A) *ZO-1*, (B) *Claudin-2*, and (C) *Occludin* in the jejunal mucosa.

and CD, and in the 3% APA partition, these increments were 60% and 63%. The ileum presented its own pattern: rises of 30% and 35% in VH and CD for 1% APA, 41% and 65% for 2% APA, and a robust 67% and 101% for the 3% APA division ($P < 0.05$).

Figure 2 depicts the noticeable alteration in Zona Occludens 1 (ZO-1) and Claudin-2 expression levels within the jejunum upon introducing a 3% APA dietary regimen, contrasted with the baseline 0% APA group, affirming statistical significance ($P < 0.05$). The manifestation of Claudin-2 ascended by 97% in the 2% APA faction and by 105% in the 3% APA contingent. On the other hand, Occludin's expression underwent only marginal changes, remaining statistically similar ($P > 0.05$).

Intestinal Chemical Barrier

Subsequently, the impact of APA on the intestinal chemical barrier was investigated. Distinct elevations in antimicrobial peptide levels within the jejunum were observed, showcasing increments of 13, 35, and 51% in the 1 APA, 2 APA, and 3% APA treatments respectively ($P < 0.05$). In parallel, lysozyme concentrations rose by 16, 38, and 62% across the same groups ($P < 0.05$). Additionally, the MCU-2 levels demonstrated an upward trend with enhancements of 11, 28, and 55% for the respective treatments ($P < 0.05$) (Figure 3).

Intestinal Immune Barrier

Figure 4 reveals variations in immunoglobulin levels due to APA supplementation. Relative to the 0% APA group, the sIgA levels in the jejunum surged by 30, 52, and 65% for the 1, 2, and 3% APA concentrations respectively ($P < 0.05$). Concurrently, IgG concentrations showcased a modest rise of 2%, followed by more pronounced increases of 17% and 52% in the subsequent concentrations ($P < 0.05$). Similarly, elevations in IgM were marked at 16, 28, and 43% across these APA treatments ($P < 0.05$).

Gut Biological Barrier

In the study, OTUs (Operational Taxonomic Unit), representative of distinct microbial taxa, were identified across various APA concentrations. The breakdown is as follows: Control (A) yielded 1,855 OTUs, 1% APA (B) had 2,087, 2% APA (C) revealed 1,840, while 3% APA (D) exhibited 2032 OTUs. Notably, a core of 853 OTUs was shared across all sample sets, as visualized in Figure 5A. A thorough examination of the dilution curve, displayed in Figure 5B, indicates that the sequence data was comprehensive enough to represent the cecal microflora composition in broilers. Turning to α-diversity measures, the 1-3% APA concentrations showed pronounced elevations in Chao1 and other

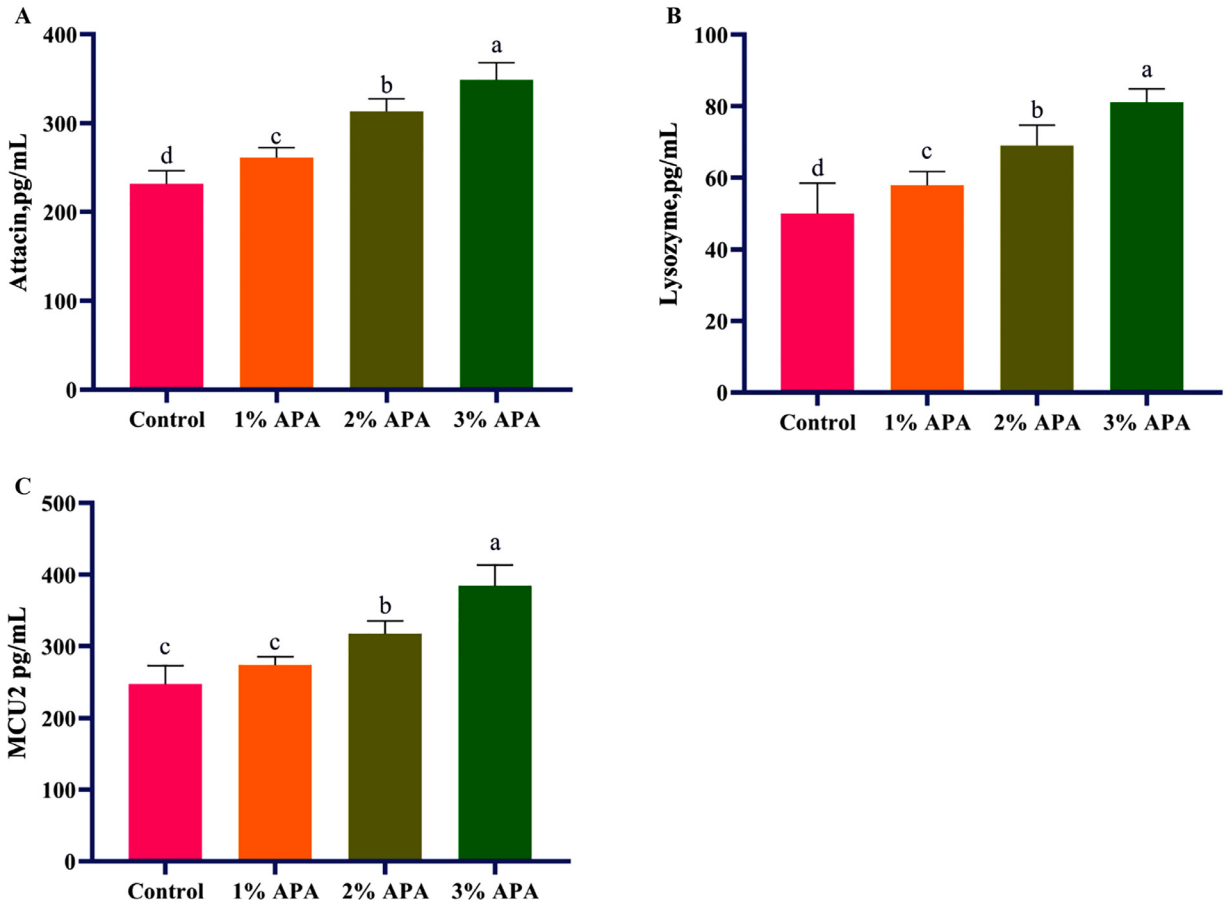

Figure 3. Effect of dietary APA on the intestinal chemical barrier of broilers. The contents of (A) antimicrobial peptides, (B) lysozyme, and (C) MCU2 in jejunal mucosa.

species indices when benchmarked against the control, a distinction that was statistically significant ($P < 0.05$). The Shannon diversity peaked in the 3% APA concentration, markedly surpassing the control and the 1 to 2% APA cohorts ($P < 0.01$). The control's Simpson index paled in comparison to its APA-supplemented counterparts ($P < 0.01$). While the Faith_PD index in APA-enhanced samples was elevated, the difference from the control was not statistically meaningful ($P > 0.05$). Interestingly, the Pielou_e index was highest in the control, lagging behind the APA groups ($P < 0.01$) as seen in Table 6.

Figure 6 illustrates the influence of APA inclusion in diets on the beta diversity of broilers' cecal microbial composition. Two prominent dimensionality-reducing analytical techniques, PCoA and NMDS, present sample distances on a bi-dimensional plane. In the PCoA visualization, the x-axis and y-axis accounted for variances of 30% and 14.7%, respectively, as seen in Figure 6A. This adequately captured the inter-sample variations. Observing a stress value of 0.114, it became evident that samples within the same cluster lacked cohesion, with no pronounced clustering discerned among groups A through D, as highlighted in Figure 6B.

By analyzing taxonomy at the phylum level, the top ten differentially abundant species were discerned. Firmicutes, Bacteroidetes, and Proteobacteria dominated in abundance, collectively making up over 90% of the microbial content across all groups as depicted in Figure 7A. There was a marked elevation in Firmicutes' relative abundance within groups 1% APA (B), 2% APA (C), and 3% APA (D) in contrast to the control group (A). Remarkably, in the 3% APA (D) cohort, Firmicutes reached a peak, making up 71.62%. Meanwhile, Bacteroidetes' relative presence dwindled in APA-supplemented groups, with the most significant drop observed in the 3% APA (D) group.

In a further analysis focused on the genus level, we identified the top ten species exhibiting notable variations in relative abundance. Specifically, Bacteroides demonstrated a diminished presence in groups supplemented with 1% APA (B), 2% APA (C), and 3% APA (D) when juxtaposed with the control group (A). Interestingly, the 2% APA and 3% APA groups displayed a comparable increase in the relative abundance of *Oscillospira* over the control group. While the concentrations of *Ruminococcus* and *Faecalibacterium* up-regulation, there was a discernible decline in *Akkermansia* within the 3% APA group in comparison to the control, as illustrated in Figure 7B.

Through the application of LEfSe analysis (seen in Figure 7C), discernible disparities in biomarkers (boasting an LDA score surpassing 2) emerged among the 1% APA (B), 2% APA (C), and 3% APA (D) groups. Within Group B (1% APA), the bacterial taxa *Pseudomonas, Pseudomonadales*, and *Pseudomonadaceae*

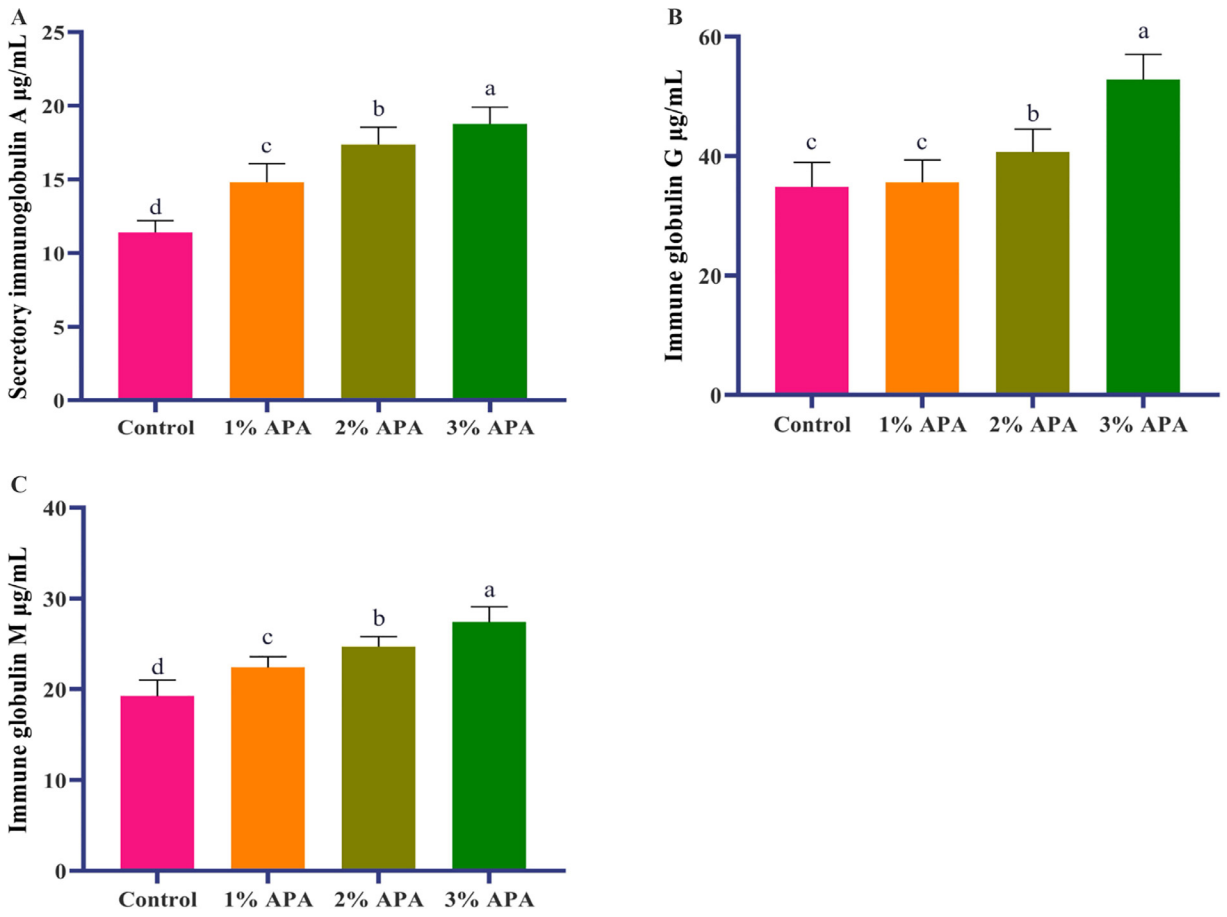

Figure 4. Effect of dietary APA on the intestinal immune barrier of broilers. The contents of (A) sIgA, (B) IgG, and (C) IgM in jejunal mucosal.

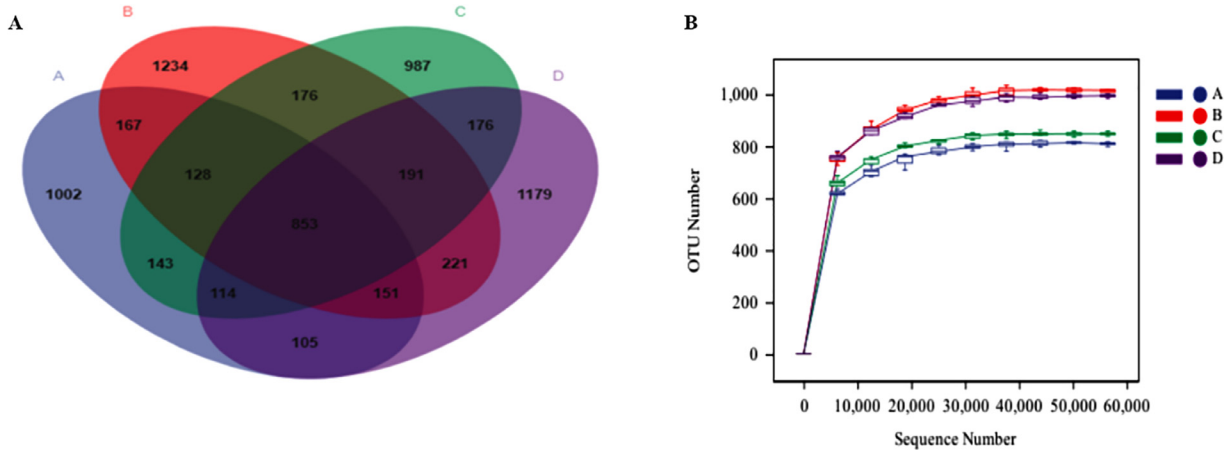

Figure 5. Caecal diversity analysis. (A) OTU Venn diagram analysis and (B) Dilution curve analysis.

Table 6. Statistical results of alpha diversity for broiler's intestinal flora.

	Groups					*P* value		
Items	Control(A)	Low(B)	Medium(C)	High(D)	SEM	ANOVA	Linear	Quadratic
Chao1	810.57^{b}	1,014.44^{a}	848.30^{ab}	995.09^{ab}	31.42	0.032	0.027	0.606
Faith_pd	50.30	56.34	54.08	60.00	2.86	0.706	0.323	0.992
Goods_coverage	0.99	0.99	0.99	0.99	0.01	0.050	0.880	0.411
Observed_species	763.88^{b}	946.35^{a}	804.02^{ab}	934.78^{ab}	28.54	0.037	0.112	0.609
Pielou_e	0.67^{c}	0.72^{b}	0.70^{b}	0.75^{a}	0.01	<0.001	<0.001	0.724
Shannon	6.39^{c}	7.08^{ab}	6.77^{bc}	7.36^{a}	0.09	<0.001	<0.001	0.685
Simpson	0.96^{b}	0.98^{a}	0.97^{ab}	0.98^{a}	0.01	0.008	0.011	0.451

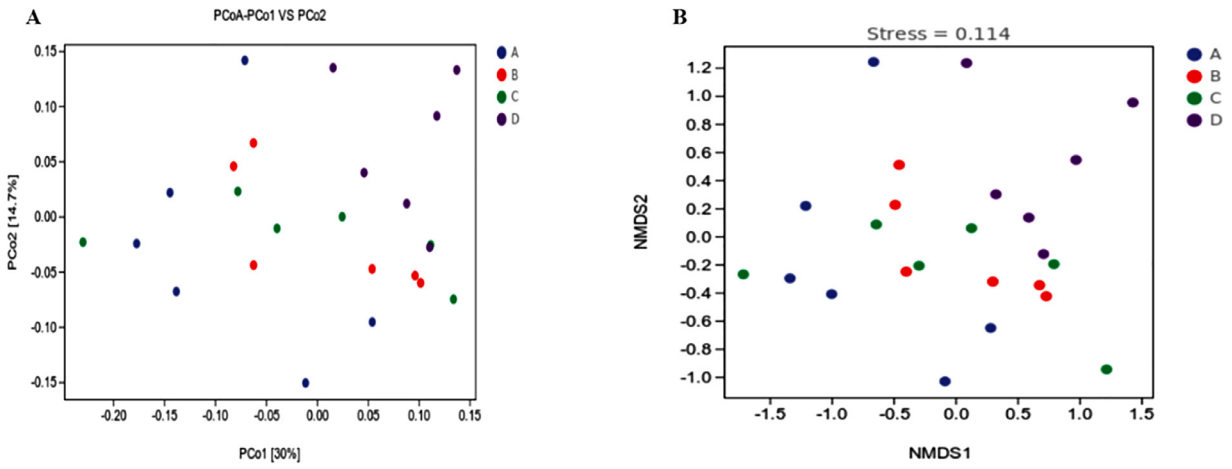

Figure 6. Statistical results of beta diversity of the broilers' intestinal flora. The results of (A) PCoA and (B) NMDS analysis.

markedly stood out. For Group C (2% APA), *Oscillospira* emerged as the taxon showing significant variance. However, for Group D (3% APA), the notable bacterial taxa comprised Firmicutes, *Clostridia, Clostridiales, Erysipelotrichi, Erysipelotrichales*, and *Ruminococcus*. Importantly, each group had distinct bacterial clades marked as significant biomarkers, as depicted in Figure 7D.

DISCUSSION

In the animal husbandry sector, the use of Chinese herbal medicine as feed additives, especially in poultry, is well established. However, the non-medicinal components remain under explored. APA, which possesses bioactive ingredients akin to the roots of Angelica sinensis, predominantly consists of elements like flavonoids, polysaccharides, terpenoids, and organic acids, as delineated by Zhou et al. (2012). These elements have been identified as potential growth enhancers in broilers. Surprisingly, there is scant literature on APA's deployment in livestock and poultry practices. A study by Guo et al. (2019) highlighted the benefits of introducing 3% astragalus stems and leaves in quail diets, leading to augmented feed consumption and weight gain. Abdulsalam et al. (2015) pointed out enhancements in broiler growth during the fattening stage upon Moringa leaf meal inclusion in their diet. Our research underscores a significant enhancement in broiler growth upon APA addition, with a pronounced effect at 3% supplementation. Wang et al. (2019) and Wang et al. (2021) have emphasized the role of flavonoids and polysaccharides, predominant in astragalus (stem and leaves), Moringa leaf meal, and APA, in livestock growth, a sentiment echoed by Chen et al. (2021) and Roy et al. (2022). Furthermore, plant-derived feed additives, as per Greene et al. (2021), elevate broiler performance by amplifying hypothalamic HSP70 expression, subsequently reducing core broiler temperature, which in turn spurs feed consumption and weight gain. Factors antagonistic to nutrition in APA might curtail broiler weight, thereby influencing the F/G ratio, as speculated by several previous studies (González-Alvarado et al., 2008; Mateos et al., 2012; Sadeghi et al., 2015).

Concerning the digestive system of broilers, food enters the glandular stomach containing gastric acid and pepsin after short-term storage in the crop. The function of the glandular stomach is to help digest protein, and subsequently, the food enters the gizzard for grinding and then in the intestine. The intestine is the main site for the digestion and absorption of food, which is strictly influenced by the state of morphological integrity of the intestinal tract (Zou et al., 2019). The intestinal epithelium has 2 parts: crypts and villi. The villi are the functional units of the digestive tract, and their height determines the food contact area and thus the activity of digestive enzymes. A shallower intestinal crypt ensures proper absorption of food nutrients by the intestine. In cases of damage to intestinal epithelial cells, the CD increases while VH decreases (Caspary, 1992). If the epithelial cells in the intestine are damaged, the height of the villi in the intestine will decrease. At the same time, the intestinal stem cells at the bottom of the crypts will accelerate the rate of proliferation and differentiation, and finally leave the crypts and move to the villi to replace the injured ones. If the crypts are getting deeper, it indicates that intestinal stem cells are undergoing frequent proliferation and differentiation, which is also a sign of intestinal epithelial damage (Sipos and Muzes, 2015; Smith et al., 2017). The VH/CD ratio serves as a benchmark in assessing the nutrient absorption and digestion capabilities within the intestine. In our findings, broilers on a 3% APA regimen exhibited a notable augmentation in the VH/CD ratio across the duodenum, jejunum, and ileum, alongside a decrement in the duodenal CD. Though there was a reduction in CD for both the jejunum and ileum, the variance was not pronounced enough to be deemed significant. A unique aspect of the broilers' inner wall is its composition: a singular layer of columnar epithelial cells. These cells, as identified by Ungewiß et al., (2017), constitute junctional complexes encompassing tight junctions, desmosomes, and adherens junctions. Such complexes are pivotal for cell polarity and permeability, playing the role of a

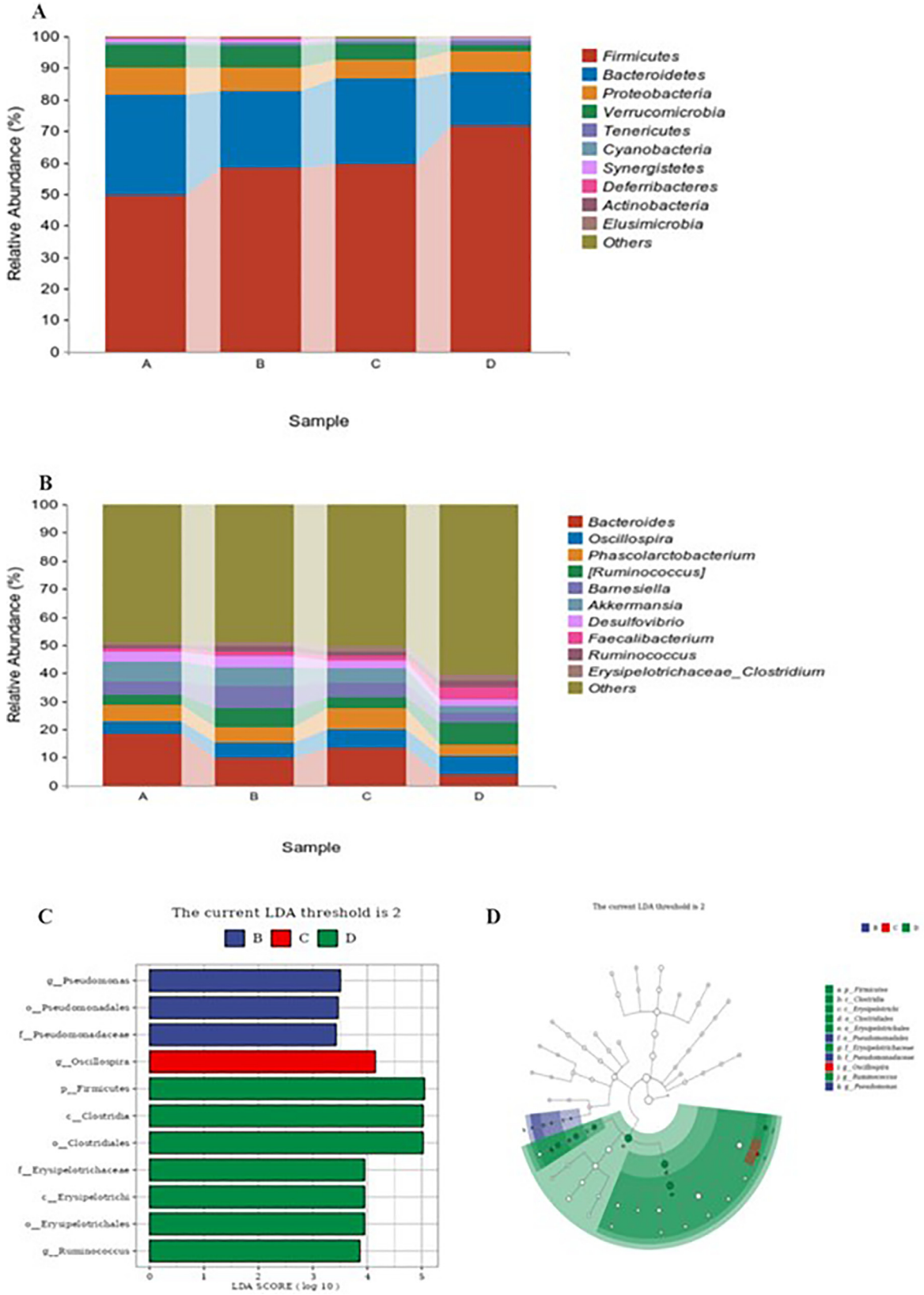

Figure 7. Intestinal flora analysis results. (A) Diagram of species distribution at the phylum level of cecal flora, (B) Diagram of species distribution at the genus level of cecal flora, (C) LDA histogram and (D) Taxonomic cladistics of LEfSe analysis of intestinal flora.

mechanical defense. The significance of tight junctions as cellular fortifications within the intestinal epithelium is emphasized by Turner (2006), and these are constituted of 4 membrane-spanning proteins, notably Occludin, Claudin, JAM, and tricellulin. Their function is enhanced through interaction with cytoplasmic scaffold proteins, like ZO, linking them to the actin cytoskeleton (Ulluwishewa et al., 2011). In our exploration, the APA regimen had a discernible impact, particularly amplifying the concentrations of ZO-1 and Claudin2. Elevated

doses of APA magnified this effect. While an increment in Occludin concentration was discerned, it lacked statistical relevance. To sum up, APA's inclusion in the diet optimizes the expression of tight junction proteins in broilers, fortifying the intestinal barrier's structural integrity.

A crucial barrier against pathogenic intruders in the gut is the intestinal chemical defense system. This system's architecture predominantly includes mucus, a secretion of the gut's mucosal lining, along with digestive agents from the intestine and antimicrobial compounds originating from gut microbiota. The robustness and unyielding nature of the epithelial cell layer act as gatekeepers, curbing the invasion while facilitating the removal of unwelcome pathogens. This not only safeguards but also stabilizes the balance of the intestinal microbial (Gao et al., 2020). Mucin in the mucosal layer is mainly regulated by the *MUC2* series of genes (Brisbin et al., 2008). Both lysozyme and antimicrobial peptides are cationic polypeptides that can stimulate the production of a large amount of lysozyme. Studies have found that antimicrobial peptides not only resist harmful microorganisms and exert their antibacterial effects but also positively stimulate intestinal immunity (Zasloff, 2002; Mansour et al., 2014). In this study, the high-dose APA group of broilers showed a significant increase in the contents of antimicrobial peptides and lysozyme and upregulated *MUC2*. This data indicated that dietary APA can help maintain the integrity of the intestinal mucosa and also have a certain positive effect on intestinal immunity.

The gut's immune barrier encompasses elements like mucosal lymphoid tissue of the intestine, intestinal-origin plasma cells, and notably, antibodies such as sIgA. Once in the gut, sIgA forms a bond with the mucosal layer. Upon a pathogenic attack, sIgA swiftly activates its immune functions, impeding these pathogens from adhering to the gut's epithelial cells. Concurrently, this action prompts enhanced mucus secretion, thwarting bacterial settlement (Pietrzak et al., 2020). Another crucial antibody, IgG, predominantly incapacitates antigens through mechanisms including the complement system and cell-mediated antibody toxicity (Hosono et al., 2020). Our research indicates that broilers' jejunum experienced elevated levels of sIgA, IgG, and IgM when supplemented with APA, with the pronounced impact evident in those given higher dosages. Higher APA concentrations seem to amplify the intestinal immune reactions, contributing to a healthier gut environment. IgM also demonstrates similar beneficial effects. The presence of compounds like terpenoids in APA is believed to invigorate macrophages, amplifying intestinal immune responses (Villar-Lorenzo et al., 2016; Shen et al., 2019).

Variations in gut microbiota have a profound effect on the intestinal ecological balance. Healthy bacteria, under optimal conditions, outcompete and eradicate harmful or unnecessary bacteria, ensuring gut homeostasis (Ricke et al., 2020). Interestingly, the microbial profile of oviparous birds, like hens, seldom influences the microbial composition of their offspring. This results in a noticeable reduction in the diversity and richness of gut microbiota in poultry. Upon hatching, the intestinal microbiota of poultry starts to evolve as they consume food, a process influenced by a myriad of internal and external determinants (Ramakrishna, 2013). Numerous research avenues indicate that Chinese medicinal components whether monomers, extracts, or complex formulations can restructure gut flora. They bolster beneficial bacterial populations while curbing detrimental ones, imparting resistance against gut-related ailments (Zhou et al., 2016). Currently, the potential of Chinese herbal extracts in poultry garners considerable attention. Reports suggest that introducing diets enriched with polysaccharides from astragalus and glycyrrhizin can enhance the diversity of cecal microbes in broilers (Qiao et al., 2022). Another study by Long et al. (2021) and colleagues posited that broilers fed with Acanthopanax senticosus extract demonstrated increased lactic acid bacteria in the ileum but decreased Escherichia coli and Salmonella. Findings from Ao and Kim (2020) mirrored these effects with Achyranthes knuckle extract. Notably, investigations focusing on non-pharmaceutical components of Chinese herbs in livestock remain scant. In this backdrop, our work sought to comprehend the ramifications of varying APA doses on the cecal microbial diversity in broilers. Our observations highlighted a surge in OTUs in the 1-3% APA dietary groups vis-a-vis controls, suggesting heightened flora diversity and richness. Despite the 3% APA cohort exhibiting lesser richness than its 1% counterpart, its species diversity metric surpassed. A thriving richness and diversity within the gut typically symbolize a mature microbiome (Konopka, 2009). Such a balanced microbial ecosystem is adept at withstanding environmental flux owing to its inherent adaptability, resilience, and steadiness (Le Chatelier et al., 2013).

Analysis of bacterial composition at the phylum rank highlighted that the primary phyla present were Firmicutes and Bacteroidetes. Interestingly, in the group fed with 3% APA, an augmentation of Firmicutes and a decline in Bacteroidetes were observed. It's worth noting that Firmicutes are known to harbor genes coding for enzymes integral to energy metabolism. These enzymes subsequently assist the host in producing various digestive enzymes that break down nutrients (Xue et al., 2016). An elevated ratio of Firmicutes to Bacteroidetes is indicative of enhanced nutrient absorption capabilities (Elokil et al., 2022). Delving deeper into the genus level, the group with high APA intake displayed an upsurge in the prevalence of *Oscillospira, Coprobacterium*, and *Ruminococcus*, but *Ackermannia* was less abundant in their cecal flora. Interestingly, *Osmospirillum's* prominence has been linked to obesity, and current research suggests it could be a promising probiotic for future applications (Yang et al., 2021). Both *Faecalibacterium* and *Ruminococcus* play roles in curbing pathogenic bacterial growth, believed to be due to their influence on short-chain fatty acid concentrations within the gut (Suchodolski, 2011). *Faecalibacterium* has also been associated with a low feed conversion ratio (Metzler-Zebeli et

al., 2019). *Akkermansia*, a bacterium in the *Verrucomicrobial* phylum whose abundance is inversely associated with obesity and diabetes (Everard et al., 2013; Shin et al., 2014), was found to be downregulated in the high-dose APA group. Furthermore, the results from LefSe analysis demonstrated that statistically and biologically distinct OTUs in each group had similar positions in the phylogenetic tree. This suggests that differential flora occupy specific niches in the cecal ecosystem.

CONCLUSIONS

The dietary supplementation with 3% APA improved the growth performance and intestinal health of broilers, including the mechanical, chemical, and immune barriers of the intestine. In addition, high-dose APA enhanced the diversity of cecal microbial flora in broilers, ensuring a dynamic and stable microecosystem.

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DISCLOSURES

The authors declare no conflict of interest.

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