Animal Nutrition 21 (2025) 472–486

Contents lists available at ScienceDirect

Animal Nutrition

journal homepage: http://www.keaipublishing.com/en/journals/aninu/

Original Research Article

Dietary supplementation with isochlorogenic acid improves growth performance and intestinal health of broilers

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ARTICLE INFO

Article history:
Received 28 January 2024
Received in revised form
18 November 2024
Accepted 20 November 2024
Available online 17 December 2024

Keywords:
Isochlorogenic acid
Broiler
Growth performance
Gut health

ABSTRACT

This study evaluated the effects of dietary supplementation with isochlorogenic acid (ICA) on growth performance and intestinal health of broilers. A total of 400 Cobb broilers (44.20 ± 0.16 g, one day old) were randomly assigned to 5 treatments (ICA supplementation at 0, 500, 1000, 2000, and 3000 mg/kg diet) with 8 replicates of 10 birds each. The experimental trial lasted for 42 d. The feed-to-gain ratio (F/G) exhibited a quadratic decrease in response to ICA supplementation during both the 1 to 42 d ($P = 0.048$) and 22 to 42 d ($P = 0.039$) periods, with the lowest F/G observed at a dietary ICA concentration of 2000 mg/kg. The apparent digestibility of calcium ($P = 0.038$) and crude protein ($P < 0.001$) exhibited a linear upward trend, and both the villus height and the villus height-to-crypt depth ratio showed a quadratic increase ($P = 0.027$) with ICA supplementation. Meanwhile, the relative mRNA expression of ileum claudin-1 ($P = 0.003$) and occludin ($P = 0.048$), along with the gastrointestinal pH ($P < 0.05$), decreased linearly, whereas the concentration of ileum secretory immunoglobulin A ($P = 0.005$) increased linearly with ICA supplementation. Additionally, the concentration of total volatile fatty acids ($P = 0.038$) in the ileum, trypsin activity ($P = 0.016$), the serum concentration of immunoglobulin G ($P = 0.005$), and the activities of serum glutathione peroxidase (GSH-Px) ($P = 0.005$) and superoxide dismutase (SOD) ($P = 0.040$) increased quadratically with ICA supplementation. Additionally, the relative mRNA expression of *SOD-1* ($P = 0.040$) and *GSH-Px* ($P = 0.040$) in the ileum increased linearly with ICA supplementation. The abundance of *Streptococcus alactolyticus* was significantly higher in broilers supplemented with ICA at 2000 mg/kg compared to the control treatment ($P = 0.030$), and the concentration of metabolites, such as 15-deoxy-delta-12,14- prostaglandin J2, was increased by ICA supplementation. Dietary ICA enhanced broilers growth performance via increased digestive enzyme activity, leading to improved feed digestibility. Additionally, ICA improved health status by maintaining gut pH and enhancing antioxidant and immune functions. The optimal supplemental level of ICA of improving growth performance and intestinal immune function of broilers was 2000 mg/kg.

1. Introduction

The escalating global demand for poultry products has driven substantial growth in the broiler industry. Broilers grow fast but have highly delicate and vulnerable intestines (Kogut et al., 2016). Besides being the main digestive organ, the intestine is the primary immune organ in poultry, serving as the first line of defense. It plays a crucial role in ensuring optimal health through its innate immune system (Kogut et al., 2017). Poultry's intestinal barrier functions

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Peer review under the responsibility of Chinese Association of Animal Science and Veterinary Medicine.

Production and Hosting by Elsevier on behalf of KeAi

https://doi.org/10.1016/j.aninu.2024.11.012

include physical, chemical, microbial, and immune barriers, jointly protecting the birds' intestinal health.

Over recent years, numerous studies have demonstrated the potential of natural plant extracts to improve animal gut health (Capasso and Di, 2020). Isochlorogenic acid (ICA) belongs to the dicaffeoylquinic acid class of compounds and is formed through the mangiferin acid pathway during plant aerobic respiration. The compound is a phenol derivative produced by the condensation reaction between quinic acid and caffeic acid and serves as an isomer of chlorogenic acid (CA) (Pamisetty et al., 2018). Multiple research studies have shown that including CA in animal feed can significantly enhance livestock and poultry production performance, immune function, antioxidant capacity, and intestinal health (Jin et al., 2023). However, CA is associated with several limitations that hamper its practical applications, including chemical instability, poor lipid solubility, low bioavailability, and enzymatic degradation by esterases (Psotová et al., 2004). ICA has a more symmetrical chemical structure than CA, as it contains two caffeoyl groups and multiple hydroxyl groups that contribute to its biological activities (Cortes-Morales et al., 2019; Kim et al., 2017; Wang et al., 2016). ICA contains phenolic acids and exhibits superior antioxidant, antibacterial, antiviral, hepatoprotective, and anti-inflammatory properties compared with CA. A limited body of research examines the impacts of ICA in broilers. The present study aimed to explore the potential advantages of incorporating ICA into broiler diets, specifically focusing on its effects on growth performance, intestinal morphology, digestive enzyme activity, volatile fatty acid (VFA) production, antioxidant capacity, immune response, intestinal microbiota composition, and metabolic pathways in broilers. This study aimed to determine the optimal dosage of ICA supplementation and provide valuable data for its practical application in broiler production.

2. Materials and methods

2.1. Animal ethics statement

This study was conducted from June to December 2022 at the Animal Husbandry Teaching Base of Hebei Agricultural University, Baoding, China. The Hebei Agricultural University Institutional Animal Care and Use Committee approved the experimental protocol (2023110).

2.2. Experimental diet

The ICA product used in the current experiment was developed and supplied by Chenguang Biotechnology Co., Ltd., Handan, China. This product was obtained from *stevia rebaudiana* after the extraction of flavonoids and CA. It contained 55% ICA, 5% CA derivatives, 5% flavonoids, 10% glycosides, 10% moisture, and 15% ash. The experimental diets were formulated according to the China National Feeding Standard of Chicken (NY/T 33–2004) to meet the nutrient requirements (Table 1).

2.3. Experimental design, animals, and management

The feeding trials were conducted at the experimental farm of Hebei Agricultural University. Four hundred healthy Cobb broilers (50% male and 50% female) at 1 d of age and with similar body weight (44.20 ± 0.16 g) were randomly divided into five treatments. The broilers in the control (CON) group were fed a basal diet without ICA supplementation, while the other four treatment groups (T1, T2, T3, and T4) were fed diets supplemented with ICA product at 500, 1000, 2000, and 3000 mg/kg, respectively. Each treatment consisted 8 replicates with 10 broilers in each replicate.

Table 1
Ingredients and nutrient levels of basal diets (%, air-dry basis).

Item	Content	
	1–21 d of age	22–42 d of age
Ingredients		
Corn	48.20	51.45
Soybean meal	41.80	36.50
Soybean oil	5.00	7.05
Premix[1]	5.00	5.00
Total	100.00	100.00
Nutrient levels		
ME, MJ/kg	12.71	13.31
CP	21.04	20.18
Ca	0.85	0.75
P	0.65	0.55
Lysine	1.59	1.45
Methionine	0.60	0.57
Threonine	1.00	0.92

ME = metabolizable energy; CP = crude protein; Ca = calcium; P = phosphorus.
[1] Provided per kilogram of diet: vitamin A, 750 IU; vitamin D_3, 3 kIU; vitamin E, 20 IU; vitamin K_3, 2 mg; vitamin B_1, 2 mg; vitamin B_2, 6 mg; vitamin B_6, 3 mg, vitamin B_{12}, 0.02 mg; calcium pantothenate, 10 mg; nicotinamide, 40 mg; biotin, 0.12 mg; Cu, 10 mg; Fe, 20 mg; Zn, 80 mg; Mn, 90 mg; Se, 0.2 mg; I, 1 mg.

The broilers were raised in cages with ad libitum access to feed and drinking water throughout the experiment, which lasted for 42 d.

2.4. Determination of growth performance

Feed intake and body weight of broilers in each cage were measured and documented at 1, 21, and 42 d. Subsequently, average daily gain (ADG), average daily feed intake (ADFI), and feed-to-weight ratio (F/G) of the broilers in each group were calculated.

ADG (g/d) = body weight gain (g)/number of days (d);

ADFI (g/d) = cumulative feed intake/(number of birds × number of days);

F/G = feed intake (g)/body weight gain (g).

2.5. Nutrient digestibility

During the final 3 d of the trial, approximately 500 g of fresh excreta samples were collected daily from pans beneath each cage. During collection, care was taken to avoid excreta contaminated with feed particles or feathers. Subsequently, the excreta was treated with diluted hydrochloric acid (prepared by mixing 1 part concentrated hydrochloric acid [36% to 38%] with 3 parts water) and dried at 65 °C for 48 h. After that, the samples were ground and stored for further analysis.

The endogenous indicator method was employed to assess the nutrient digestibility of broilers in each treatment. Nutrient analysis of samples followed AOAC (2019) procedures: dry matter (DM; method 930.15), crude protein (CP; method 990.03). Phosphorus (P) and calcium (Ca) concentrations were determined using an atomic absorption spectrophotometer (ZA4000; Hitachi High-Technologies, Tokyo, Japan) using method 985.01 according to AOAC (2019). Amino acid profiles of feed samples were analyzed by ultra-performance liquid chromatography (LA8080; Hitachi High-Technologies, Tokyo, Japan) using method 982.30 according to AOAC (2019). Gross energy (GE) content in feed and excreta samples was analyzed using a bomb calorimeter (Model 6300; Parr Instruments, Moline, IL, USA). Metabolizable energy (ME) values of experimental diets were calculated based on established criteria (Xiong et al., 2020).

2.6. Measurement of the pH of the gastrointestinal tract

At the termination of the experiment, one broiler with a body weight closest to the replicate mean was selected from each cage.

Digesta samples were immediately collected from the stomach, duodenum, jejunum, ileum, and cecum. The pH values were measured at three randomly selected locations in each sample using a Testo-205 pH meter (Testo AG, Germany).

2.7. Digestive enzyme assay

Approximately 1 g of the terminal ileum contents was placed into a 5-mL centrifuge tube. Then, an appropriate volume of saline was added, and the contents homogenized. Subsequently, the it was centrifuged at 850 × *g* for 10 min. The levels of amylase, trypsin, and lipase in the supernatant were quantified using enzyme-linked immunosorbent assay (ELISA) kits (Jiangsu Meimian Industrial Co., Ltd., Jiangsu, China) following the manufacturer's instructions. The coefficients of variation for both inter-assay and intra-assay were <10% and <12%, respectively.

2.8. Intestinal morphometry

Ileum tissues were fixed in formalin for 48 h, sectioned, and paraffin-embedded. Then, the tissue samples were stained with hematoxylin and eosin and encapsulated with a neutral gel. Subsequently, the sections were scanned using a panoramic slide scanner (DESK/MIDI/250/1000, Wuhan Servicebio Technology Co., Ltd., Wuhan, China), and the intestinal villus height and crypt depth were measured using scanning and viewing software. Finally, the ratio of villus height to crypt depth ratio was calculated.

2.9. VFAs

Approximately 1 g of ileal digesta was transferred to a 5-mL centrifuge tube, and 2.5 mL of pre-chilled ultrapure water was added, vortexed for 30 s, and then centrifugated at 10,000 × *g* at 4 °C for 10 min. The resulting supernatant (5 mL) was mixed with 1 mL metaphosphoric acid (25%, wt/vol). The mixture was vortexed for 30 s, and then placed in an ice bath for 40 min. It was centrifuged again under the same conditions (10,000 × *g*, 4 °C, 10 min). The final supernatant was collected and the concentrations of acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid, and valeric acid were quantified using a gas chromatograph (GC-2014; Shimadzu Corporation, Kyoto, Japan).

2.10. Measurement of serum immunity indexes

One broiler was randomly selected from each replicate cage, and about 10 mL of blood was collected from the wing vein. The blood samples were centrifuged at 850 × *g* for 10 min at 4 °C, and the resulting supernatant was stored at −20 °C until analysis. The serum concentrations of immunoglobulins (IgM, IgG, IgA), complement components (C3, C4), and tumor necrosis factor-α (TNF-α) were measured using commercially available ELISA kits (Meimian Industrial Co., Ltd., Jiangsu, China) according to the manufacturer's instructions.

2.11. Determination of antioxidant index

The activities of total antioxidant capacity (T-AOC), catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GSH-Px), as well as the contents of malondialdehyde (MDA) in serum, were analyzed using the respective ELISA kits (Meimian Industrial Co., Ltd., Jiangsu, China) according to the manufacturer's instructions.

2.12. Measurement of the ileum secretory IgA index

The ileum samples were retrieved from the refrigerator at −80 °C, and the content of secretory immunoglobulin A (SIgA) in the ileum was analyzed using the corresponding ELISA kit (Jiangsu Meimian Industrial Co., Ltd., Jiangsu, China) according to the manufacturer's instruction.

2.13. The expression of antioxidant- and intestinal permeability-related genes in ileum tissue

The mRNA levels of target genes in ileum tissue samples were quantified using quantitative real-time PCR (qRT-PCR). The primers utilized for amplification were designed with the assistance of Primer 6.0 software based on the gene sequences retrieved from GenBank for occludin, claudin-1, zonula occludens-1 (*ZO-1*), superoxide dismutase-1 (*SOD-1*), and glutathione peroxidase (*GSH-Px*). These primers were synthesized by Shanghai Personal Biotechnology Co., Ltd. (Shanghai, China). The specific primer sequences utilized in this research are shown in Table 2. The mRNA sequences were obtained from the National Center for Biotechnology Information (NCBI).

2.14. Assessment of microbial diversity

The ileum chyme samples were retrieved from a refrigerator at −80 °C. Nucleic acids were extracted using the OMEGA Soil DNA Kit (D5635–02; Omega Bio-Tek Inc., Norcross, GA, USA), following the manufacturer's instructions. The extracted DNA was subjected to by 0.8% agarose gel electrophoresis to assess molecular size integrity. Additionally, DNA concentration was quantified using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

Using total DNA as a template, the forward primer 338F (5'-ACTCCTACGGGAGGCAGCA-3') and reverse primer 806R (5'-GGACTACHVGGGTWTCTAAT-3') were combined with a junction sequence, a barcode sequence to amplify the V3–V4 region of the bacterial 16S rRNA gene. High-throughput sequencing of all the bacteria (16S rRNA) in the samples was performed on the Illumina NovaSeq 6000 platform. The diversity and abundance of the microbial communities were analyzed by Shanghai Personal Biotechnology Co., Ltd., Shanghai, China.

2.15. Untargeted metabolomics

Digesta from the terminal ileum were immediately transferred to pre-chilled 5-mL cryovials and snap-frozen in liquid nitrogen,

Table 2
Sequence of primers for real-time PCR.

Target genes	Primer sequence (5′ to 3′)	GenBank accession no.
β-Actin	Forward: CTTCCAGCCATCTTTCTT Reverse: ATATCCACATCACACTTCAT	ENSGALG00010021232
ZO-1	Forward: AGACAGCAGACATACATC Reverse: CAGATTCAGGAGGAGTTC	ENSGALG00015013331
Occludin	Forward: TTCGTGGCAAAGTGGCAG Reverse: GGTCGTACTCATGGATCTGTG	ENSGALG00010028576
Claudin-1	Forward: TGGTTGGTGTGTTTGTTG Reverse: CATCCGCATCTTCTTCAC	ENSGALG00010013646
SOD-1	Forward: TTCTGTCATTTCTCTTAC Reverse: TAGTTTGCTCTCATTATC	ENSGALG00010011746
GSH-Px	Forward: GATGAGATCCTGAGAGTGGTGGAC Reverse: TCATCAGGTAAGGTGGGCACAA	NM_000581.4

ZO-1 = zonula occludens-1; *SOD-1* = superoxide dismutase 1; *GSH-Px* = glutathione peroxidase.

then stored at −80 °C until metabolomic analysis. The analysis was conducted by Shanghai Personal Biotechnology Co., Ltd. (Shanghai, China). The liquid chromatography column of the ACQUITY UPLC HSS T3 (2.1 × 150 mm, 1.8 μm; Waters Co., Milford, MA, USA) model was adopted, with the flow rate set at 0.25 mL/min, the column temperature at 40 °C, and the injection volume at 2 μL. To prepare the samples, frozen digesta were thawed on ice. Five hundred milligrams of digesta were accurately weighed and transferred into a 2-mL centrifuge tube, and 600 μL methanol MeOH, containing 4 μL/L 2-amino-3-(2-chloro-phenyl)-propionic acid, was added. Steel balls (2 mm diameter) were added, and the mixture was vortexed vigorously for 30 s. Subsequently, the samples were ground using a tissue grinder at 50 Hz for 120 s. After that, ultrasonication was performed on the samples at room temperature for 10 min, followed by centrifugation at 10,000 × *g* and at 4 °C for another 10 min. The resulting supernatant was then filtered through a membrane with a pore size of 0.22 μm before being transferred to detection bottles for untargeted metabolomic analysis using liquid chromatography-mass spectrometry (LC-MS).

2.16. Statistical analysis

Data were statistically analyzed using the mixed model in SAS (SAS Inst. Inc., Cary, NC) as follows:

$$Y_{ij} = \mu + T_i + P_j + e_{ij},$$

where Y_{ij} is the dependent variable; μ is the mean; *Ti* is the fixed treatment effect; P_j is the random effect; e_{ij} is the error residual.

The normality of data among groups was confirmed and screened using the MIXED procedure of SAS. Treatments were the fixed effects, and replicates were the random effects. The linear and quadratic ICA dose responses were analyzed using specific pre-planned contrasts. $P \leq 0.05$ was considered a significant difference, and trends were discussed at $0.05 < P \leq 0.10$. Microbial diversity data were assessed using non-parametric tests in GraphPad Prism.

Untargeted metabolomics data processing and multivariate analysis: the Ropls software was utilized for all multivariate data analysis and modeling (Boulesteix and Strimmer, 2007). The data were standardized by centering them around the mean and scaling. Subsequently, the model was constructed by applying principal component analysis (PCA), orthogonal partial least-square discriminant analysis (PLS-DA), and orthogonal partial least-square discriminant analysis (OPLS-DA). Finally, metabolites exhibiting a significance level of $P < 0.05$ and variable importance in projection (VIP) values > 1 were identified as having differential abundance.

Pathway analysis of the identified differential metabolites was performed using the online tools (MetaboAnalyst5.0, https://www.metaboanalyst.ca/MetaboAnalyst/home.xhtml), which integrates pathway enrichment analysis with pathway topological analysis. The differentially abundant metabolites were then linked to the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway to better understand the higher-level systemic functions. These metabolites and their corresponding pathways were visualized by the KEGG Mapper tool.

3. Results

3.1. The effect of ICA on broiler growth performance

As shown in Table 3, ICA had a limited effect on ADFI. However, ADG increased linearly with ICA supplementation from 22 to 42 d ($P = 0.015$) and 1 to 42 d ($P = 0.047$). F/G decreased linearly from 1 to 21 d ($P = 0.048$) and decreased quadratically from 22 to 42 d ($P = 0.039$) and 1 to 42 d ($P = 0.048$) with ICA supplementation.

Table 3
Effect of ICA supplementation on growth performance of broilers.

Item	ICA, mg/kg					SEM	*P*-value	
	0	500	1000	2000	3000		Linear	Quadratic
ADFI, g/d								
1–21 d	34.17	33.27	33.91	32.33	33.36	0.800	0.317	0.370
22–42 d	122.06	117.69	117.39	116.28	117.99	1.963	0.207	0.096
1–42 d	81.09	78.85	79.31	77.48	78.49	1.431	0.174	0.289
ADG, g/d								
1–21 d	20.95	21.45	21.28	21.93	21.52	0.453	0.291	0.386
22–42 d	77.53^{b}	77.52^{b}	77.93^{b}	80.62^{a}	79.26^{ab}	0.808	0.015	0.312
1–42 d	49.24^{b}	49.48^{b}	49.60^{b}	51.27^{a}	49.89^{b}	0.448	0.047	0.064
F/G								
1–21 d	1.63	1.56	1.6	1.48	1.55	0.036	0.048	0.122
22–42 d	1.58^{a}	1.52^{ab}	1.51^{ab}	1.44^{b}	1.49^{b}	0.027	0.014	0.039
1–42 d	1.65^{a}	1.59^{ab}	1.60^{ab}	1.51^{b}	1.57^{ab}	0.029	0.019	0.048

ICA = isochlorogenic acid; SEM = standard error of the mean; ADFI = average daily feed intake; ADG = average daily gain; F/G = feed-to-gain ratio.
Within a row, values with different superscripts indicate a significant difference ($n = 8$, $P < 0.05$).

3.2. The effect of ICA on the apparent digestibility of nutrients in broilers

Although the digestibility of DM and GE was not affected by ICA supplementation, a quadratic increasing trend was observed in the digestibility of CP ($P = 0.073$) and Ca ($P = 0.085$) (Table 4). Concurrently, the digestibility of P tended to increase linearly ($P = 0.051$) with ICA supplementation. This phenomenon indicates a potential relationship between ICA supplementation and nutrient digestibility alterations.

3.3. The effects of ICA on the physical barrier of broilers

As shown in Table 5, with ICA supplementation, the villus height and the ration of villus height to crypt depth ($P = 0.027$) increased quadratically, while the crypt depth tended to decrease quadratically ($P = 0.051$).

As shown in Fig. 1, although the relative mRNA expression of *ZO-1* was not affected, the relative mRNA expression of claudin-1 ($P = 0.003$) and occludin ($P = 0.048$) increased linearly with ICA supplementation.

3.4. The effects of ICA on the chemical barrier in broilers

As shown in Table 6, the pH of the stomach, duodenum, jejunum, ileum, and cecum decreased linearly ($P < 0.05$).

Table 4
Effect of ICA on apparent digestibility of nutrients in broilers (%).

Item	ICA, mg/kg					SEM	*P*-value	
	0	500	1000	2000	3000		Linear	Quadratic
DM	60.82	60.75	60.83	60.98	60.78	0.069	0.511	0.155
CP	62.37^{c}	63.43^{bc}	64.51^{ab}	65.97^{a}	65.50^{ab}	0.762	<0.001	0.073
Ca	61.72	63.94	63.71	64.70	64.26	0.797	0.038	0.085
P	63.48	63.47	63.84	63.62	63.85	0.127	0.051	0.681
GE	64.43	64.48	65.26	65.35	65.16	0.478	0.159	0.295

ICA = isochlorogenic acid; DM = dry matter; CP = crude protein; Ca = calcium; P = phosphorus; GE = gross energy.
Within a row, values with different superscripts indicate a significant difference ($n = 8$, $P < 0.05$).

Table 5
Effect of ICA on the morphology of broiler ileum.

Item	ICA, mg/kg					SEM	*P*-value	
	0	500	1000	2000	3000		Linear	Quadratic
Villus height, μm	1054.88^{c}	1072.20^{bc}	1076.22^{abc}	1095.09^{a}	1086.00^{ab}	6.543	0.003	0.038
Crypt depth, μm	205.57	184.41	171.89	170.95	171.35	8.920	0.017	0.051
Villus height/crypt depth	5.19^{b}	5.86^{ab}	6.26^{a}	6.41^{a}	6.35^{a}	0.271	0.005	0.027

ICA = isochlorogenic acid.
Within a row, values with different superscripts indicate a significant difference ($n = 5$, $P < 0.05$).

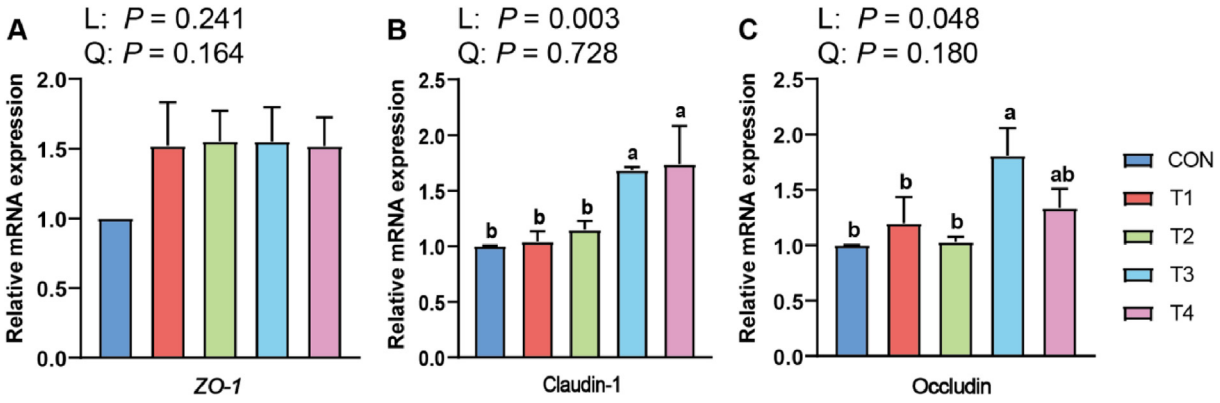

Fig. 1. Effect of ICA supplementation on mRNA relative expression of tight junction protein-related genes in the ileum of broilers. (A) *ZO-1*, (B) claudin-1, (C) occludin. Letters L and Q represent linear and quadratic effects of ICA supplementation dose, respectively. ICA = isochlorogenic acid; *ZO-1* = zonula occludens-1. T1, T2, T3, and T4 represent the supplementation of the ICA product at 0, 500, 1000, 2000, and 3000 mg/kg, respectively. Bars with different letters indicate a significant difference ($n = 5$, $P < 0.05$).

Table 6
Effect of ICA on the pH of different segments of the gastrointestinal tract in broilers.

pH value	ICA, mg/kg					SEM	*P*-value	
	0	500	1000	2000	3000		Linear	Quadratic
Stomach	3.33^{a}	3.31^{ab}	3.29^{abc}	3.27^{bc}	3.25^{c}	0.016	<0.001	0.588
Duodenum	6.46	6.40	6.38	6.37	6.36	0.023	0.010	0.154
Jejunum	6.52^{a}	6.44^{b}	6.43^{b}	6.42^{b}	6.41^{b}	0.026	0.013	0.109
Ileum	6.76^{a}	6.67^{ab}	6.59^{b}	6.58^{b}	6.53^{b}	0.054	0.006	0.238
Cecum	6.81	6.69	6.62	6.60	6.55	0.064	0.008	0.266

ICA = isochlorogenic acid.
Within a row, values with different superscripts indicate a significant difference ($n = 8$, $P < 0.05$).

Table 8
Effect of ICA on VFAs content in the ileum of broilers (mmol/L).

Item	ICA, mg/kg					SEM	*P*-value	
	0	500	1000	2000	3000		Linear	Quadratic
TVFAs	38.02^{b}	45.06^{ab}	42.76^{ab}	49.48^{a}	44.45^{ab}	2.286	0.046	0.038
Acetic acid	26.02^{b}	31.21^{ab}	29.14^{ab}	34.86^{a}	31.02^{ab}	1.798	0.045	0.058
Propionic acid	6.71	7.63	7.40	7.65	7.13	0.543	0.737	0.257
Isobutyric acid	0.66	0.72	0.67	0.72	0.68	0.133	0.970	0.833
Butyric acid	3.18	3.67	3.84	3.69	3.38	0.234	0.841	0.051
Isovaleric acid	0.84	0.94	0.90	1.46	1.36	0.175	0.012	0.637
Valeric acid	0.62	0.89	0.80	1.10	0.86	0.198	0.327	0.277

ICA = isochlorogenic acid; TVFAs = total volatile fatty acids.
Within a row, values with different superscripts indicate a significant difference ($n = 5$, $P < 0.05$).

As shown in Table 7, although the amylase activity was not affected, the pancrelipase activity ($P < 0.001$) increased linearly with ICA supplementation. Trypsin activity ($P = 0.016$) showed a quadratic increase in response to ICA supplementation, with peak activity observed at a dietary concentration of 2000 mg/kg.

As shown in Table 8, the effects of ICA on the concentrations of propionic acid, iso-butyric acid, iso-valeric acid, and valeric acid were limited. With the increasing ICA supplementation, the concentration of total volatile fatty acids (TVFAs) increased quadratically ($P = 0.038$), the concentrations of acetic acid ($P = 0.058$) and butyric acid ($P = 0.051$) tended to increase quadratically.

3.5. The effect of ICA on the immune barrier in broilers

The serum concentrations of C4, IgA, IgM, and TNF-α were not affected by ICA supplementation (Table 9), whereas the serum concentration of C3 ($P = 0.004$) increased linearly, and the serum

Table 7
Effect of ICA on digestive enzymes in ileal contents of broilers (U/mg).

Item	ICA, mg/kg					SEM	*P*-value	
	0	500	1000	2000	3000		Linear	Quadratic
Amylase	82.99	98.73	104.39	115.51	110.22	12.878	0.128	0.305
Trypsin	98.60^{b}	122.91^{a}	130.61^{a}	139.53^{a}	131.50^{a}	7.765	0.008	0.016
Pancrelipase	32.89^{c}	34.46^{c}	39.28^{bc}	55.22^{a}	49.05^{ab}	3.931	<0.001	0.150

ICA = isochlorogenic acid.
Within a row, values with different superscripts indicate a significant difference ($n = 5$, $P < 0.05$).

Table 9
Effect of ICA on serum immunological properties of broilers (μg/mL, unless otherwise stated).

Item	ICA, mg/kg					SEM	*P*-value	
	0	500	1000	2000	3000		Linear	Quadratic
C3	989.10^{b}	1035.30^{ab}	1034.18^{ab}	1072.79^{a}	1064.16^{a}	16.550	0.004	0.102
C4	360.91	389.71	386.22	412.39	410.19	40.410	0.498	0.752
IgA	8.86	8.67	8.74	9.28	8.71	0.622	0.775	0.642
IgG	106.98^{b}	108.35^{b}	109.55^{b}	113.35^{a}	109.15^{b}	1.182	0.022	0.005
IgM	5.28	5.41	5.31	5.51	5.43	0.348	0.729	0.833
TNF-α, ng/mL	62.34	55.82	54.53	51.28	52.30	4.601	0.135	0.327

ICA = isochlorogenic acid; C3 = complement 3; C4 = complement 4; IgA = immunoglobulin A; IgG = immunoglobulin G; IgM = immunoglobulin M; TNF-α, = tumor necrosis factor α.
Within a row, values with different superscripts indicate a significant difference ($n = 5$, $P < 0.05$).

concentration of IgG ($P = 0.005$) increased quadratically with ICA supplementation. A linear increase was also observed in the ileum SIgA concentration ($P = 0.005$) with ICA supplementation (Table 10).

3.6. The effects of ICA on antioxidant capacity

As shown in Table 11, although the serum activity of CAT was not affected by ICA supplementation, the activities of GSH-Px ($P = 0.005$) and SOD ($P = 0.040$) increased quadratically, while the content of MDA tended to decrease quadratically ($P = 0.058$) with ICA supplementation. The serum activity of T-AOC showed a linearly increase ($P = 0.003$) in response to ICA supplementation, with peak activity observed at a dietary concentration of 2000 mg/kg.

As shown in Fig. 2, the relative mRNA expression of *SOD-1* ($P = 0.089$) and *GSH-Px* ($P = 0.067$) both tended to increase quadratically with ICA supplementation.

3.7. The effects of ICA on microbial barriers in broilers

As depicted in Fig. 3A, the sparse curve exhibited a consistent trend, indicating that the amount of data sequenced was appropriate and that the diversity of all the samples tended to be saturated. Additionally, nearly all the samples in the OTU rank abundance curve approached saturation (Fig. 3B), suggesting that the data possessed sufficient depth to capture the diversity information of most of the samples. There were 819 OTUs common to all the subgroups, while 6421 OTUs were specific to the CON group, 6608 were specific to the T1 treatment, 6673 were specific to the T2 treatment, 8009 were specific to the T3 group, and 7721 were specific to the T4 treatment (Fig. 3C). Furthermore, no significant difference was found in α diversity ($P > 0.05$, Fig. 3D). The result of the PCA is presented in Fig. 3E. Principal component one (PC1) accounted for approximately 51.4%, and principal component two (PC2) explained approximately 21.3% of the floral communities in the terminal ileum. Taken together, they accounted for 72.7% of all the flora. Moreover, the groups exhibited clear differentiation, indicating that ICA supplementation pronounced an impact on terminal ileum microbial colonization in broilers.

The microorganisms in the terminal ileum of broilers at the phylum level were predominantly composed of four phyla: Firmicutes, Bacteroidetes, Proteobacteria, and Tenericutes (Fig. 4A). The combined proportion of these four phyla in each treatment was 97.03%, 98.79%, 99.14%, 98.64%, and 99.01%, respectively. Family-level (Fig. 4B), genus-level (Fig. 4C), and species-level (Fig. 4D) compositions are shown separately. Further analysis of the microbial community composition is presented in Fig. 4E. The abundance of Firmicutes was significantly higher in the ICA groups than in the CON treatment ($P = 0.008$). In contrast, the abundance of Bacteroidetes was significantly lower in both the T1 and T3 groups than in the CON treatment ($P = 0.024$). Additionally, the abundance of Streptococcaceae was significantly higher in the T3 treatment than in the T2 and CON treatments ($P = 0.026$). Meanwhile, Lactobacillaceae displayed significantly

Table 10
Effects of ICA on ileal SIgA of broilers (ng/g).

Item	ICA, mg/kg					SEM	*P*-value	
	0	500	1000	2000	3000		Linear	Quadratic
SIgA	1498.09^{b}	1592.17^{ab}	1648.34^{ab}	1803.08^{a}	1753.93^{a}	79.993	0.005	0.187

ICA = isochlorogenic acid; SIgA = secretory immunoglobulin A.
Within a row, values with different superscripts indicate a significant difference ($n = 5$, $P < 0.05$).

Table 11
Effect of ICA on serum antioxidation in broilers (U/mL, unless otherwise stated).

Item	ICA, mg/kg					SEM	*P*-value	
	0	500	1000	2000	3000		Linear	Quadratic
SOD	479.63^{b}	482.07^{b}	539.23^{a}	556.69^{a}	544.77^{a}	14.784	< 0.001	0.040
GSH-Px	381.29^{b}	414.37^{ab}	434.01^{a}	449.75^{a}	432.84^{a}	11.515	0.003	0.005
CAT	79.12	79.05	80.82	83.33	82.09	1.712	0.087	0.438
T-AOC	21.89^{c}	22.32^{bc}	23.19^{bc}	26.85^{a}	25.15^{ab}	0.958	0.003	0.154
MDA, nmol/mL	3.73^{a}	3.31^{ab}	3.42^{ab}	2.90^{b}	3.11^{b}	0.160	0.002	0.058

ICA = isochlorogenic acid; SOD = superoxide dismutase; GSH-Px = glutathione peroxidase; CAT = catalase; T-AOC = total antioxidant capacity; MDA = malondialdehyde.
Within a row, values with different superscripts indicate a significant difference ($n = 5$, $P < 0.05$).

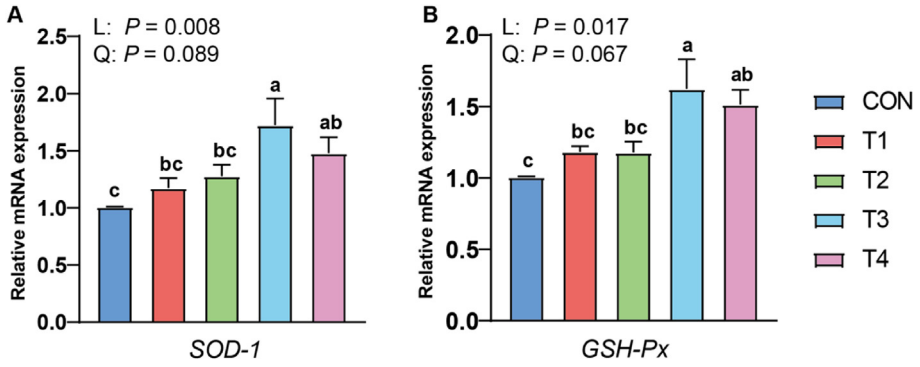

Fig. 2. Effect of ICA on mRNA relative expression of antioxidant-related genes in the ileum of broilers ($n = 5$). (A) The mRNA expression of *SOD-1* in the ileum; (B) The mRNA expression of *GSH-Px* in the ileum. ICA = isochlorogenic acid; *SOD-1* = superoxide dismutase 1; *GSH-Px* = glutathione peroxidase. Letters L and Q represent linear and quadratic effects of ICA supplementation dose, respectively. T1, T2, T3, and T4 represent the supplementation of the ICA product at 0, 500, 1000, 2000, and 3000 mg/kg, respectively. Bars with different letters indicate a significant difference ($n = 5$, $P < 0.05$).

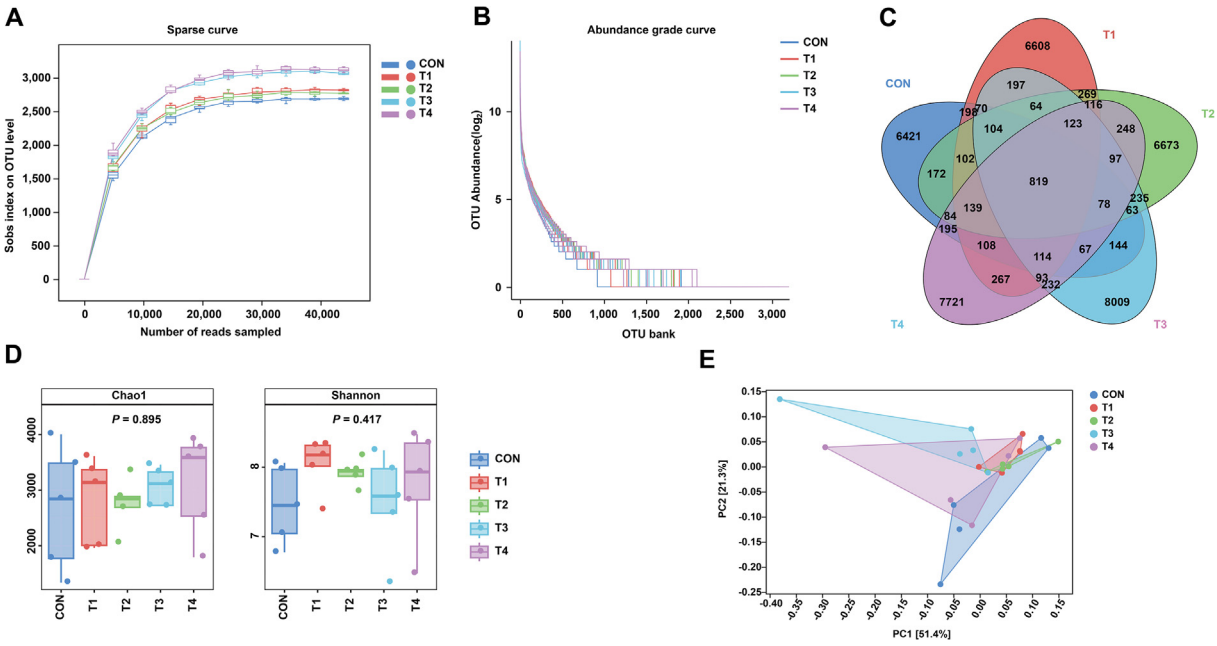

Fig. 3. The effects of ICA on the diversity of the intestinal flora in broilers ($n = 5$). (A) Sparse curve. (B) Abundance grade curve. (C) The Venn analysis of amplicon sequence variants (ASV). (D) Alpha diversity. (E) Principal component analysis. ICA = isochlorogenic acid. T1, T2, T3, and T4 represent the supplementation of the ICA product at 0, 500, 1000, 2000, and 3000 mg/kg, respectively.

higher abundance in the T3 treatment than in the T4 treatment ($P = 0.025$). Furthermore, *Streptococcus* demonstrated a significantly higher abundance in both the T3 and CON treatments than in the T2 treatment ($P = 0.026$), whereas *Lactobacillus* displayed significantly higher abundance in both the T3 and CON treatments than in the T4 treatment ($P = 0.025$). Moreover, the abundance of *Dehalobacterium* was significantly lower in the T3 treatment than in the T1 group ($P = 0.047$). *Streptococcus alactolyticus* showed significantly higher abundance in both the T3 and CON treatments than in the T2 treatment ($P = 0.047$), while *Lactobacillus helveticus* displayed a significantly higher abundance in the T3 and CON treatments than in the T4 treatment ($P = 0.029$). The Krona circle diagram in Fig. 4F illustrates the taxonomic levels, namely domain, kingdom, phylum, class, order, family, genus, and species, arranged in a hierarchical manner from the innermost to the outermost regions. The proportional size of each sector accurately represents the relative abundance of the distinct taxonomic units.

To further clarify the dose-dependent effect of ICA on intestinal microorganisms, a "one-against-all" linear discriminant analysis (LDA) effect size (LEfSe) analysis of the ICA treatment and the CON treatment (LDA >2.0) was conducted. The results showed that the ICA treatments significantly enriched p_Firmicutes, f_Ruminococcaceae, g_*Oscillospira*, o_Lactobacillales, and *g_Streptococcus* (Fig. 5A–D).

3.8. The effects of ICA on metabolites in broilers

Overall, the T3 treatment demonstrated superior growth performance, immune performance, and antioxidant function compared with the other treatments. Accordingly, the CON and T3 treatments were selected for further metabolomics analysis. There was a significant overlap in peak retention time and signal intensity of the base peak chromatogram in LC-MS, indicative of minimal variation caused by instrumental error throughout the testing process. This suggested excellent instrument stability and reliable results. The

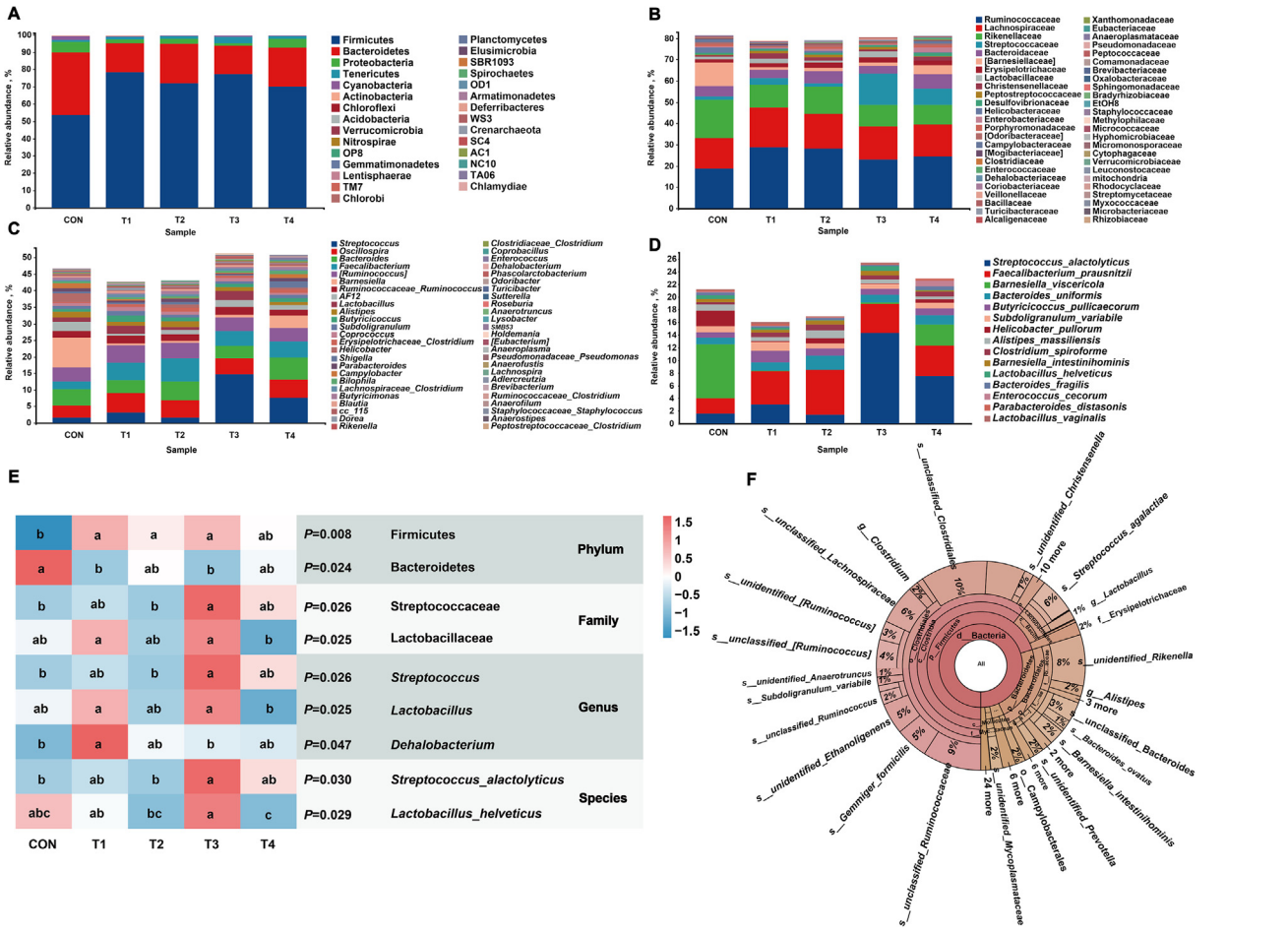

Fig. 4. The effects of ICA on species composition analysis of broilers ($n = 5$). The relative abundance of gut microbial community at the (A) phylum level, (B) family level, (C) genus level, and (D) species level; (E) heat maps of the differential bacteria; (F) Krona loop graph. ICA = isochlorogenic acid. T1, T2, T3, and T4 represent the supplementation of the ICA product at 0, 500, 1000, 2000, and 3000 mg/kg, respectively. Values with different letters indicate a significant difference ($P < 0.05$).

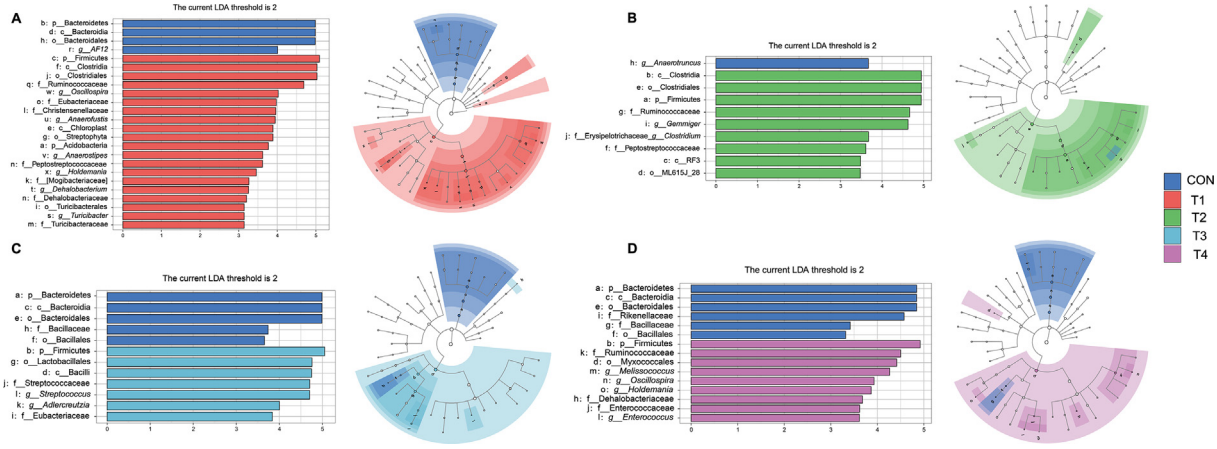

Fig. 5. The effect of ICA on LEfSe analysis of broilers ($n = 5$). (A) Differences in ileum microbes enriched between CON and T1 groups. (B) Differences in ileum microbes enriched between CON and T2 groups. (C) Differences in ileum microbes enriched between CON and T3 groups. (D) Differences in ileum microbes enriched between CON and T4 groups. CON = control; ICA = isochlorogenic acid. T1, T2, T3, and T4 represent the supplementation of the ICA product at 0, 500, 1000, 2000, and 3000 mg/kg, respectively.

OPLS-DA score plots in Fig. 6C and D demonstrated that samples were well-scattered between treatments while clustered within their respective treatments. Meanwhile, all the blue Q2 points from left to right were lower than the original blue Q2 points on the far right (Fig. 6 E and F). Additionally, the regression line crossed below zero on the vertical coordinate axis less frequently. Both plots confirmed the reliability and validity of the results. As shown in Fig. 6 G, a total of 901 metabolites were detected, with 85 differential metabolites being identified ($P < 0.05$, VIP > 1) in the terminal ileum. Among these, 65 were upregulated, while 20 were downregulated.

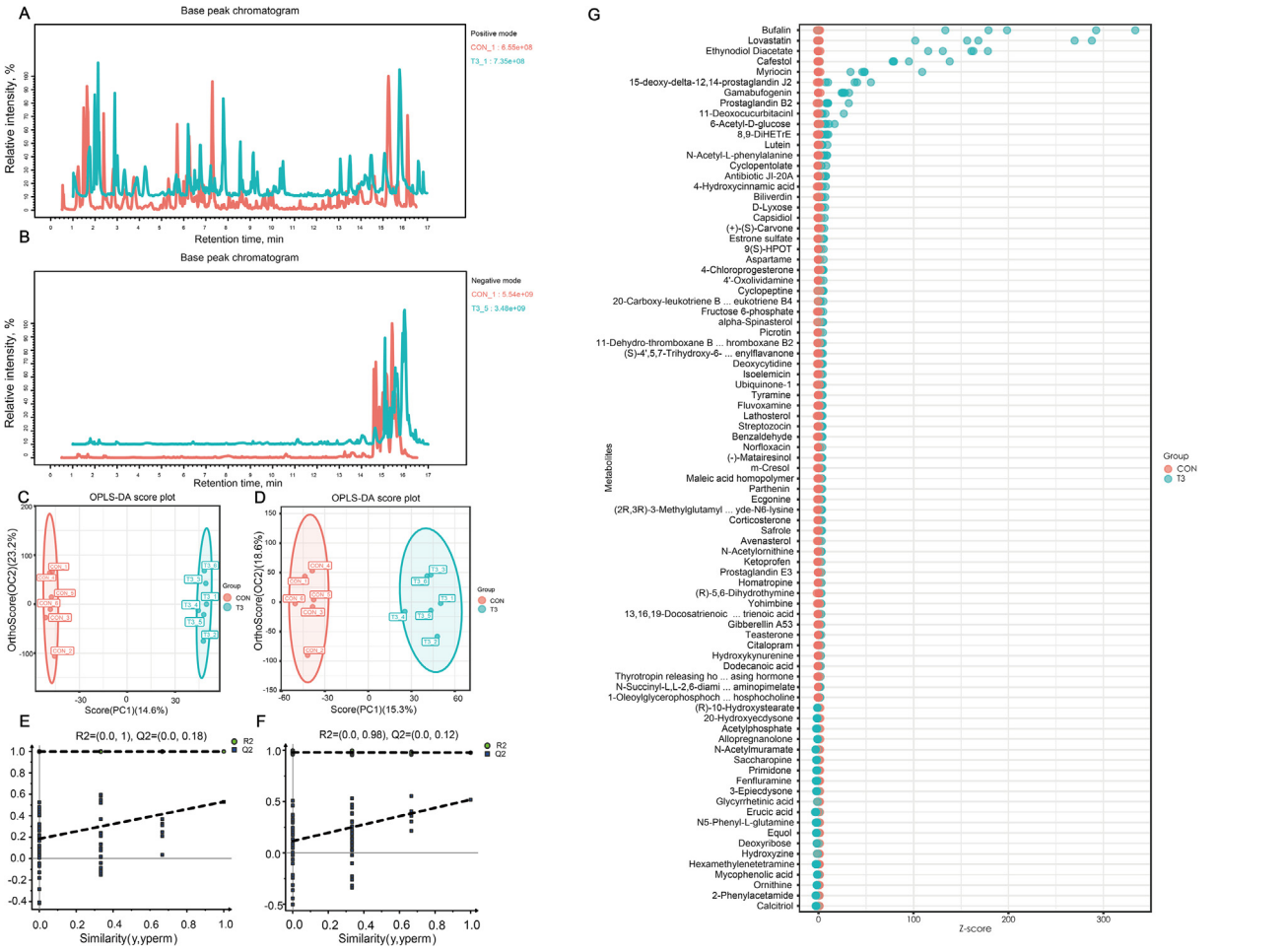

Fig. 6. The effects of ICA on metabolites in broilers. (A) Base peak chromatogram positive mode. (B) Base peak chromatogram negative mode. (C) OPLS-DA sore plot positive mode. (D) OPLS-DA sore plot negative mode. (E) OPLS-DA displacement chart positive mode. (F) OPLS-DA displacement chart negative mode. (G) Z-score plot. ICA = isochlorogenic acid; OPLS-DA = orthogonal partial least-square discriminant analysis. T3 represent the supplementation of the ICA product at 2000 mg/kg. The vertical coordinate is the name of the metabolite. The color of the points represents different treatments, and the horizontal coordinate is the relative metabolite content in the group obtained by Z-score conversion. The farther to the right, the more metabolites there are in the group.

To derive the metabolic pathway enrichment results, a pathway enrichment analysis was performed using the KEGG ID of differential metabolites and it was found that the six metabolic pathways that were enriched in the terminal ileum were arachidonic acid metabolism, lysine biosynthesis, serotonergic synapse, arginine biosynthesis, steroid biosynthesis (Fig. 7).

The heat map in Fig. 8 A shows the correlation of the differential metabolites with gut microbial, antioxidant, and immune indicators. Streptococcaceae, *Streptococcus*, and *Streptococcus_alactolyticus* were positively correlated with 8,9-diHETrE, 11-dehydro-thromboxane B2, 15-deoxy-delta-12,14-prostaglandin J2, N-succinyl-L, L-2,6-diaminopimelate, and (2R,3R)-3-methylglutamyl-5-semialdehyde-N6-lysine, while negatively correlated with 20-carboxy-leukotriene B4, saccharopine, and calcitriol. Fig. 8B shows a heat map of the association between differential metabolites and antioxidant indexes. Serum SOD and CAT were positively correlated with 8,9-diHETrE, 15-deoxy-delta-12,14-prostaglandin J2, and N-succinyl-L, L-2,6-diaminopimelate. Serum GSH-Px was positively correlated with 15-deoxy-delta-12,14-prostaglandin J2 and (2R,3R)-3-methylglutamyl-5-semialdehyde-N6-lysine, while negatively correlated with saccharopine. Serum T-AOC was positively correlated with 20-carboxy-leukotriene B4, N-succinyl-L, L-2,6-diaminopimelate, and (2R,3R)-3-methylglutamyl-5-semialdehyde-N6-lysine, while negatively correlated with saccharopine. Serum MDA was positively correlated with saccharopine, while negatively correlated with 8,9-diHETrE, N-succinyl-L, L-2,6-diaminopimelate, and (2R,3R)-3-methylglutamyl-5-semialdehyde-N6-lysine. The gene *SOD-1* was positively correlated with 15-deoxy-delta-12,14- prostaglandin J2 while negatively correlated with 20-hydroxyecdysone. The gene *GSH-Px* was positively correlated with prostaglandin B2 while negatively correlated with 20-hydroxyecdysone. Complement C3 was positively correlated with 8,9-diHETrE, 11-dehydro-thromboxane B2, and 15-deoxy-delta-12,14- prostaglandin J2, while negatively correlated with calcitriol. IgG was positively correlated with 15-deoxy-delta-12,14- prostaglandin J2. A heat map of the association of significantly differential microorganisms with antioxidant indicators is shown in Fig. 8C. Serum SOD, CAT, IgG, and the *SOD-1* gene in the ileum were positively correlated with Streptococcaceae, *Streptococcus*, and *Streptococcus_alactolyticus*. The genes *SOD-1* and *GSH-Px* were positively correlated with Firmicutes while negatively correlated with Bacteroidetes.

4. Discussion

Large-scale intensive farming practices have contributed to stress and impaired intestinal health of broilers. These can result in gut disorders and suboptimal intestinal health during the breeding

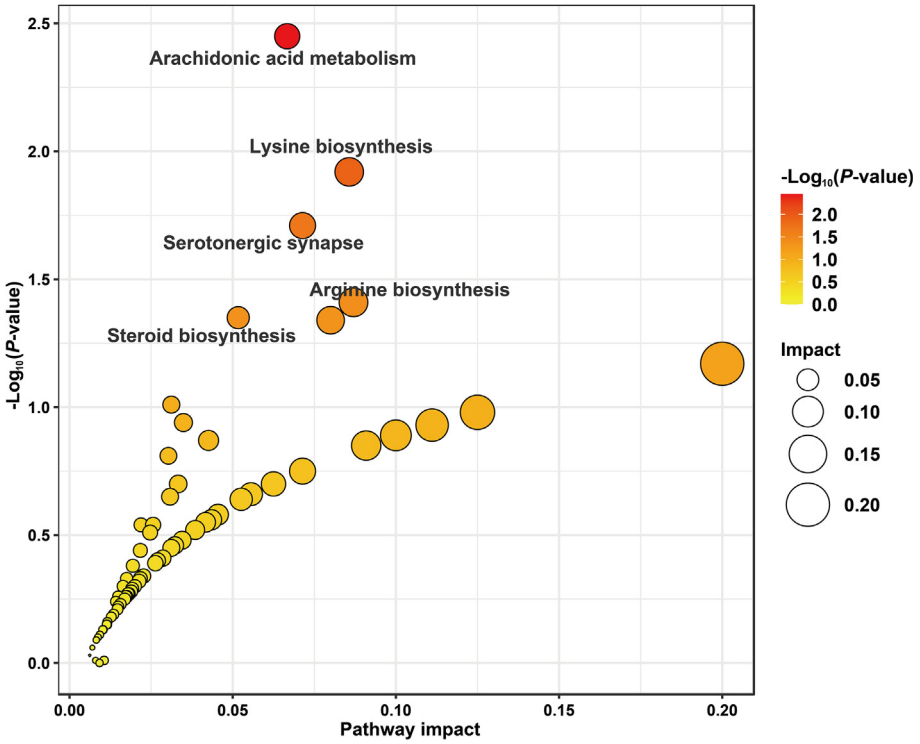

Fig. 7. Bubble map of metabolic pathway influencing factors. The larger the dot, the greater the influence on the metabolic pathway; the darker the color, the smaller the *P*-value.

process, ultimately affecting growth performance (Ducatelle et al., 2023). Growth performance serves as a crucial indicator of the growth and development of poultry as well as the effectiveness of feed. It also represents the outcomes of nutrient intake in terms of digestion, absorption, and metabolic deposition. ICA has superior physiological activity compared to its isomer CA (Cortes-Morales et al., 2019). Chen et al. (2022) demonstrated that incorporating varying levels of CA into animal diets enhanced the growth performance. Liu et al. (2023),b revealed that the supplementation broilers diets with CA increased ADG while reduced the F/G ratio. In this study, ADG exhibited a quadratic increase in response to ICA supplementation during both the 1 to 42 d and 22 to 42 d periods, with the peak performance observed at a dietary concentration of 2000 mg/kg. With ICA supplementation, the F/G decreased linearly from 1 to 21 d, decreased quadratically from 22 to 42 d, and decreased quadratically from 1 to 42 d.

The supplementation of acidifying agents to feed or drinking water has been demonstrated to moderately improve animal growth performance (Hamid et al., 2018). In this regard, ICA can serve as a biologically active feed acidifying agent that increases ration acidity, potentially inhibiting the growth of harmful substances in the ration and reducing gastrointestinal pH. Moreover, ICA can regulate the structure of the intestinal flora and create an optimal intestinal environment in broilers (Abdel-Fattah et al., 2008). Consequently, ICA supplementation can lead to an increased daily weight gain, reduced F/G ratio, improved apparent digestibility, and promote a healthy growth rate in broilers.

Maintaining mucosal homeostasis and its nutrient absorption capacity is critically dependent on structural and functional integrity. In broilers, parameters such as intestinal villus height, crypt depth, and the villus height-to-crypt depth ratio are key indicators for evaluating intestinal development and nutrient absorption efficiency. Increased intestinal villus height expands the absorptive surface area, improving nutrient absorption efficiency. A shallower crypt depth is associated with accelerated maturation of intestinal epithelial cells and enhanced secretory activity. Moreover, a higher villus height-to-crypt depth ratio reflects a more structurally intact intestinal mucosa (Ząbek et al., 2020). This study demonstrated a dose-dependent quadratic increase in both villus height and the ratio of villus height to crypt depth, concomitantly with a tendency for a quadratic decrease in crypt depth. Consistent with our findings, Ma et al. (2021),b observed that dietary supplementation with mixed organic acids significantly increased ileal villus height and the villus height-to-crypt depth ratio in broilers, suggesting a promotive effect on intestinal epithelial cell development. Similarly, Goh et al. (2022) reported that organic acids enhanced villus morphology and growth performance in weaned piglets. These findings align with the present study, indicating that ICA supplementation promotes intestinal development and nutrient absorption in broilers. Additionally, short-chain fatty acids stimulate intestinal epithelial cell proliferation, improve small intestinal morphology, and maintain gut integrity (Tan and Coussens, 2007).

Tight junctions play a critical role in regulating epithelial cell permeability and maintaining cellular polarity, thereby preventing the passage of pathogens and macromolecules (Sánchez de Medina et al., 2014). Occludin plays a crucial role in regulating the selective permeability of macromolecules through the paracellular pathway and maintaining the integrity of tight junctions. Additionally, the phosphorylation of claudin-1 enhances the barrier function of tight junctions (Heinemann and Schuetz, 2019). It was demonstrated that the intragastric infusion of short-chain fatty acids significantly increased the relative mRNA expression levels of claudin-1 and occludin in both the duodenum and ileum of weaned piglets (Diao et al., 2019). This finding suggests that short-chain fatty acids promote intestinal barrier function and facilitate intestinal development in these animals. In this study, the relative mRNA expression levels of claudin-1 and occludin increased linearly with the increasing ICA supplementation. It is plausible that ICA modulates the intestinal barrier physically by reducing the adherence of

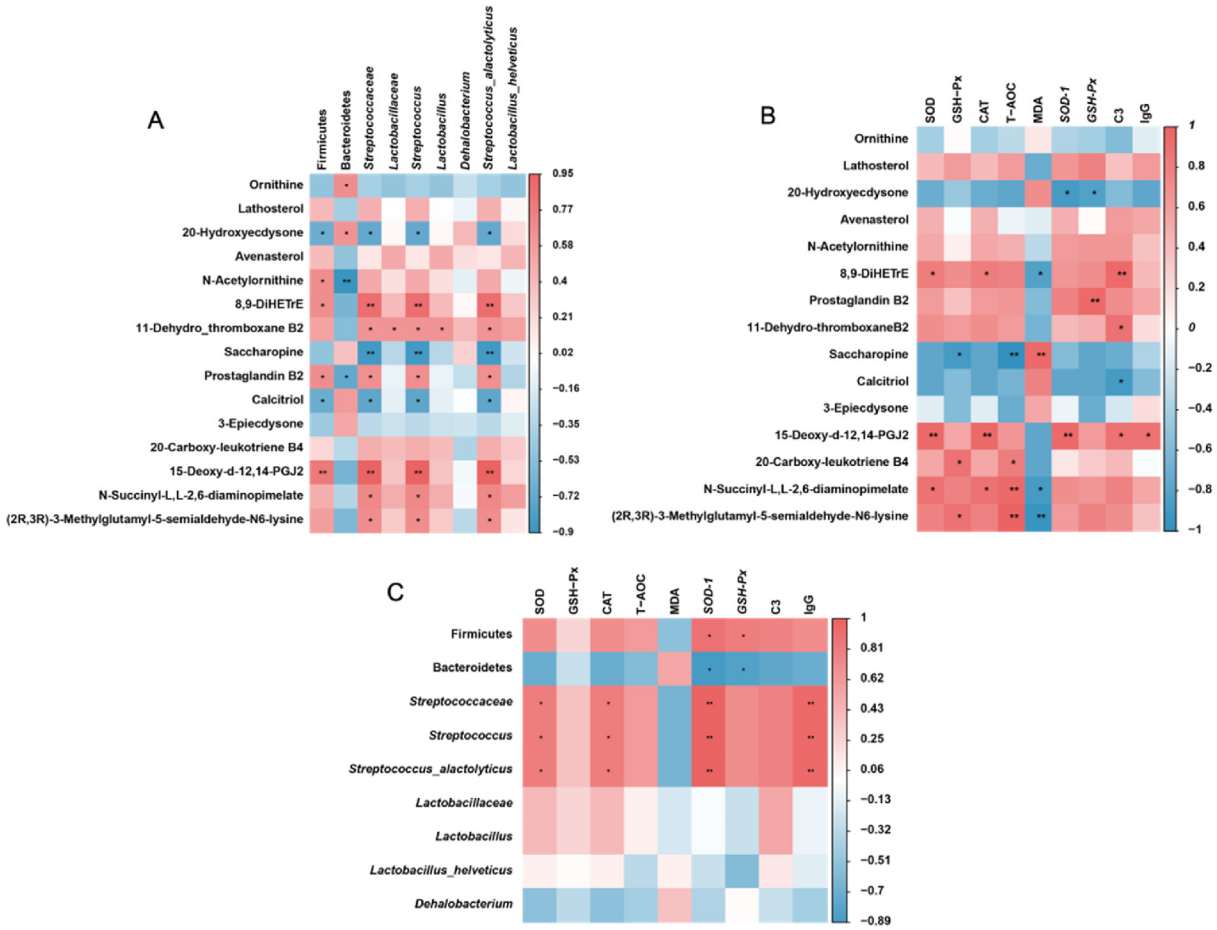

Fig. 8. Antioxidant and immune–microbiome–metabolome associations in the ileum. The color depth is positively correlated with the correlation coefficient. In the figure, red circles indicate positive correlation and blue indicates negative correlation. (A) Correlation analysis between ileum differential microbes and DAMs. (B) Correlation analysis between antioxidant indices and ileum DAMs. (C) Correlation analysis between antioxidant indices and ileum differential microbes. DAMs = differential accumulated metabolites; SOD = superoxide dismutase; *SOD-1* = superoxide dismutase 1; *GSH-Px* = glutathione peroxidase; CAT = catalase; T-AOC = total antioxidant capacity; MDA = malondialdehyde; C3 = complement 3; IgG = immunoglobulin G. *$P < 0.05$; **$P < 0.001$.

pathogenic bacteria, thereby preventing their invasion (Ma et al., 2022). This mechanism enhances ileum barrier function in broilers, contributing to maintaining internal environment homeostasis and supporting intestinal health.

The mucus layer's chemical barrier consists of a gel-like substance secreted by intestinal epithelial cells and digestive glands, which is crucial for maintaining intestinal mucosal integrity. Evaluation of digestive enzyme activity serves as a key indicator of the intestinal tract's digestive and absorptive capabilities, reflecting overall gastrointestinal health (Chen and Zhao, 2019). The presence of caffeic acid and quinic acid in the structure of ICA enables ICA to act as an acidifier, and the results of the present experiment also demonstrated that the addition of ICA to the ration significantly reduced the pH of the stomach, jejunum, and ileum, thereby creating an acidic environment within the gastrointestinal tract. Adding mixed organic acids to broiler diets significantly increased pancreatic amylase activity (Ma et al., 2021,b), and supplementing lactic acid in the diet significantly increased pancreatic lipase activity, thereby improving the apparent digestibility of CP, Ca, P, DM and GE (Bai et al., 2019). This indicates that a decrease in intestinal pH within a certain range can improve digestive enzyme activity, thereby improving the efficiency of nutrient digestion and absorption. The results of the present study showed a linear increase in trypsin and lipase activities, a quadratic increase of trypsin activity (peaked at a dose of 2000 mg/kg), and a tendency of quadratic increase of the digestibility of CP and Ca, with ICA supplementation.

The increase in trypsin levels may be attributed to the ability of ICA, an organic acid, to effectively lower the gastrointestinal pH of broilers within a specific range. There is an improvement in pancreatic secretion and enhanced activity of digestive enzymes, which aids in the digestion of dietary proteins, and a concomitant increase in average daily weight gain.

The observed increase in pancreatic lipase activity may stem from multiple mechanisms. ICA likely stimulates proteolytic enzyme secretion, enhances nutrient breakdown and absorption in chyme, promotes intestinal development, facilitates lipase production, and augments digestive capacity (Owens et al., 2008). Volatile fatty acids are short-chain fatty acids primarily produced through anaerobic bacterial fermentation of undigested carbohydrates in food. They typically comprise carbon chains ranging from 1 to 6 carbons, encompassing acetic acid, propionic acid, butyric acid, isobutyric acid, valeric acid, and isovaleric acid. The primary outcomes arising from the breakdown of dietary fiber by gut bacteria are VFAs (Corrêa-Oliveira et al., 2016; Yi et al., 2021).

Incorporating a combination of diverse organic acids in the diet led to a notable decrease in ileum pH and an elevation in the concentrations of acetic acid, butyric acid, and TVFAs among

mature pigs (Li et al., 2019,b). The current experiment showed that supplementing broiler diets with different levels of ICA enhanced the concentrations of propionic acid, butyric acid, isobutyric acid, and isovaleric acid in the gut. Moreover, the concentration of TVFA increased quadratically, and the concentrations of acetic acid and butyric acid tended to increase quadratically with ICA supplementation at 2000 mg/kg. Dietary supplementation with 15 to 30 nmol/L acetic acid could provide energy for anaerobic bacteria while reducing gastrointestinal pH and promoting the growth of *Bifidobacterium* (Pastell et al., 2009). Volatile fatty acids, as bacterial post-fermentation products, could energize the intestinal mucosal epithelial cells and accelerate the formation of intestinal barriers, which was beneficial to broilers in resisting the invasion of pathogens and further enhancing their growth performance (Ma et al., 2021,b).

The immune system is a crucial defense mechanism against environmental pathogens, mitigating reproductive losses. Serum immunoglobulins and complement proteins are vital constituents of the immune system, with their levels in the bloodstream serving as crucial indicators of overall immune function (Tan and Coussens, 2007). IgA binds antigens, aiding in their clearance without causing inflammation. IgG contributes to humoral immunity, phagocytosis, agglutination, and precipitation. IgM regulates bacterial infections during the initial stages (Bai et al., 2021). ICA, functioning as a feed acidifier, has been shown to enhance immune function in livestock and poultry, thereby improving broiler health (Dittoe et al., 2018). Caffeic acid supplementation significantly increased immunoglobulin levels in beluga whales (Ahmadifar et al., 2022). In this study, IgG levels exhibited a quadratic increase with ICA supplementation at 2000 mg/kg. This finding suggests that ICA supplementation may enhance humoral immune response in broilers, potentially contributing to improved immune function and overall health.

ICA enhances immune performance through various mechanisms, including activation of the calcium-modulated phosphatase-mediated signaling pathway in neurons, enhancement of macrophage phagocytosis, and promotion of B- and T-lymphocyte proliferation and differentiation, thereby improving overall immune function (Wu et al., 2004). The serum level of C3, a crucial complement protein situated at the convergence point of all complement activation pathways (Martin et al., 2022), was significantly increased in broilers when ICA was supplemented to the diet, indicating an augmentation in specific immune function. Consequently, including ICA in broiler diets significantly increased serum immunoglobulin and complement content, ultimately improving overall immunity. SIgA is secreted by plasma cells within the intestinal mucosa and is a barrier against bacterial adhesion on the mucosal surface. Additionally, SIgA can form complexes with antigens and thus prevent their entry into the mucosa, as well as stimulate the secretion of large amounts of mucus by goblet cells to protect the intestinal tract (He et al., 2020). Our findings demonstrated that dietary supplementation with ICA significantly elevated SIgA levels in the ileum of broilers, suggesting that ICA may enhance intestinal immune status in these birds.

Enhancing antioxidant capacity is pivotal for improving immune function. T-AOC and the activities of SOD and GSH-Px are critical indicators of the body's ability to neutralize oxygen-free radicals, playing a vital role in regulating and protecting cellular metabolism (Suair et al., 2018). In this study, the activities of SOD and GSH-Px showed a quadratic increase in response to dietary ICA supplementation at 2000 mg/kg, whereas malondialdehyde (MDA) concentration showed a quadratic decrease, indicating that dietary supplementation with ICA at 2000 mg/kg significantly enhances the organism's antioxidant capacity. Similarly, dietary supplementation with CA, extracted from honeysuckle, markedly elevated serum levels of T-AOC, SOD, and GSH-Px while reducing MDA concentrations in fattening pigs, further supporting its role in bolstering antioxidative potential (Liu et al., 2023,b).

Dietary supplementation with CA extracted from stevia residue significantly enhanced serum superoxide dismutase (SOD) activity and reduced malondialdehyde (MDA) concentrations in fattening pigs (Xiong et al., 2022). The antioxidant capacity of piglets was enhanced through dietary supplementation with CA, resulting in improved growth performance and a decrease in the occurrence of diarrhea (Chen et al., 2018). ICA contains phenolic hydroxyl groups that readily react with free radicals, providing protons and electrons for neutralization, thereby protecting against oxidative damage (Hou et al., 2017). The phenolic hydroxyl group of ICA can also chelate with oxidation-inducing transition metal ions, such as Fe^{2+} and Cu^{2+}, forming complexes that inhibit chain oxidation reactions. This indirectly provides a defense mechanism against peroxidation induced by reactive oxygen species (Cruciani et al., 2019). The conformational effect of the cyclohexane skeleton of ICA also influences its antioxidant capacity. At the same time, the relative positions of its two caffeic acid moieties explain the variations in its antioxidant capacities (Liu et al., 2019).

Based on these findings, the impact of ICA on the mRNA expression levels of antioxidant-related genes in broiler ileum tissues was further investigated. The results indicate that the relative mRNA expression of *SOD-1* and *GSH-Px* tended to increase quadratically with ICA supplementation, peaking at ICA at 2000 mg/kg diet. The previous study found that CA activates the Nrf2 pathway by suppressing the expression of CYP2E1 and CYP4A, leading to an increase in the levels of heme oxygenase-1 (HO-1) and NADPH quinone dehydrogenase-1 expression, and this effect reduces ROS production and helps to alleviate oxidative damage (Liu et al., 2018). The hydrogen atoms in caffeoylquinic acid likely contribute to scavenging free radicals and inhibiting oxidative reactions (Liang and Kitts, 2015).

Maintaining the host's metabolic health heavily relies on stabilizing the intestinal microbiota (Guevarra et al., 2019). Disturbance to the microbiota can negatively impact the well-being of animals and undermine their ability to mount an effective immune defense (Fan and Pedersen, 2021). Organic acids significantly affect poultry gastrointestinal tract function by depolarizing bacterial membranes and increasing internal bacterial acidity, thereby modulating microbial populations. Nutrient digestibility also influences gut microbiota composition (Yin et al., 2018). In this experiment, an increase in the relative abundance of Firmicutes at the phylum level was observed, accompanied by a decrease in the presence of Bacteroidetes. The Firmicutes play a crucial role in breaking down external peptides and amino acids, while the members of Bacteroidetes specialize in degrading cellulose, hemicellulose, and pectin (Li et al., 2019,b; Yan et al., 2017). It has been demonstrated that caffeic acid is the most potent inhibitor of the growth of Bacteroidetes anisopliae (Cueva et al., 2010). Studies investigating organic acid supplementation in feed have reported inconsistent findings due to variations in fecal fluid source and dosage (Bruno et al., 2019; Yang et al., 2020). At the level of species, there was a rise in the proportionate prevalence of *Streptococcus_alactolyticus*, which was initially recognized as a prevailing bacterial species responsible for producing lactic acid in the gastrointestinal tracts of chickens and pigs (Czerwiński et al., 2010). Short peptides produced through fermentation by lactic acid bacteria can lower cholesterol levels while enhancing antioxidant capacity and immune functions. Proteins undergo degradation into short peptides and amino acids during cow milk fermentation using a combination of *Streptococcus hygroscopicus* and *Lactobacillus* colony cultures, which enhances the probiotic properties of yogurt (Ghosh et al., 2013; Hagi et al., 2016). This implies that *Streptococcus_alactolyticus* contributes to improved antioxidant capacity

and immune functions. The antimicrobial properties of phenolic acids are of paramount importance in preventing the formation of intestinal bacterial biofilms by inhibiting the growth and adhesion of harmful bacteria, thus preventing intestinal infections, increasing nutrient utilization, and improving poultry performance.

In the current study, an untargeted metabolomic analysis of the CON and T3 treatments was undertaken, revealing that ICA primarily affected two metabolic pathways: arachidonic acid metabolism and lysine biosynthesis. Arachidonic acid is a polyunsaturated fatty acid consisting of a chain of 20 carbon atoms and characterized by four double bonds, making it prone to oxidation. This leads to the excessive production of metabolites crucial for the functioning of the immune system, suppression of inflammation, and regulation of feed intake (Hanna and Hafez, 2018). The lysine biosynthesis pathway differs from other amino acids and varies according to the microorganism. Bacteria require diamino-heptane dioic acid synthesis for lysine production, while yeast and molds require α-aminoadipic acid synthesis (Xu et al., 2019). Lysine has been proven to boost animal growth, enhance immune response and antioxidant ability, and optimize intestinal structure (Alabi et al., 2017; Konieczka et al., 2022; Menegat et al., 2020). The microbiota and metabolites mentioned above are associated with immunity and antioxidants, so the assessment of the correlation of differential metabolites in distinct metabolic pathways with antioxidant indicators and differentially abundant microorganisms suggested that ICA influences the levels of these metabolites by modulating gut microorganisms. This affects the uptake of these metabolites and their transportation by intestinal epithelial cells to the liver, ultimately impacting organismal physiological functions.

In the terminal ileum, positive correlations were found between microorganisms (*Streptococcus_alactolyticus*) and metabolites (15-deoxy-delta-12,14- prostaglandin J2; N-succinyl-L, L-2,6-diaminopimelate; and (2R,3R)-3-methylglutamyl-5-semialdehyde-N6-lysine). This result suggested that the increase in *Streptococcus_alactolyticus* abundance in the terminal ileum was associated with the positive correlation found between this species and 15-deoxy-delta-12,14- prostaglandin J2; N-succinyl-L, L-2,6-diaminopimelate; (2R,3R)-3-methylglutamyl-5-semialdehyde-N6-lysine; and other metabolites, resulting in increased antioxidant and immune functions. 15-Deoxy-delta-12,14-prostaglandin J2 is a prostaglandin derived from arachidonic acid that exerts anti-inflammatory, antioxidant, antitumor, and other biological activities by activating the PPAR-γ signaling pathway (Ueno et al., 2001). The previous study found that 15-deoxy-delta-12,14- prostaglandin J2 inhibits the NF-κB inflammatory pathway by reducing TNF-α levels through modulating proteasome activity (Marcone et al., 2016). Studies have shown that 15-deoxy-delta-12,14- prostaglandin J2 attenuates lung infections in rats by inhibiting the expression of pro-inflammatory factors, such as TNF-α and IL-10, and chemokines, such as CCL2, CCL3, and CCLA (Cloutier et al., 2012; Maehara et al., 2019). The previous study showed that the administration of 15-deoxy-delta-12,14- prostaglandin J2 to mice suffering from unilateral ureteral obstruction (UUO) resulted in an increase in the protein expression of Nrf2 and HO-1 in the animals and also prevented protein carbonylation, a UUO-induced marker of oxidative stress (Nilsson et al., 2017). In addition, it has been demonstrated that oxidative stress induces intracellular heat shock 70 (HSP70) and that 15-deoxy-delta-12,14- prostaglandin J2 modulates the anti-inflammatory pathway by regulating HSP70, decreasing the levels of NF-κB and COX-2, and mitigating oxidative stress in chondrocytes (Bianchi et al., 2014). N-Succinyl-L, L-2,6-diaminopimelate and (2R,3R)-3-methylglutamyl-5-semialdehyde-N6-lysine exhibited significant upregulation within the lysine biosynthetic pathway. The N-succinyl-L, L-diaminopimelate pathway involves the enzymatic action of desuccinylase to produce L, L -diaminopimelate and succinate from N-succinyl-L, L-diaminopimelate (Born et al., 1998). This indicates that ICA influences gut microflora, thereby regulating metabolite levels and impacting metabolic pathways associated with antioxidant and immune properties. However, the mechanism underlying these interactions requires further exploration.

5. Conclusion

In conclusion, this study demonstrated that dietary supplementation with ICA could increase feed digestibility, optimize intestinal morphology, enhance antioxidant capacity, and augment immune responses in broilers, consequently leading to improved growth performance. Specifically, ICA fortified the intestinal barrier by upregulating tight junction proteins, improved digestive enzyme activity and VFAs production, boosted SIgA secretion, and modulated gut microbiota and metabolites. Furthermore, ICA improved intestinal immune function through its antioxidative properties. Considering broilers' growth performance and intestinal immune function, the group supplemented with ICA at 2000 mg/kg showed the best performance in this experiment.

Credit Author Statement

Siyuan Zhou: Writing – original draft, Software, Methodology, Investigation, Formal analysis, Conceptualization. **Haotian Jiang:** Writing – original draft, Data curation. **Yujia Wang:** Visualization, Conceptualization. **An Yan:** Investigation. **Guohua Liu:** Supervision, Resources. **Shudong Liu:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization. **Baojiang Chen:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

Acknowledgments

This study was supported by the Natural Science Foundation of Hebei Province, China (C2024204217 and C2022204218), the earmarked fund for CARS-41 of China, and the Innovation Program of the Chinese Academy of Sciences, China (CAAS-IFR-ZDRW202401).

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