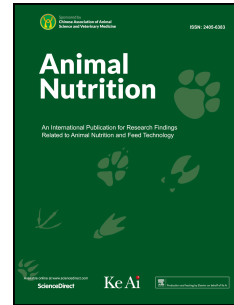


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Dietary supplementation of isochlorogenic acid improved growth performance and intestinal health of broilers

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1 **Dietary supplementation of isochlorogenic acid improved growth performance**
2 **and intestinal health of broilers**

3

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19

20 **Abstract**

21 In this research, we examined the impact of incorporating isochlorogenic acid (ICA)
22 into broiler diets on growth performance and intestinal health. A total of 400 Cobb
23 broilers (44.20 ± 0.16 g, one day old) were randomly divided into 5 treatments with 8
24 replicates of 10 birds each, including a control, and four ICA treatments. The ICA was
25 supplemented at 0, 500, 1000, 2000, and 3000 mg/kg feed, respectively. The
26 experiment lasted for 42 d. The broilers were fed ad libitum and had free access to
27 water. The feed-to-gain ratio (F/G) decreased quadratically with increasing ICA
28 supplementation from 22 to 42 d ($P = 0.039$) and 1 to 42 d ($P = 0.048$), and was the
29 lowest at the supplementation of 2000 mg/kg ICA. The apparent total tract
30 digestibility of calcium ($P = 0.038$) and crude protein ($P < 0.001$) increased linearly
31 with increasing supplementation of ICA. The height of the villi and the ratio of villus
32 height to crypt depth increased quadratically ($P = 0.027$) with increasing ICA
33 supplementation. The relative mRNA expression of ileum claudin-1 ($P = 0.003$) and
34 occludin ($P = 0.048$), and gastrointestinal pH ($P < 0.05$) decreased linearly with
35 increasing supplementation of ICA. The ileum SIgA concentration ($P = 0.005$)
36 increased linearly, whereas the concentration of total volatile fatty acid ($P = 0.038$) in
37 the ileum, trypsin activity ($P = 0.016$) and blood concentration of IgG ($P = 0.005$)
38 increased quadratically with increasing ICA supplementation. The activities of serum
39 GSH-Px ($P = 0.005$) and SOD ($P = 0.040$) increased quadratically, while the relative
40 mRNA expression of *SOD-1* ($P = 0.040$) and *GSH-Px* ($P = 0.040$) in the ileum
41 increased linearly with increasing ICA supplementation. *Streptococcus alactolyticus*
42 abundance was significantly higher in the broilers supplemented with ICA at 2000
43 mg/kg than in the control treatment ($P = 0.030$) and the concentration of metabolites
44 such as 15-deoxy-d-12,14-PGJ2 was increased by ICA supplementation. The dietary

45 supplementation of ICA enhanced growth performance of broilers by increasing the
46 activity of digestive enzymes, which resulted in greater feed digestibility and better
47 health status by maintaining gut pH and increasing antioxidant and immune status.
48 The optimal supplemental level of ICA was from 2016.42 to 2431.91 mg/kg for
49 maximising growth performance and economic benefits in broilers.

50

51 **Keywords:** Isochlorogenic acid; Broiler; Growth performance; Gut health

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68 1. Introduction

69 The broiler industry is undergoing rapid and stable development as the demand for
70 chicken meat has gradually increased. Broilers exhibit a rapid growth rate, alongside a
71 highly delicate and vulnerable intestinal tract (Kogut et al., 2016). The intestinal tract,
72 in addition to its role as the largest digestive organ, also functions as the primary
73 immune organ in poultry, serving as their foremost line of defense. It plays a crucial
74 role in ensuring optimal health through its innate immune system (Kogut et al., 2017).
75 The intestinal barrier functions of poultry include physical, chemical, microbial, and
76 immune barriers, which synergistically safeguard the intestinal well-being of the
77 birds.

78 Over recent years, numerous studies have demonstrated the potential of natural plant
79 extracts to improve gut health in animals (Capasso and Di, 2020). Isochlorogenic acid
80 (ICA) belongs to the dicaffeoylquinic acid class of compounds and is formed through
81 the mangiferic acid pathway during plant aerobic respiration. The compound is a
82 derivative of phenol and is produced by the condensation reaction between quinic acid
83 and caffeic acid, and serves as an isomer of chlorogenic acid (Pamisetty et al., 2018).
84 Multiple research studies have provided evidence that the inclusion of chlorogenic
85 acid in animal feed can significantly enhance livestock and poultry production
86 performance, immune function, antioxidant capacity, and intestinal health (Jin et al.,
87 2023). However, chlorogenic acids have limitations that restrict their application,
88 including chemical instability, limited solubility in lipids, suboptimal bioavailability,
89 and susceptibility to degradation mediated by esterases (Psotová et al., 2004).
90 Isochlorogenic acid has a more symmetrical chemical structure than chlorogenic acid
91 as it contains two caffeoyl groups and multiple hydroxyl groups that contribute to its

92 biological activities (Cortes-Morales et al., 2019; Kim et al., 2017; Wang et al., 2016).
93 Isochlorogenic acid contains phenolic acids and exhibits superior antioxidant,
94 antibacterial, antiviral, hepatoprotective, and anti-inflammatory properties compared
95 with chlorogenic acid.

96 There is a limited body of research examining the impacts of ICA in broilers.
97 The present study aimed to explore the potential advantages of incorporating ICA into
98 broiler diets, specifically focusing on its effects on growth performance, intestinal
99 morphology, digestive enzyme activity, volatile fatty acid production, antioxidant
100 capacity, immune response, intestinal microbiota composition, and metabolic
101 pathways in broilers. Our objective was to determine the optimal dosage of ICA
102 supplementation and provide valuable data for its practical application in broiler
103 production.

104 2. Materials and methods

105 2.1 Animal ethics

106 This study was conducted between June 2022 and December 2022 at the Animal
107 Husbandry Teaching Base of Hebei Agricultural University in Baoding, P. R. China.
108 The experimental protocol (2023110) was approved by the Institutional Animal Care
109 and Use Committee of Hebei Agricultural University.

110 2.2 Experimental diet

111 The ICA (with a purity of $\geq 55\%$) used in the present experiment was developed
112 and provided by Chenguang Biotechnology Co., Ltd., China. It contained
113 approximately 10% crude fiber, 8% ash, and 7% moisture (measured and provided by

114 the company). The experimental diets were formulated according to China National
115 Feeding Standard of Chicken (NY/T 33-2004) to meet the nutrient requirements as
116 shown in Table 1.

117 2.3 Experimental design, animals, and management

118 The feeding trials were conducted at the experimental farm of Hebei Agricultural
119 University. Four hundred healthy Cobb broilers (half male and half female) at 1 d of
120 age and with similar body weight (44.20 ± 0.16 g) were randomly divided into five
121 treatments. The broilers in the control (CON) group were fed a basal diet with no ICA
122 and the remaining four treatments (T1, T2, T3 and T4) were fed diets supplemented
123 with ICA at 500, 1000, 2000, and 3000 mg/kg, respectively. Every treatment had 8
124 replicates with 10 broilers in each replicate. They were raised in cages. Feed and
125 drinking water were supplied freely to all the experimental groups throughout the
126 experiment and the feeding experiment lasted for 42 d.

127 2.4 Determination of growth performance

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

132 $ADG (g/d) = \text{body weight change (g)}/\text{number of days (d)};$

133 $ADFI (g/d) = \text{cumulative feed intake}/(\text{number of birds} \times \text{number of days});$

134 $F/G = \text{feed intake (g)}/\text{weight gain (g)}.$

135 2.5 Nutrient digestibility

136 The endogenous indicator method was employed to assess the nutrient
137 digestibility of broilers in each treatment (acid-insoluble ash, AIA). The dry matter
138 (DM, method 930.15), calcium (Ca, method 978.02), phosphorus (P, method 946.06),
139 and crude protein (CP, method 990.03) of feed and feces were determined according
140 to AOAC (2019) standards. The gross energy (GE) was determined using an
141 automatic bomb calorimeter (Parr 6300 Calorimeter; Parr Instrument Company,
142 Moline, IL, USA). The feed amino acid composition was analysed using ultra-
143 performance liquid chromatography (Agilent 1260, Agilent Technologies Co., Ltd.,
144 Beijing, China) according to China National Standard (GB/T 18246-2019). The
145 metabolic energy (ME) of the dietary formulation was calculated according to
146 previously prescribed criteria (Xiong et al, 2020). Formulation consisted of corn (ME
147 = 13.56 MJ/kg), soybean meal (ME = 10.58 MJ/kg) and soybean oil (ME = 35.02
148 MJ/kg) as major ingredients mixed in a certain proportion. If the proportion of each
149 raw material in the formula was a%, b% and c%, the ME value of the formula was
150 calculated as:

$$151 \quad \text{ME} = 13.56 \times a\% + 10.58 \times b\% + 35.02 \times c\%$$

152 2.6 Measurement of the pH of the gastrointestinal tract

153 At the conclusion of the experiment, xx number of broilers were selected from
154 each replicate cage based on their body weight which closely matched the average
155 weight. The digesta of the stomach, duodenum, jejunum, ileum, and cecum were
156 quickly removed, and pH values were recorded at 3 different points using a Testo-205
157 pH meter.

158 2.7 Digestive enzyme assay

159 Approximately 1 g of the contents of the terminal ileum was placed in a 5-mL
160 centrifuge tube. Ileum contents were mashed by adding an appropriate amount of
161 saline and then centrifuged ($850 \times g$, 10 min). The levels of amylase, trypsin, and
162 lipase in the supernatant were measured by using enzyme linked immunosorbent
163 assay (ELISA) kits obtained from Jiangsu Meimian industrial Co., Ltd. (Jiangsu,
164 China) as per the manufacturer's instructions. The coefficients of variation for both
165 inter-assay and intra-assay were observed to be below 10% and 12%, respectively.

166 2.8 Intestinal morphometry

167 Ileum tissues were fixed in formalin for 48 h, sectioned, and paraffin-embedded.
168 The tissue samples were treated with hematoxylin and eosin staining followed by
169 encapsulation in a neutral gel. Subsequently, the sections underwent scanning using a
170 panoramic section scanner (DESK/MIDI/250/1000, Wuhan Servicebio Technology
171 Co., Ltd, Wuhan, China), and the intestinal villus height and crypt depth were
172 measured with scanning and viewing software. Subsequently, the villus height-to-
173 crypt depth ratio was calculated.

174 2.9 Volatile fatty acids

175 Approximately 1 g of the contents of the terminal ileum was placed in a 5-mL
176 centrifuge tube. Then, 2.5 mL of pre-cooled ultrapure water was added followed by
177 vortexing and centrifugation at $10000 \times g$ for 10 min at 4°C . Then, 25%
178 metaphosphoric acid was added to the supernatant at a ratio of 1:5. The mixture was
179 vortexed and placed in an ice bath for 40 min, and centrifuged at $10,000 \times g$ for 10

180 min at 4 °C. The supernatant was aspirated and the levels of acetic acid, propionic
181 acid, isobutyric acid, butyric acid, isovaleric acid, and valeric acid in the ileum were
182 determined using gas chromatography (GC-2014 model, Shimadzu, Japan).

183 2.10 Measurement of serum immunity indices

184 One chicken was randomly drawn from each repeat and blood (10 mL) was
185 collected from the wing vein, centrifuged at $850 \times g$ for 10 min, and the supernatant
186 was stored at -20 °C for further analysis. The concentrations of immunoglobulin M
187 (IgM), immunoglobulin G (IgG), immunoglobulin A (IgA), complement 3 (C3),
188 complement 4 (C4), and tumor necrosis factor- α (TNF- α) in blood were determined
189 using the respective ELISA kits (Meimian industrial Co., Ltd, Jiangsu, P. R. China)
190 according to the manufacturer's instructions.

191 2.11 Determination of antioxidant index

192 The activities of total antioxidant capacity (T-AOC), catalase (CAT), superoxide
193 dismutase (SOD), glutathione peroxidase (GSH-Px), and the contents of
194 malondialdehyde (MDA) in blood were determined using the respective ELISA kits
195 (Meimian industrial Co., Ltd, Jiangsu, P. R. China) according to the manufacturer's
196 instructions.

197 2.12 Measurement of the ileum secretory IgA index

198 The ileum samples were taken from the -80 °C refrigerator and the content of
199 SIgA in the ileum was determined by ELISA kit (Jiangsu Meimian industrial Co., Ltd,
200 Jiangsu, P. R. China). The specific operation was followed according to the
201 instructions.

202 2.13 The expression of antioxidant- and intestinal permeability-related genes in
203 ileum tissue

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

212 2.14 Assessment of microbial diversity

213 The ileum chyme samples were taken out from a freezer set at -80 °C, and the
214 nucleic acids were extracted using the OMEGA Soil DNA Kit (D5635-02, Omega
215 Bio-Tek, Norcross, GA, USA) kit. To determine their molecular size, the extracted
216 DNA was analyzed through 0.8% agarose gel electrophoresis. Additionally, Nanodrop
217 was used to quantify the DNA concentration.

218 Using total DNA as a template, the forward primer 338F (5'-
219 ACTCCTACGGGGAGGCAGCA-3') and reverse primer 806R (5'-
220 GGACTACHVGGGTWTCTAAT-3') were combined with a junction sequence, a
221 barcode sequence to amplify the V3-V4 region of the bacterial 16S RNA gene. High-
222 throughput sequencing of all the bacteria (16S RNA) in the samples was performed
223 by the Illumina NovaSeq 6000 platform, and the diversity and abundance of the

224 microbial communities were analyzed by Shanghai Personal Biotechnology Co., Ltd
225 (Shanghai, P. R. China).

226 2.15 Untargeted metabolomics

227 The contents from the terminal ileum were placed in sterile tubes with a volume
228 of 5 mL and stored at a temperature of -80 °C for metabolomic analysis. The analysis
229 was conducted by Shanghai Personal Biotechnology Co., Ltd (Shanghai, P.R. China).
230 To prepare the samples, they were transferred to centrifuge tubes with a volume of 2
231 mL. Then, 600 µL of methanol containing 4 µL/L of 2-amino-3-(2-chloro-phenyl)-
232 propionic acid was added to the samples. The mixture was vortexed for 30 seconds.
233 Subsequently, the samples were ground using a tissue grinder at a frequency of 50 Hz
234 for approximately 120 seconds. After that, ultrasonication was performed on the
235 samples at room temperature for 10 minutes followed by centrifugation at $10,000 \times g$
236 and at a temperature of 4°C for another 10 minutes. The resulting supernatant was
237 then filtered through a membrane with a pore size measuring about 0.22 µm before
238 being transferred to detection bottles for untargeted metabolomic analysis using liquid
239 chromatography–mass spectrometry (LC-MS).

240 2.16 Statistical analysis

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

243 $Y_{ij} = \mu + T_i + P_j + e_{ij}$, where Y_{ij} is the dependent variable; μ is the mean; T_i is the
244 fixed treatment effect; P_j is the random effect; e_{ij} is the error residual.

245 The normality of data among groups was confirmed and screened using the

246 MIXED procedure of SAS. Treatments were the fixed effects, and broilers were the
247 random effects. The linear and quadratic ICA dose responses were determined by
248 using specific preplanned contrasts. $P \leq 0.05$ was considered a significant difference,
249 and trends were discussed at $0.05 < P \leq 0.10$. Microbial diversity data were assessed
250 using non-parametric tests in GraphPad Prism.

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

259 Pathway analysis: differential metabolites were analyzed using MetaboAnalyst
260 (Trygg and Wold, 2002), which integrates pathway enrichment analysis with pathway
261 topological analysis. The differentially abundant metabolites were then linked to the
262 Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway for a better
263 understanding of higher-level systemic functions. The visualization of these
264 metabolites and their corresponding pathways was achieved through the utilization of
265 the KEGG Mapper tool.

266 3. Results

267 3.1 The effect of ICA on broiler growth performance

268 As shown in Table 3, the effect of ICA on ADFI was limited, while the ADG
269 increased linearly with increasing ICA supplementation at 22 to 42 d ($P = 0.015$) and
270 1 to 42 d ($P = 0.047$). The F/G decreased linearly ($P = 0.048$) at 1 to 21 d, and
271 quadratically at 22 to 42 d ($P = 0.039$) and 1 to 42 d ($P = 0.048$) with the increasing
272 ICA supplementation.

273 As shown in Table 4, the relevant indicators of growth performance were carried
274 out twice (d 22 to 42 ADG, d 22 to 42 F/G, d 1 to 42 ADG and d 1 to 42 F/G), and
275 based on these results, the optimal supplemental level of ICA in diets of broilers was
276 from 2016.42 to 2431.91 mg/kg.

277 3.2 The effect of ICA on the apparent digestibility of nutrients in broilers

278 As shown in Table 5, although the DM and GE digestibility were not affected by
279 ICA supplementation, the digestibility of CP ($P = 0.073$) and Ca ($P = 0.085$) tended to
280 increase quadratically and P digestibility tended to increase linearly ($P = 0.051$) with
281 the increasing ICA supplementation.

282 3.3 The effects of ICA on the physical barrier of broilers

283 As shown in Table 6, with the increasing ICA supplementation, the villus height
284 and villus height/crypt depth ($P = 0.027$) were increased quadratically, whereas the
285 crypt depth tended to decrease quadratically ($P = 0.051$).

286 As shown in Fig. 1, although the relative mRNA expression of *ZO-1* was not
287 affected, the relative mRNA expression of claudin-1 ($P = 0.003$) and occludin ($P =$
288 0.048) increased linearly with the increasing ICA supplementation.

289 3.4 The effects of ICA on the chemical barrier in broilers

290 As shown in Table 7, the pH of the stomach, duodenum, jejunum, ileum and
291 cecum were all decreased linearly ($P < 0.05$).

292 As shown in Table 8, although the activity of amylase was not affected, the lipase
293 activity increased linearly ($P < 0.001$) with the increasing ICA supplementation. The
294 trypsin activity ($P = 0.016$) increased quadratically and reached a maximum value at
295 the supplementation of 2000 mg/kg.

296 As shown in Table 9, the effect of ICA on the concentrations of propionic acid,
297 iso-butyric acid, iso-valeric acid and valeric acid were limited. With the increasing
298 ICA supplementation, the concentration of TVFA increased quadratically ($P = 0.038$),
299 the concentration of acetic ($P = 0.058$) and butyric acid ($P = 0.051$) tended to increase
300 quadratically.

301 3.5 The effect of ICA on the immune barrier in broilers

302 The serum concentrations of C4, IgA, IgM and TNF- α were not affected by ICA
303 supplementation (Table 10), whereas serum concentration of C3 ($P = 0.004$) increased
304 linearly and IgG ($P = 0.005$) quadratically with the increasing ICA supplementation.

305 A linear increase was also observed in the ileum SIgA concentration ($P = 0.005$) with
306 the increasing ICA supplementation (Table 11).

307 3.6 The effects of ICA on antioxidant capacity

308 As shown in Table 12, although the serum activity of CAT was not affected by
309 ICA supplementation, the GSH-Px ($P = 0.005$) and SOD ($P = 0.040$) activity
310 increased quadratically, the contents of MDA tended to decrease quadratically ($P =$
311 0.058) with the increasing ICA supplementation. The serum activity of T-AOC

312 increased linearly ($P = 0.003$) with the increasing ICA supplementation, peaking at a
313 supplementation level of 2000 mg/kg.

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

317 3.7 The effects of ICA on microbial barriers in broilers

318 As depicted in Fig. 3 A, the sparse curve exhibited a consistent trend, indicating
319 that the amount of data sequenced was appropriate and that the diversity of all the
320 samples tended to be saturated. Additionally, nearly all the samples in the OTU rank
321 abundance curve approached saturation (Fig. 3 B) suggesting that the data possessed
322 sufficient depth to capture the diversity information of most of the samples. There
323 were 819 OTUs common to all the subgroups, while 6,421 OTUs were specific to the
324 CON group, 6,608 were specific to the T1 treatment, 6,673 were specific to the T2
325 treatment, 8,009 were specific to the T3 group, and 7,721 were specific to the T4
326 treatment (Fig. 3 C). Furthermore, no significant difference was found in alpha
327 diversity ($P > 0.05$, Fig. 3 D). The results of the PCA are presented in Fig. 3 E.
328 Principal component one (PC1) accounted for approximately 51.4% and principal
329 component two (PC2) explained approximately 21.3% of the floral communities in
330 the terminal ileum. Together, they accounted for 72.7% of all the flora. Moreover, the
331 groups exhibited clear differentiation, indicating that ICA supplementation had a
332 pronounced impact on terminal ileum microbial colonization in broilers.

333 The microorganisms in the terminal ileum of broilers at the phylum level were

334 predominantly composed of four phyla—Firmicutes, Bacteroidetes, Proteobacteria,
335 and Tenericutes (Fig. 4 A). The combined proportion of these four phyla in each
336 treatment was respectively 97.03%, 98.79%, 99.14%, 98.64%, and 99.01%. Family-
337 level (Fig. 4 B), genus-level (Fig. 4 C), and species-level (Fig. 4 D) compositions are
338 shown separately. Further analysis of the microbial community composition is
339 presented in Fig. 4 E. The abundance of Firmicutes was significantly higher in the
340 ICA groups than in the CON treatment ($P = 0.008$), while the abundance of
341 Bacteroidetes was significantly lower in both the T1 and T3 groups than in the CON
342 treatment ($P = 0.024$). Additionally, the abundance of *Streptococcaceae* was
343 significantly higher in the T3 treatment than in the T2 and CON treatments ($P =$
344 0.026). Meanwhile, *Lactobacillaceae* displayed significantly higher abundance in the
345 T3 treatment than in the T4 treatment ($P = 0.025$). Furthermore, *Streptococcus*
346 demonstrated a significantly higher abundance in both the T3 and CON treatments
347 than in the T2 treatment ($P = 0.026$), whereas *Lactobacillus* displayed significantly
348 higher abundance in both the T3 and CON treatments than in the T4 treatment ($P =$
349 0.025). Moreover, the abundance of *Dehalobacterium* was significantly lower in the
350 T3 treatment than in the T1 group ($P = 0.047$). *Streptococcus alactolyticus* showed
351 significantly higher abundance in both the T3 and CON treatments than in the T2
352 treatment ($P = 0.047$), while *Lactobacillus helveticus* displayed a significantly higher
353 abundance in the T3 and CON treatments than in the T4 treatment ($P = 0.029$). The
354 Krona circle diagram in Fig. 4 F illustrates the hierarchical arrangement of taxonomic
355 levels, kingdom, phylum, class, order, family, genus, and species from the innermost

356 to the outermost regions. The proportional size of each sector accurately represents
357 the relative abundance of the distinct taxonomic units.

358 To further elucidate the dose-dependent effect of ICA on intestinal
359 microorganisms, we conducted a “one-against-all” linear discriminant analysis (LDA)
360 effect size (LEfSe) analysis comparing the ICA treatments with the CON treatment
361 (LDA > 2.0). Our results showed that p_*Firmicutes*, f_*Ruminococcaceae*,
362 g_*Oscillospira*, o_*Lactobacillales*, and g_*Streptococcus* were significantly enriched in
363 the ICA treatments (Fig. 5 A to D).

364 3.8 The effects of ICA on metabolites in broilers

365 Overall, the T3 treatment demonstrated superior growth performance, immune
366 performance, and antioxidant function compared with the other treatments.
367 Accordingly, the CON and T3 treatments were selected for further metabolomics
368 analysis. There was a significant overlap in both peak retention time and signal
369 intensity of the base peak chromatogram in LC-MS, indicative of minimal variation
370 caused by instrumental error throughout the entire testing process. This suggested an
371 excellent instrument stability and reliable results. The OPLS-DA score plots in Fig. 6
372 C and D demonstrated that samples were well-scattered between treatments while
373 being clustered within their respective treatments. Meanwhile, all the blue Q2 points
374 from left to right were lower than the original blue Q2 points on the far right (Fig. 6 E
375 and F). Additionally, the regression line crossed below zero on the vertical coordinate
376 axis less frequently. Both plots confirmed the reliability and validity of the results. As
377 shown in Fig. 6 G, a total of 901 metabolites were detected, with 85 differential

378 metabolites being identified ($P < 0.05$, $VIP > 1$) in the terminal ileum. Among these,
379 65 were upregulated while 20 were downregulated.

380 We further undertook a KEGG pathway enrichment analysis of the differential
381 metabolites and found that the six metabolic pathways enriched in the terminal ileum
382 were arachidonic acid metabolism, lysine biosynthesis, 3,5-hydroxytryptophan
383 synapses, arginine biosynthesis, steroid biosynthesis, and entomohormone
384 biosynthesis (Fig. 7).

385 The heat map in Fig. 8 A shows the correlation of the differential metabolites
386 with gut microbial, antioxidant, and immune indicators. *Streptococcus*,
387 *Streptococcaceae*, and *Streptococcus alactolyticus* were positively correlated with
388 8,9-diHETrE; 11-dehydro-thromboxane B2; 15-deoxy-d-12,14-PGJ2; N-succinyl-
389 L,L-2,6-diaminopimelate; and (2R,3R)-3-methylglutamyl-5-semialdehyde-N6-lysine
390 were positively correlated and negatively correlated with 20-carboxy-leukotriene B4,
391 saccharopine, and calcitriol. Fig. 8 B shows a heat map of the association between
392 differential metabolites and antioxidant indices. Serum SOD and CAT were positively
393 correlated with 8,9-diHETrE; 15-deoxy-d-12,14-PGJ2; and N-succinyl-L,L-2,6-
394 diaminopimelate. Serum GSH-Px was positively correlated with 15-deoxy-d-12,14-
395 PGJ2 and (2R,3R)-3-methylglutamyl-5-semialdehyde-N6-lysine and negatively
396 correlated with saccharopine. Serum T-AOC was positively correlated with 20-
397 carboxy-leukotriene B4; N-succinyl-L,L-2,6-diaminopimelate; and (2R,3R)-3-
398 methylglutamyl-5-semialdehyde-N6-lysine, and negatively correlated with
399 saccharopine. Serum MDA was positively correlated with saccharopine and

400 negatively correlated with 8,9-diHETrE, N-succinyl-L,L-2,6-diaminopimelate, and
401 (2R,3R)-3-methylglutamyl-5-semialdehyde-N6-lysine. The gene *SOD-1* was
402 positively correlated with 15-deoxy-d-12,14-PGJ2 and negatively correlated with 20-
403 hydroxyecdysone. The gene *GSH-Px* showed a positive correlation with prostaglandin
404 B2 and a negative correlation with 20-hydroxyecdysone. Complement C3 was
405 positively correlated with 8,9-diHETrE; 11-dehydro-thromboxane B2; and 15-deoxy-
406 d-12,14-PGJ2 and negatively correlated with calcitriol. IgG was positively correlated
407 with 15-deoxy-d-12,14-PGJ2. A heat map of the association of significantly
408 differential microorganisms with antioxidant indicators is shown in Fig. 8 C. Blood
409 SOD, CAT, the *SOD-1* gene, and IgG were positively correlated with *Streptococcus*,
410 *Streptococcaceae*, and *Streptococcus alactolyticus*. The *SOD-1* gene and *GSH-Px*
411 were positively correlated with Firmicutes and negatively correlated with
412 Bacteroidetes.

413 4. Discussion

414 Large-scale intensive farming practices have contributed to stress and impaired
415 intestinal health of broilers. These can result in disorders in the gut as well as
416 suboptimal intestinal health during the breeding process, ultimately affecting growth
417 performance (Ducatelle et al., 2023). Growth performance serves as a crucial
418 indicator of the growth and development of poultry as well as the effectiveness of
419 feed. It also represents the outcomes of nutrient intake in terms of digestion,
420 absorption, and metabolic deposition. Isochlorogenic acid has superior physiological
421 activity compared to its isomer chlorogenic acid (Cortes-Morales et al., 2019). Studies

422 have demonstrated that incorporating varying levels of chlorogenic acid into animal
423 diets enhance their growth performance (Chen et al., 2022). Liu et al. (2023) revealed
424 that the supplementation broilers diets with chlorogenic acid increased ADG while
425 reducing the F/G ratio. In this study, the ADG increased quadratically with the
426 increasing ICA supplementation at 22 to 42 d and 1 to 42 d and peaked at the
427 supplementation of 2000 mg/kg. The F/G decreased linearly at 1 to 21 d, whereas
428 quadratically at 22 to 42 d and 1 to 42 d, with the increasing ICA supplementation.

429 The addition of acidifying agents to feed rations or drinking water has been
430 demonstrated to enhance the growth performance of animals to a certain extent
431 (Hamid et al., 2018). In this regard, ICA can serve as a biologically active feed
432 acidifying agent that increases ration acidity, potentially inhibiting the growth of
433 harmful substances in the ration and reducing gastrointestinal pH. Moreover, ICA can
434 regulate the structure of the intestinal flora and create an optimal intestinal
435 environment in broilers (Abdel-Fattah et al., 2008). Consequently, ICA
436 supplementation can lead to an increased daily weight gain, reduced F/G ratio,
437 improved apparent digestibility, and the promotion of healthy growth rate in broilers.

438 The maintenance of operational equilibrium in the mucosa and its nutrient
439 absorption capacity hinges significantly on the presence of tangible obstructions. In
440 broilers, indicators such as intestinal villi height, crypt depth, and the villus height-to-
441 crypt depth ratio play crucial roles in assessing development and nutrient digestion
442 capabilities. An increase in the height of the intestinal villi enhances the surface area
443 available for intestinal absorption, thereby improving the efficiency of digestion and

444 nutrient absorption. Shallower crypt depth indicates an increased maturation rate of
445 intestinal epithelial cells and improved secretion function. Moreover, a higher villus
446 height-to-crypt depth ratio reflects a more structurally intact intestinal mucosa (Ząbek
447 et al., 2020). This study revealed a quadratic increase in both villus height and the
448 ratio of villus height to crypt depth, while there was a tendency for crypt depth to
449 exhibit a quadratic decrease. Consistent with our findings, Ma et al. (2021) observed
450 that the incorporation of mixed organic acids into the diet significantly enhance ileum
451 villus height and villus height-to-crypt depth ratio in broilers. This suggests that
452 organic acids promote the development of intestinal epithelial cells. Similarly, Goh et
453 al. (2022) demonstrated the positive effects of organic acids on villus height and the
454 villus height-to-crypt depth ratio in weaned piglets, while also improving their growth
455 performance. These findings align with this study, indicating that the addition of ICA
456 promotes intestinal development in broilers and enhances their nutrient digestion and
457 absorption capabilities. Short-chain fatty acids effectively stimulate the proliferation
458 of intestinal epithelial cells, improving small intestine morphology in broilers while
459 maintaining overall gut integrity (Tan and Coussens, 2007).

460 Tight junctions play a critical role in regulating epithelial cell permeability and
461 maintaining cellular polarity, thereby preventing the passage of pathogens and
462 macromolecules (Sánchez de Medina et al., 2014). Occludin plays a crucial role in
463 regulating the selective permeability of macromolecules through the paracellular
464 pathway and maintaining the integrity of tight junctions. Additionally, the
465 phosphorylation of claudin-1 enhances the barrier function of tight junctions

466 (Heinemann and Schuetz, 2019). Diao et al. (2019) demonstrated that the intragastric
467 infusion of short-chain fatty acids significantly increased the relative mRNA
468 expression levels of claudin-1 and occludin in both the duodenum and ileum of
469 weaned piglets. This finding suggests that short-chain fatty acids promote intestinal
470 barrier function and facilitate intestinal development in these animals. In this study,
471 the relative mRNA expression levels of claudin-1 and occludin increased linearly with
472 the increasing ICA supplementation. It is plausible that ICA modulates the intestinal
473 barrier physically by reducing the adherence of pathogenic bacteria, thereby
474 preventing their invasion (Ma et al., 2022). This mechanism enhances ileum barrier
475 function in broilers, contributing to the maintenance of internal environment
476 homeostasis and supporting intestinal health.

477 The mucus layer's chemical barrier consists of a gel-like substance secreted by
478 intestinal epithelial cells and digestive glands which is crucial for maintaining
479 intestinal mucosal integrity. Evaluation of digestive enzyme activity serves as a key
480 indicator of the intestinal tract's digestive and absorptive capabilities, reflecting
481 overall gastrointestinal health (Chen and Zhao, 2019). The presence of caffeic acid
482 and quinic acid in the structure of ICA enables ICA to act as an acidifier, and the
483 results of the present experiment also demonstrated that the addition of ICA to the
484 ration significantly reduced the pH of the stomach, jejunum, and ileum, thereby
485 creating an acidic environment within the gastrointestinal tract. Ma et al. (2021) found
486 that adding mixed organic acids to broiler diets significantly increased pancreatic
487 amylase activity. Bai et al. (2019) reported that supplementing lactic acid in the diet

488 significantly increased pancreatic lipase activity, thereby improving the apparent
489 digestibility of CP, Ca, P, DM and GE. This indicates that a decrease in intestinal pH
490 within a certain range can improve digestive enzyme activity, thereby improving the
491 efficiency of nutrient digestion and absorption. The results of the present study
492 showed a linear increase in trypsin and lipase activities and trypsin activity increased
493 quadratically with the addition of 2000 mg/kg ICA as the amount of added ICA
494 increased, and the digestibility of CP and Ca tended to increase quadratically.

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

500 The increase in pancreatic lipase activity observed in this study may be attributed
501 to several factors, including the stimulation of proteolytic enzyme secretion by ICA,
502 the enhanced breakdown and absorption of nutrients by chyme, promotion of
503 intestinal development, facilitation of increased lipase production, and augmentation
504 of digestive capacity (Owens et al., 2008). Volatile fatty acids are short-chain fatty
505 acids that are primarily produced through anaerobic bacterial fermentation of
506 undigested carbohydrates in food. They typically comprise carbon chains ranging
507 from 1 to 6 carbons, encompassing acetic acid, propionic acid, butyric acid, isobutyric
508 acid, valeric acid, and isovaleric acid. The primary outcomes arising from the
509 breakdown of dietary fiber by gut bacteria are volatile fatty acids (Corrêa-Oliveira et

510 al., 2016; Yi et al., 2021).

511 Li et al. (2019) study revealed that the incorporation of a combination of diverse
512 organic acids in the diet led to a notable decrease in ileum pH and an elevation in the
513 concentrations of acetic acid, butyric acid, and total volatile fatty acids among mature
514 pigs. Our results indicated that supplementing broiler diets with different levels of
515 ICA enhanced the concentrations of propionic acid, butyric acid, isobutyric acid, and
516 isovaleric acid in the gut. Moreover, the results of this experiment showed that the
517 concentration of TVFA increased quadratically, the concentration of acetic and butyric
518 acid tended to increase quadratically with the addition of 2000 mg/kg. Pastell et al.
519 (2009) reported that supplementing the ration with 15 to 30 nmol/L acetic acid could
520 provide energy for anaerobic bacteria, while also reducing gastrointestinal pH and
521 promoting the growth of *Bifidobacterium*. Ma et al. (2021) found that VFAs, as
522 bacterial post-fermentation products, could energize the intestinal mucosal epithelial
523 cells and accelerate the formation of intestinal barriers, which was beneficial to
524 broilers in resisting the invasion of pathogens and further enhancing their growth
525 performance.

526 The immune system serves as a crucial defense mechanism against
527 environmental pathogens, mitigating reproductive losses. Serum immunoglobulins
528 and complement proteins are vital constituents of the immune system, with their
529 levels in the bloodstream serving as crucial indicators of overall immune function
530 (Tan and Coussens, 2007). IgA binds antigens, aiding in their clearance without
531 causing inflammation. IgG contributes to humoral immunity, phagocytosis,

532 agglutination and precipitation. IgM regulates bacterial infections during the initial
533 stages (Bai et al., 2021). Isochlorogenic acid, functioning as a feed acidifier, has been
534 shown to enhance immune function in livestock and poultry, thereby improving
535 broiler health (Dittoe et al., 2018). Ahmadifar et al. (2022) demonstrated that caffeic
536 acid supplementation significantly increased immunoglobulin levels in beluga whales.
537 In this study, IgG levels exhibited a quadratic increase with the addition of 2000
538 mg/kg ICA in the diet. This finding suggests that ICA supplementation may enhance
539 humoral immune response in broilers, potentially contributing to improved immune
540 function and overall health.

541 Isochlorogenic acid enhances immune performance through various mechanisms,
542 including activation of the calcium-modulated neural phosphatase-mediated signaling
543 pathway, enhancement of macrophage phagocytosis, and promotion of B- and T-
544 lymphocyte proliferation and differentiation, thereby improving overall immune
545 function (Wu et al., 2004). The serum level of C3, a crucial complement protein
546 situated at the convergence point of all complement activation pathways (Martin et
547 al., 2022), was found to be significantly increased in broilers when ICA was added to
548 the diet, indicating an augmentation in specific immune function. Consequently, the
549 inclusion of ICA in broiler diets led to a significant increase in serum immunoglobulin
550 and complement content, ultimately improving overall immunity. SIgA is secreted by
551 plasma cells within the intestinal mucosa and serves as a barrier against bacterial
552 adhesion on the mucosal surface. Additionally, SIgA can form complexes with
553 antigens and thus prevent their entry into the mucosa, as well as stimulate the

554 secretion of large amounts of mucus by goblet cells for the protection of the intestinal
555 tract (He et al., 2020). Our findings demonstrated that dietary supplementation with
556 ICA significantly elevated SIgA levels in the ileum of broilers, suggesting that ICA
557 may enhance intestinal immune status in these birds.

558 Enhancing antioxidant capacity can effectively improve immune function. T-
559 AOC and the levels of SOD and GSH-Px activities reflect the body's ability to
560 scavenge oxygen radicals and play a crucial role in regulating and protecting cellular
561 metabolism (Suair et al., 2018). The results of this experiment showed that the
562 activities of SOD and GSH-Px increased quadratically with the addition of 2000
563 mg/kg ICA, and the activity of MDA concentration tended to decrease quadratically,
564 which indicated that the addition of 2000 mg/kg ICA to the ration could enhance the
565 antioxidant capacity of the organism. One study revealed that supplementing
566 chlorogenic acid extracted from honeysuckle to the diet of fattening pigs substantially
567 increased serum T-AOC and serum levels of SOD and GSH-Px while decreasing
568 MDA concentrations, indicative of its potential for improving antioxidative capability
569 in these animals (Liu et al., 2023).

570 Xiong et al. (2022) demonstrated that including chlorogenic acid extracted from
571 stevia residue in the diet of fattening pigs significantly increased serum SOD activity
572 and decreased malondialdehyde (MDA) levels. Chen et al. (2018) discovered that the
573 antioxidant capacity of piglets was enhanced through dietary supplementation with
574 chlorogenic acid, resulting in improved growth performance and a decrease in the
575 occurrence of diarrhea. Isochlorogenic acid contains phenolic hydroxyl groups that

576 readily react with free radicals, providing protons and electrons for their
577 neutralization, thereby protecting against oxidative damage (Hou et al., 2017). The
578 phenolic hydroxyl group of ICA can also chelate with oxidation-inducing transition
579 metal ions, such as Fe^{2+} and Cu^{2+} , forming complexes that inhibit chain oxidation
580 reactions. This indirectly provides a defense mechanism against peroxidation induced
581 by reactive oxygen species (Cruciani et al., 2019). The conformational effect of the
582 cyclohexane skeleton of ICA also influences its antioxidant capacity, while the
583 relative positions of its two caffeic acid moieties explain the variations in its
584 antioxidant capacities (Liu et al., 2019). Based on these findings, we further
585 investigated the impact of ICA on the mRNA expression levels of antioxidant-related
586 genes in broiler ileum tissues. The results indicated that the relative mRNA expression
587 of *SOD-1* and *GSH-Px* tended to increase quadratically with increasing ICA
588 supplementation and peaking at 2000 mg/kg. Isochlorogenic acid activates the Nrf2
589 pathway by suppressing the expression of CYP2E1 and CYP4A, leading to an
590 increase in the levels of heme oxygenase-1 (HO-1) and NADPH quinone
591 dehydrogenase-1 (NQO-1) expression. This effect reduces ROS production and helps
592 to alleviate oxidative damage (Liu et al., 2018). The hydrogen atoms present in
593 caffeoylquinic acid likely contribute to the scavenging of free radicals and inhibition
594 of oxidative reactions (Liang and Kitts, 2015).

595 The maintenance of the host's metabolic health heavily relies on stabilizing the
596 intestinal microbiota (Guevarra et al., 2019). Disturbance to the microbiota can
597 negatively impact the well-being of animals and undermine their ability to mount an

598 effective immune defense (Fan and Pedersen, 2021). Organic acids exert a significant
599 effect on poultry gastrointestinal tract function by depolarizing bacterial membranes
600 and increasing internal bacterial acidity, thereby modulating microbial populations.
601 Nutrient digestibility also influences gut microbiota composition (Yin et al., 2018). In
602 this work, we observed an increase in the relative abundance of Firmicutes at the
603 phylum level, accompanied by a decrease in the presence of Bacteroidetes. The
604 Firmicutes play a crucial role in breaking down external peptides and amino acids,
605 while the members of Bacteroidetes specialize in degrading cellulose, hemicellulose,
606 and pectin (Li et al., 2019; Yan et al., 2017). It has been demonstrated that caffeic acid
607 is the strongest inhibitor of the growth of *Bacteroidetes anisopliae* (Cueva et al.,
608 2010). Studies investigating organic acid supplementation in feed have reported
609 inconsistent findings due to variations in fecal fluid source and dosage (Bruno et al.,
610 2019; Yang et al., 2020). At the level of species, there was a rise in the proportionate
611 prevalence of *Streptococcus alactolyticus*, which was initially recognized as a
612 prevailing bacterial species responsible for producing lactic acid in the gastrointestinal
613 tracts of chickens and pigs (Czerwiński et al., 2010). Short peptides produced through
614 fermentation by lactic acid bacteria can lower cholesterol levels while enhancing
615 antioxidant capacity and immune functions. Proteins undergo degradation into short
616 peptides and amino acids during cow milk fermentation using a combination of
617 *Streptococcus hygroscopicus* and *Lactobacillus* colony cultures, which enhances the
618 probiotic properties of yoghurt (Ghosh et al., 2013; Hagi et al., 2016). This implies
619 that *Streptococcus alactolyticus* contributes to improved antioxidant capacity as well

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

624 In this study, we undertook an untargeted metabolomic analysis of the CON and
625 T3 treatments, revealing that ICA primarily affected two metabolic pathways, namely,
626 arachidonic acid metabolism and lysine biosynthesis. Arachidonic acid is a
627 polyunsaturated fatty acid consisting of a chain of 20 carbon atoms and characterized
628 by the presence of four double bonds, which makes it prone to oxidation. This leads to
629 the excessive production of metabolites that are crucial for the functioning of the
630 immune system, suppression of inflammation, and regulation of feed intake (Hanna
631 and Hafez, 2018). The lysine biosynthesis pathway differs from that of other amino
632 acids and varies according to microorganism. Bacteria require diaminohexanedioic
633 acid synthesis for lysine production, while yeast and molds require α -amino adipic
634 acid synthesis (Xu et al., 2019). Lysine has been proven to boost animal growth,
635 enhance immune response and antioxidant ability, as well as optimize intestinal
636 structure (Alabi et al., 2017; Konieczka et al., 2022; Menegat et al., 2020). The
637 microbiota and metabolites mentioned above are associated with immunity and
638 antioxidants, so our assessment of the correlation of differential metabolites in distinct
639 metabolic pathways with antioxidant indicators and differentially abundant
640 microorganisms suggested that ICA influences the levels of these metabolites by
641 modulating gut microorganisms. This affects the uptake of these metabolites and their

642 transportation by intestinal epithelial cells to the liver, ultimately impacting
643 organismal physiological functions.

644 In the terminal ileum, positive correlations were found between microorganisms
645 (*Streptococcus alactolyticus*) and metabolites (15-deoxy-d-12,14-PGJ2; N-succinyl-
646 L,L-2,6-diaminopimelate; and (2R,3R)-3-methylglutamyl-5-semialdehyde-N6-lysine).
647 This result suggested that the increase in *Streptococcus alactolyticus* abundance in the
648 terminal ileum was associated with the positive correlation found between this species
649 and 15-deoxy-d-12,14-PGJ2; N-succinyl-L,L-2,6-diaminopimelate; (2R,3R)-3-
650 methylglutamyl-5-semialdehyde-N6-lysine; and other metabolites, resulting in
651 increased antioxidant and immune functions. 15-Deoxy-d-12,14-PGJ2 is a
652 prostaglandin derived from arachidonic acid that exerts anti-inflammatory,
653 antioxidant, antitumor, and other biological activities by activating the PPAR- γ
654 signaling pathway (Ueno et al., 2001). Marcone et al. found that 15-deoxy-d-12,14-
655 PGJ2 inhibits the NF- κ B inflammatory pathway by reducing TNF- α levels through
656 modulating proteasome activity (Marcone et al., 2016). Studies have shown that 15-
657 deoxy-d-12,14-PGJ2 attenuates lung infections in rats by inhibiting the expression of
658 pro-inflammatory factors, such as TNF- α and IL-10, and chemokines, such as CCL2,
659 CCL3, and CCLA (Cloutier et al., 2012; Maehara et al., 2019). Nilsson et al. (2017)
660 showed that the administration of 15-deoxy-d-12,14-PGJ2 to mice suffering from
661 unilateral ureteral obstruction (UUO) resulted in an increase in the protein expression
662 of Nrf2 and HO-1 in the animals, and also prevented protein carbonylation, a UUO-
663 induced marker of oxidative stress. In addition, it has been demonstrated that

664 oxidative stress induces intracellular heat shock 70 (HSP70) and that 15-deoxy-d-
665 12,14-PGJ2 modulates the anti-inflammatory pathway by regulating HSP70 and
666 decreasing the levels of NF- κ B and COX-2 and mitigating oxidative stress in
667 chondrocytes (Bianchi et al., 2014). N-Succinyl-L,L-2,6-diaminopimelate and
668 (2R,3R)-3-methylglutamyl-5-semialdehyde-N6-lysine exhibited significant
669 upregulation within the lysine biosynthetic pathway. The N-succinyl-L,L-
670 diaminopimelate pathway involves the enzymatic action of desuccinylase to produce
671 L,L -diaminopimelate and succinate from N-succinyl-L,L-diaminopimelate (Born et
672 al., 1998). This indicates that ICA influences gut microflora, thereby regulating
673 metabolite levels and impacting metabolic pathways associated with antioxidant and
674 immune properties. However, the mechanism underlying these interactions requires
675 further exploration.

676 5. Conclusion

677 In conclusion, our study demonstrates that supplementation with ICA enhances feed
678 digestibility, intestinal morphology, antioxidant capacity, and immune responses in
679 broilers, leading to improved growth performance. Specifically, ICA strengthens the
680 intestinal barrier by upregulating tight junction proteins, enhances digestive enzyme
681 activity and volatile fatty acid production, boosts SIgA secretion, and modulates gut
682 microbiota and metabolites. Additionally, ICA improves intestinal immune function
683 through its antioxidative properties. Based on the growth performance and economic
684 benefits, the optimal supplemental level of ICA was from 2016.42 to 2431.91 mg/kg
685 in broilers.

686 **Credit Author statement**

687 **Siyuan Zhou**: Conceptualization, Methodology, Software, Investigation, Formal
688 Analysis, Writing-Original Draft; **Haotian Jiang**: Data Curation, Writing - Original
689 Draft; **Yujia Wang**: Visualization, Investigation; **An Yan**: Investigation; Guohua Liu:
690 Resources, Supervision; **Shudong Liu** and **Baojiang Chen**: Conceptualization,
691 Funding Acquisition, Resources, Supervision, Writing - Review & Editing. All
692 authors have read and agreed to the published version of the manuscript.

693 **Declaration of competing interests**

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

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- 933 combination with hops and a mixture of a free butyrate acidifier and gluconic acid
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- 935 10(8): 1335.

936 Table 1 Composition and nutrient levels of basal diets (% air-dry basis)

Item	Content	
	1 to 21 d of age	22 to 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 ²		
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

937 CP = crude protein; Ca = calcium; P = phosphorus; ME = metabolizable energy.

938 ¹Provided per kilogram of diet: vitamin A, 750 IU; vitamin D₃, 3 kIU; vitamin E,
939 20 IU; vitamin K₃, 2 mg; vitamin B₁, 2 mg; vitamin B₂, 6 mg; vitamin B₆, 3 mg,
940 vitamin B₁₂, 0.02 mg; calcium pantothenate, 10 mg; nicotinamide, 40 mg; biotin, 0.12
941 mg; Cu, 10 mg; Fe, 20 mg; Zn, 80 mg; Mn, 90 mg; Se, 0.2 mg; I, 1 mg.

942 ²Metabolizable energy was calculated, while all other nutrient levels were
943 analysed.

944 Table 2 Sequence of primers for real-time PCR.

Target genes	Primer sequence (5'to 3')	GenBank accession no.
β -actin	Forward: CTTCCAGCCATCTTTCTT	ENSGALG00010021232
	Reverse: ATATCCACATCACACTTCAT	
<i>ZO-1</i>	Forward: AGACAGCAGACATACATC	ENSGALG00015013331
	Reverse: CAGATTCAGGAGGAGTTC	
Occludin	Forward: TTCGTGGCAAAGTGGCAG	ENSGALG00010028576
	Reverse: GGTCGTACTIONCATGGATCTGTG	
Claudin-1	Forward: TGGTTGGTGTGTTTGTG	ENSGALG00010013646
	Reverse: CATCCGCATCTTCTTCAC	
<i>SOD-1</i>	Forward: TTCTGTCATTTCTCTTAC	ENSGALG00010011746
	Reverse: TAGTTTGCTCTCATTATC	
<i>GSH-Px</i>	Forward: GATGAGATCCTGAGAGTGGTGGAC	NM_000581.4
	Reverse: TCATCAGGTAAGGTGGGCACAA	

945 *ZO-1* = zonula occludens-1; *SOD-1* = superoxide dismutase 1; *GSH-Px* =

946 glutathione peroxidase.

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

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

948 ICA = isochlorogenic acid; SEM = standard error of the mean; ADFI = average

949 daily feed intake; ADG = average daily gain; F/G = feed-to-gain ratio.

950 ^{a,b} Within a row, values with different superscripts indicate a significant951 difference ($n = 8$, $P < 0.05$).

952

953 Table 4 Estimation of the optimal supplemental level of ICA in broiler diets based on the regression model

Item	Quadratic curve equation	<i>P</i> -value	<i>R</i> ²	Optimal supplemental level, mg/kg
22 to 42 d				
ADG	$y = 77.003 + 0.002x - 0.0000004122x^2$	0.036	0.165	2431.91
F/G	$y = 1.578 - 0.000119x + 0.00000002934x^2$	0.006	0.242	2027.95
1 to 42 d				
ADG	$y = 48.934 + 0.002x - 0.0000004264x^2$	0.036	0.165	2345.22
F/G	$y = 1.652 - 0.000113x + 0.00000002802x^2$	0.019	0.193	2016.42

954 ICA = isochlorogenic acid; ADFI = average daily feed intake; ADG = average daily gain; F/G = feed-to-gain ratio.

955 Table 5 Effect of ICA on apparent digestibility of nutrients in broilers (%)

Item	ICA levels, mg/kg					SEM	<i>P</i> -value	
	0	500	1000	2000	3000		Liner	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

956 ICA = isochlorogenic acid; CP = crude protein; Ca = calcium; P = phosphorus; GE = gross energy; DM = dry matter.

957 ^{a,b,c} Within a row, values with different superscripts indicate a significant difference ($n = 8$, $P < 0.05$).

958 Table 6 The effect of ICA on the morphology of broiler ileum

Item	ICA levels, mg/kg					SEM	P-value	
	0	500	1000	2000	3000		Liner	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

959 ICA = isochlorogenic acid.

960 ^{a,b,c} Within a row, values with different superscripts indicate a significant difference ($n = 5$, $P < 0.05$).

961 Table 7 Effect of ICA on the pH of different segments of the gastrointestinal tract in broilers

pH value	ICA levels, mg/kg					SEM	P-value		962
	0	500	1000	2000	3000		Liner	Quadratic	963
Stomach	3.33 ^a	3.31 ^{ab}	3.29 ^{abc}	3.27 ^{bc}	3.25 ^c	0.016	<0.001	0.588	964
Duodenum	6.46	6.40	6.38	6.37	6.36	0.023	0.010	0.154	965
Jejunum	6.52 ^a	6.44 ^b	6.43 ^b	6.42 ^b	6.41 ^b	0.026	0.013	0.109	966
Ileum	6.76 ^a	6.67 ^{ab}	6.59 ^b	6.58 ^b	6.53 ^b	0.054	0.006	0.238	967
Cecum	6.81	6.69	6.62	6.60	6.55	0.064	0.008	0.266	968
									969

970 ICA = isochlorogenic acid.

971 ^{a,b,c} Within a row, values with different superscripts indicate a significant difference ($n = 8$, $P < 0.05$).

972 Table 8 Effect of ICA on digestive enzymes in ileal contents of broilers (U/mg)

Item	ICA levels, mg/kg					SEM	P-value	
	0	500	1000	2000	3000		Liner	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

973 ICA = isochlorogenic acid.

974 ^{a,b,c} Within a row, values with different superscripts indicate a significant difference ($n = 5$, $P < 0.05$).

975 Table 9 Effect of ICA on volatile fatty acid content in the ileum of broilers (mmol/L)

Item	ICA levels, mg/kg					SEM	P-value	
	0	500	1000	2000	3000		Liner	Quadratic
TVFA	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

976 ICA = isochlorogenic acid; TVFA = total volatile fatty acids.

977 ^{a,b} Within a row, values with different superscripts indicate a significant difference ($n = 5$, $P < 0.05$).

978 Table 10 Effect of ICA on serum immunological properties of broilers

Item, $\mu\text{g/mL}$	ICA levels, mg/kg					SEM	<i>P</i> -value	
	0	500	1000	2000	3000		Liner	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

979 ICA = isochlorogenic acid; C3 = complement 3; C4 = complement 4; IgA = immunoglobulin A; IgG = immunoglobulin G; IgM =

980 immunoglobulin M; TNF- α = tumor necrosis factor α .

981 ^{a,b} Within a row, values with different superscripts indicate a significant difference ($n = 5$, $P < 0.05$).

982 Table 11 Effects of ICA on ileal SIgA of broilers

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

983 ICA = isochlorogenic acid; SIgA = secretory immunoglobulin A.

984 ^{a,b} W Within a row, values with different superscripts indicate a significant difference ($n = 5, P < 0.05$).

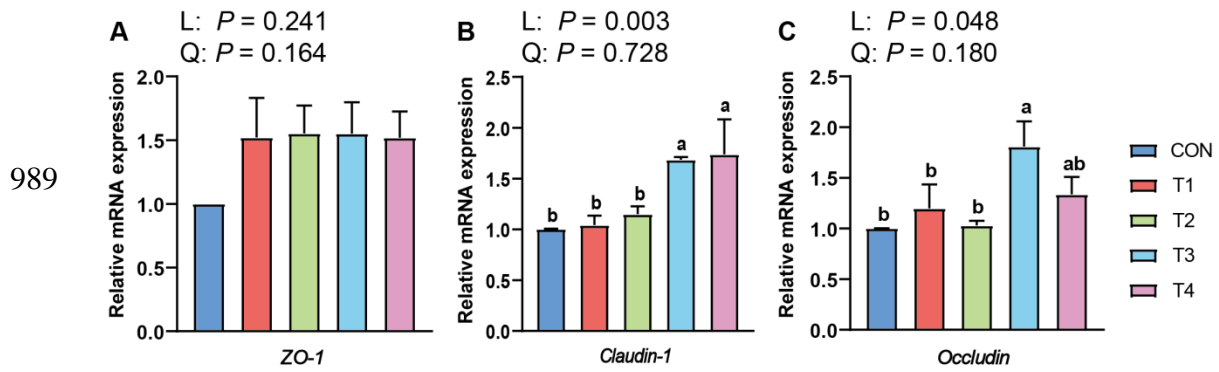
985 Table 12 Effect of ICA on serum antioxidation in broilers

Item, U/mL	ICA levels (mg/kg)					SEM	<i>P</i> -value	
	0	500	1000	2000	3000		Liner	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

986 ICA = isochlorogenic acid; SOD = superoxide dismutase; GSH-Px = glutathione peroxidase; CAT = catalase; T-AOC = total antioxidant

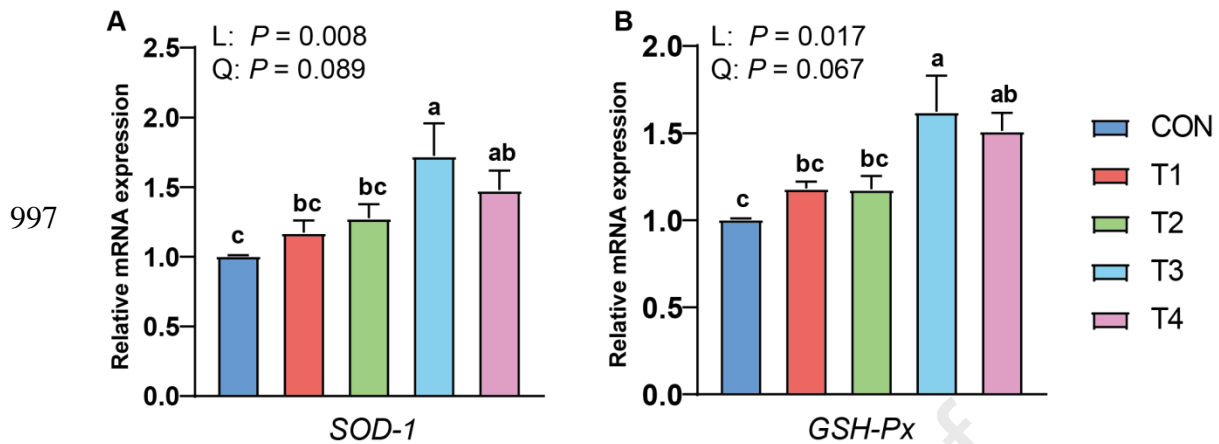
987 capacity; MDA = malondialdehyde.

988 ^{a,b,c} Within a row, values with different superscripts indicate a significant difference ($n = 5$, $P < 0.05$).



990 Fig. 1 Effect of ICA on mRNA relative expression of tight junction protein-
 991 related genes in the ileum of broilers. Letters L and Q represent liner and quadratic
 992 effects of ICA supplementation dose, respectively. ICA = isochlorogenic acid; *ZO-1* =
 993 zonula occludens-1. T1, T2, T3 and T4 represent ICA at 0, 500, 1000, 2000 and 3000
 994 mg/kg, respectively. ^{a,b} Bars with different superscripts indicate a significant
 995 difference ($n = 5$, $P < 0.05$).

996

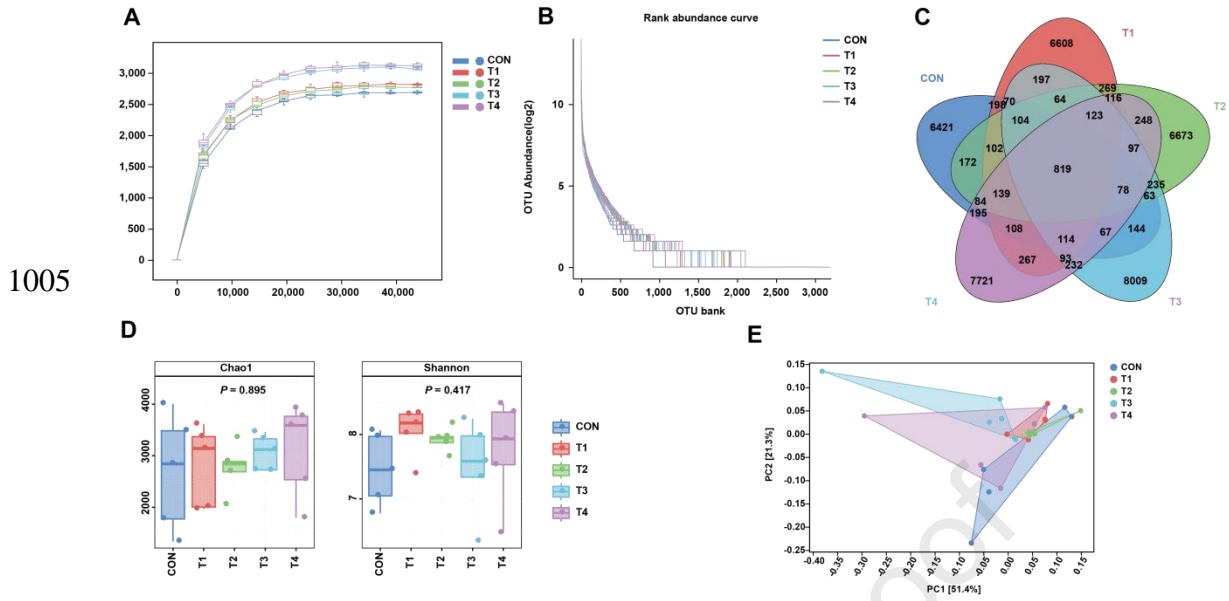


998 Fig. 2 Effect of ICA on mRNA relative expression of antioxidant-related genes in

999 the ileum of broilers ($n = 5$). (A) The mRNA expression of *SOD-1* in the ileum; (B)1000 The mRNA expression of *GSH-Px* in the ileum. ICA = isochlorogenic acid; *SOD-1* =1001 superoxide dismutase 1; *GSH-Px* = glutathione peroxidase. Letters L and Q represent

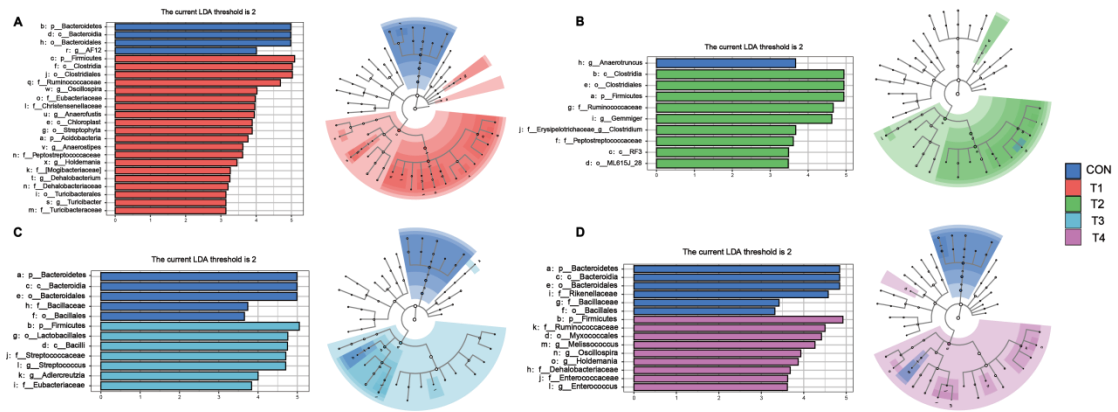
1002 liner and quadratic effects of ICA supplementation dose, respectively. T1, T2, T3 and

1003 T4 represent ICA at 0, 500, 1000, 2000 and 3000 mg/kg, respectively. ^{a,b} Bars with1004 different superscripts indicate a significant difference ($n = 5$, $P < 0.05$).



1005

1006 Fig. 3. The effects of ICA on the diversity of the intestinal flora in broilers ($n =$
 1007 5). (A) Sparse curve. (B) Abundance grade curve. (C) The Venn analysis of amplicon
 1008 sequence variants (ASV). (D) Alpha diversity. (E) Principal component analysis. ICA
 1009 = isochlorogenic acid. T1, T2, T3 and T4 represent ICA at 0, 500, 1000, 2000 and
 1010 3000 mg/kg, respectively.



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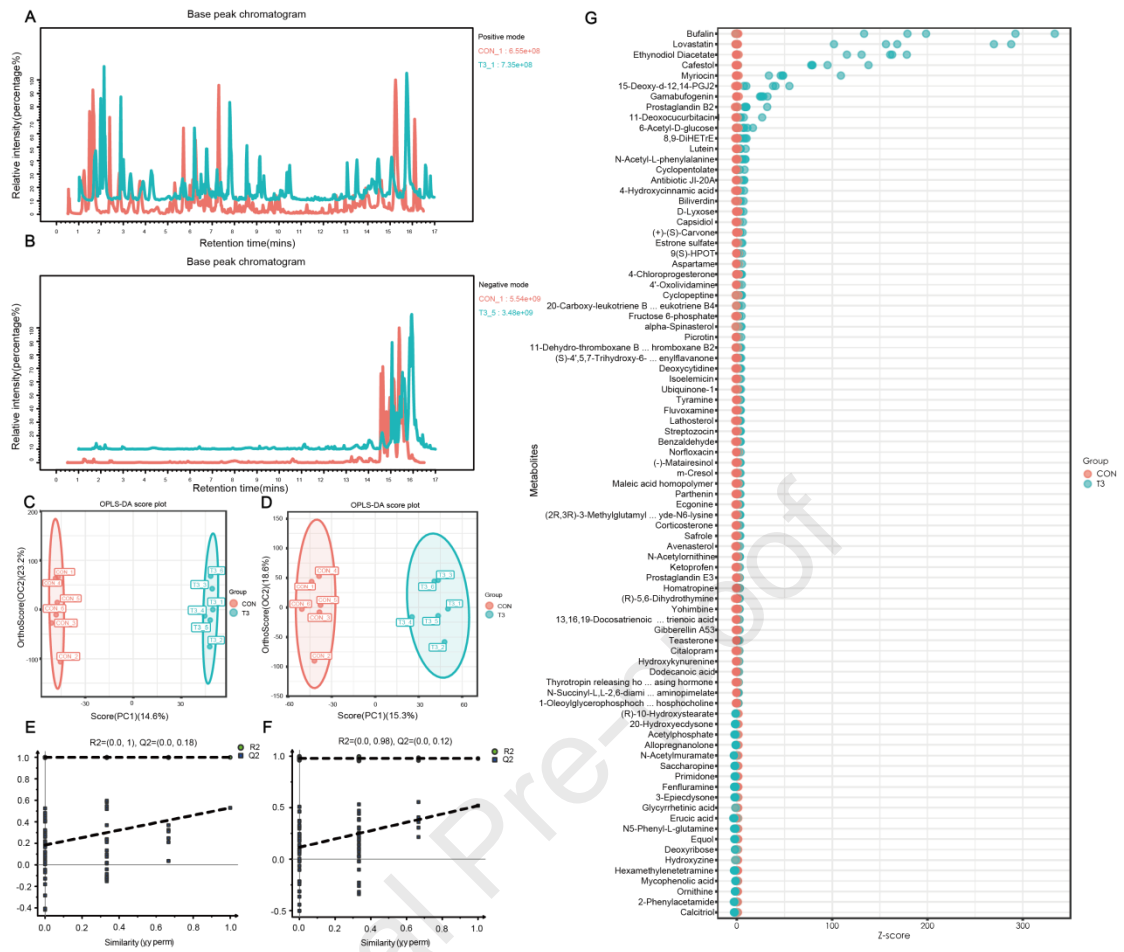
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Fig.5 The effect of ICA on LefSe analysis of broilers ($n = 5$). (A) Differences in ileum microbes enriched between CON and T1 groups. (B) Differences in ileum microbes enriched between CON and T2 groups. (C) Differences in ileum microbes enriched 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 ICA at 0, 500, 1000, 2000 and 3000 mg/kg, respectively.



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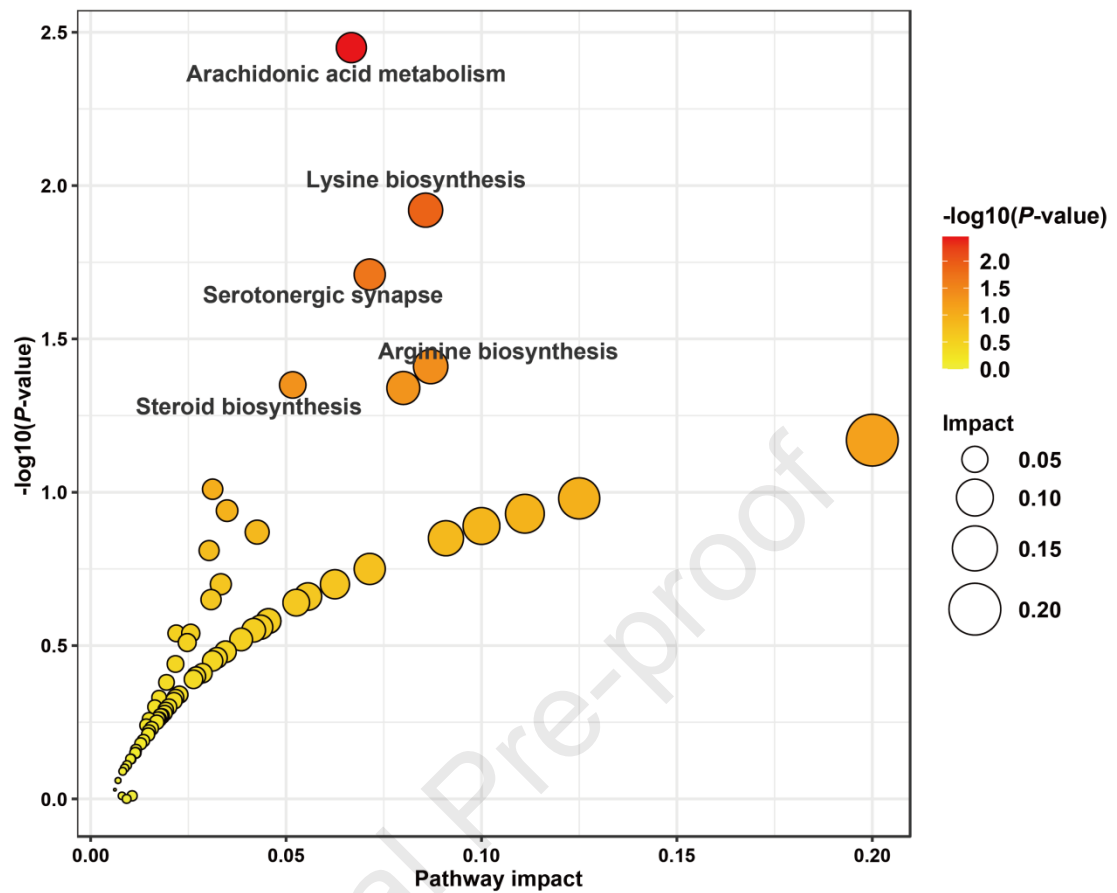
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Fig. 6 The effects of ICA on metabolites in broilers. (A) Base peak chromatogram positive mode. (B) Base peak chromatogram negative mode. (C) OPLS-DA score plot positive mode. (D) OPLS-DA score plot negative mode. (E) OPLS-DA displacement chart positive mode. (F) OPLS-DA displacement chart negative mode. (G) Z-score plot. ICA = isochlorogenic acid; OPLS-DA = orthogonal partial least-square discriminant analysis. T3 represents ICA supplementation 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.

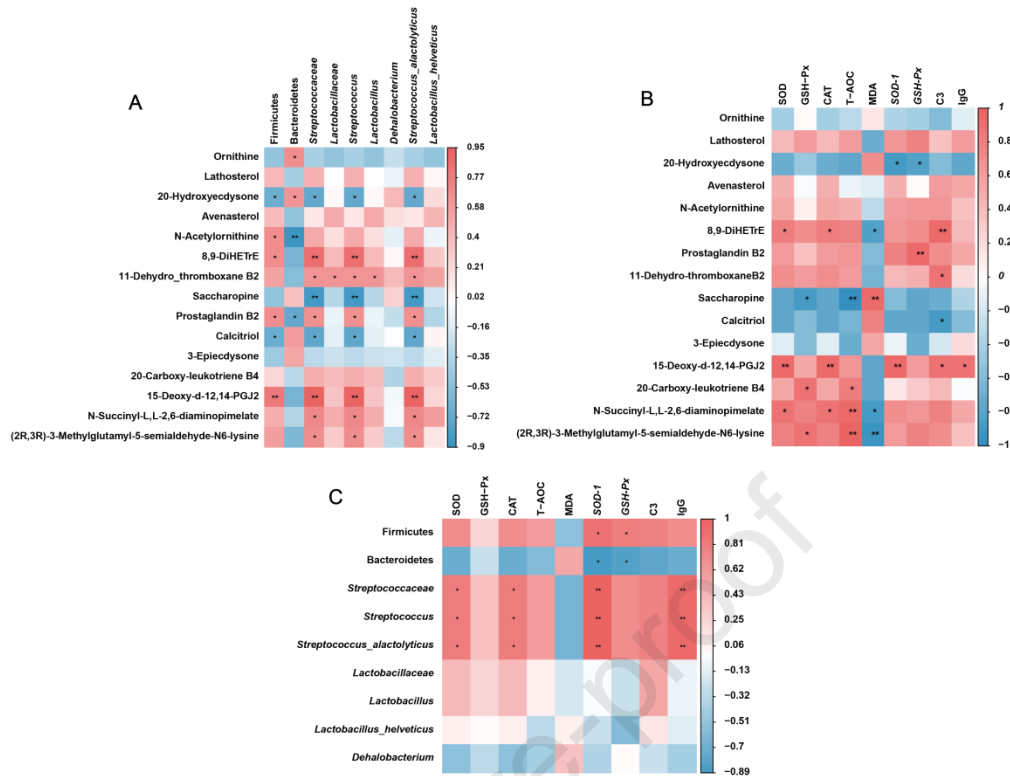


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1037 Fig.7 Bubble map of metabolic pathway influencing factors. The larger the dot,

1038 the greater the influence on the metabolic pathway; the darker the color, the smaller

1039 the P -value.



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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. * $P < 0.05$; ** $P < 0.001$.

Declaration of competing interests

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.

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