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Dietary resistant starch protects against post-antibiotic intestinal damage by restoring microbial homeostasis and preserving intestinal barrier function in meat duck

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ABSTRACT

Resistant starch (**RS**) is recognized as a nutritional strategy that supports gut and overall host health by modulating gut microbiota. To directly assess the effects of RS on gut microbiota and its role in improving intestinal barrier function in meat ducks, this study first established an antibiotic-induced microbial dysbiosis model, which was characterized by reduced gut microbial diversity, intestinal dysfunction, and an inflammatory outburst following antibiotic exposure. Whereafter, in addition to the control group, ducks treated with antibiotics for 7 consecutive days were further allocated to two groups and fed the basal diet and RS diet that derived from 12 % raw potato starch until 21 d. The results demonstrated that dietary RS supplementation reversed the antibiotic-induced reduction in microbial diversity and restored the Firmicutes-to-Bacteroidetes ratio. Additionally, RS inclusion enriched beneficial bacterial genera, including *Coprobacter, Odoribacter*, and *Faecalibacterium* (LDA score > 3). Post-antibiotic intervention led to a reduction in villus density and muscular thickness, accompanied by a significant downregulation ($P < 0.05$) of *zonula occludens-1* and *mucin-2* expression, along with increased serum pro-inflammatory cytokine levels ($P < 0.05$). Notably, dietary RS supplementation significantly enhanced ($P < 0.05$) the expression of *glucagon-like peptide receptor* and the anti-apoptotic factor *Bcl-2*, while suppressing caspase transcription. This resulted in increased villus height and muscular thickness in the ileum ($P < 0.05$). Furthermore, RS intervention remarkably reduced ($P < 0.05$) pro-inflammatory cytokine levels, particularly interleukin-1β and tumor necrosis factor-α, in both the ileum and serum. These effects were likely linked to alterations in cecal microbiota, including increased abundances of *Barnesiella, Ruminiclostridium 9, Megamonas, Faecalitalea, Adlercreutzia, Coprobacter* and *Collinsella*. In conclusion, dietary RS supplementation mitigated antibiotic-induced cecal microbial dysbiosis and restored intestinal structure by promoting enterocyte proliferation and reducing apoptosis. Consequently, RS supplementation helped alleviate systemic inflammation in meat ducks following antibiotic treatment.

Introduction

The poultry industry has become one of the fastest growing meat producing animal industries in response to the increasing requirement for animal protein (Yadav and Jha, 2019). Acting as one of important animal-protein sources, modern meat ducks are now the fastest growing birds among the numerous poultry species (Bentley et al., 2020; El Sabry and Almasri, 2023; Quaresma, et al., 2024). This might be related to some unique characteristics possessed by ducks, including adaptability to various feedstuffs and higher resistance to disease compared to some other poultry species (El Sabry and Almasri, 2023; Qin et al., 2018). Accordingly, in practice, unconventional raw materials usually characterized by fiber-rich are developed and utilized in duck farming, which may be partly attributable to their relatively well-developed cecal fermentability, as evidenced by the total bacteria reach $\log_{10}$ 12.45 colony-forming unit (**CFU**) per gram the cecal digesta in 14-d-old birds (Li, et al., 2024a; Liao, et al., 2018). In addition to the roles in degradation indigestible ingredients, a diverse microbiota in the intestinal

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lumen serves as an effective barrier against pathogens colonization (Kogut, et al., 2018). Alterations in the composition of cecal microbiota can lead to long-lasting deleterious effects in birds (Becattini, et al., 2016; Yadav and Jha, 2019). Perturbations in host-microbiota crosstalk during early life may contribute to chronic inflammation, compromise host health, and pose serious threats to food safety and animal welfare (Aruwa and Sabiu, 2024). Therefore, maintaining cecal microbial homeostasis is essential for intestinal health and optimal performance in duck production.

Hitherto, substantial research has verified the interaction between diet intervention and changes in microbial diversity and community composition (Ignatiou and Pitsouli, 2024). These studies have provided significant insights into the role of nondigestible dietary components, such as probiotics, prebiotics, and dietary fiber, in shaping microbial architecture and functionality (An, et al., 2022; Li, et al., 2024b; Noguera-Fernández, et al., 2024). Resistant starch (**RS**), a non-viscous and soluble fiber, has been shown to be readily fermented by gut microbiota (Holscher, 2017), and provide significant benefits to gut health and the resident microbiota of birds (Regassa and Nyachoti, 2018). Clinical data indicate that RS intake enhances the biochemical functions of the human intestinal tract (Maier, et al., 2017). Furthermore, long-term RS consumption has been associated with positive alterations in intestinal morphology and improved colon barrier integrity (Nofrarías, et al., 2007). Using raw potato starch (**RPS**) as a source of RS, our recent dose-dependent trial demonstrated that a diet containing 12 % RPS enhanced ileal barrier function in 14- and 35-day-old meat ducks (Qin, et al., 2019). Consistent with other studies on RPS and intestinal homeostasis (Zhang, et al., 2022a, 2022b), the protective effects of dietary RPS on barrier function and inflammatory response were observed under both conventional conditions and systemic inflammatory models, which were accompanied by an increased abundance of Firmicutes and elevated production of short-chain fatty acids (**SCFAs**) in the cecal content of ducks (Qin, et al., 2022, 2020, 2023). Since dietary RS is barely digested by the host and instead serves as a substrate for colonic microbiota in a mutualistic relationship with the host tract (Maier, et al., 2017), it is likely that the beneficial effects of RS on intestinal health in meat ducks are primarily mediated through its direct influence on cecal microbiota. Notably, RS-induced microbial modulation may extend beyond gut health to influence stress-related behaviors via the microbiota-gut-brain axis, as shown in mice supplemented with RS diet (Lyte, et al., 2016), suggesting broader welfare implications of dietary RS via microbial modulation in poultry.

Antibiotic-induced gut microbiota dysbiosis has been widely used to understand the mechanism underlying microbiota-host interactions following microbial disruption, and strategies for remediating these perturbations (Fishbein, et al., 2023). By causing a significant reduction in microbial diversity and the depletion of specific taxa, antibiotics can further disrupt gut signaling and trigger pathological inflammation (Brussow, 2020; Fishbein, et al., 2023; Zarrinpar, et al., 2018). Given that the enhancement of intestinal barrier function by RS is closely associated with the direct modulation of cecal microbiota, dietary RS supplementation may help reverse antibiotic-induced intestinal dysfunction. To validate this, the current study first established an antibiotic-induced microbial disruption model, followed by dietary RS supplementation, to assess its impact on intestinal development and barrier function. Given the prohibition on synthetic antibiotic growth promoters in feed, this approach underscores the potential of RS as a nutritional strategy to enhance animal welfare and food safety.

Materials and methods

Animals and treatments

All procedures of this experiment were approved by the Animal Care and Use Committee of Sichuan Agricultural University (Certification No. SYXK2019-187). Birds were housed in a temperature- and humidity-controlled room. The temperature was controlled at 32°C for 1 to 3 d and then gradually lowered to 22°C by 21 d with 23L:1D lighting system. A total of 144 7-d-old male ducks (367.3 ± 10.38 g) from the Cherry Valley meat-type strain were allocated to the control group (CON, $n = 8$) or antibiotic (AB, $n = 16$) groups, which were provided either without or with broad-spectrum antibiotics (ampicillin 1.0 g/L and neomycin 0.8 g/L, based on (Carvajal-Aldaz, et al., 2017)) in drinking water supply daily and fed a basal diet for 7 d. The study focused on RS-mediated cecal microbiota reshaping post-antibiotics, as RS effects in healthy ducks were previously established (Qin, et al., 2023, 2019). Thus, on 14 d of age, antibiotic treatment was stopped, ducks from AB groups were further divided into two groups based on average body weight and fed the basal diet (Post AB, $n = 8$) or a RS diet (Post AB_RS, $n = 8$) until to 21d, *i.e.*, the study included CON, Post AB, and Post AB_RS groups, with 8 replicates of 6 ducks each treatment, as shown in Fig. 1A. Birds had *ad libitum* access to feed and water, and the RS diet contained 12 % raw potato starch (RPS, Type 2) in native granular form based on our previous work (Qin, et al., 2019). The basal and RS diets were isonitrogenous and isocaloric, and formulated to meet the nutrient requirements of meat ducks, according to National Research Council (1994) and meat-type duck recommendations (Table 1). The numbers of death and culling birds were recorded during the whole study.

To evaluate the establishment of the antibiotic-induced microbial disruption model, six ducks were randomly selected from the CON and AB groups at 14 d of age. Blood samples were collected from the jugular vein into vacuum tubes, allowed to clot, and centrifuged at 3,000 × *g* for 15 min to separate the serum, which was then stored at −20°C for further analysis. Then, these birds were euthanized via anesthetization and exsanguination. About 5-cm ileum segment was excised and the mucosa was collected by PBS-perfused segment scraping with sterile glass slides, while cecum was incised with surgical scissors, and luminal contents transferred into RNase-free cryovials using disposable spatulas. All samples were snap-frozen in liquid nitrogen and stored at −80°C until analysis.

To evaluate the effects of post-antibiotic treatment and dietary RS supplementation on intestinal development and barrier function, one bird per replicate was selected from the CON, Post AB, and Post AB_RS groups based on average body weight for sampling at 21 d. Blood samples were collected from the jugular vein and the serum was separating by centrifugation. Following euthanasia, the whole ileum was dissected for determination of length and weight. Whereafter, the mid-ileum samples were excised from the middle third of the ileum, spanning equidistantly between the jejuno-ileal junction and ileocecal

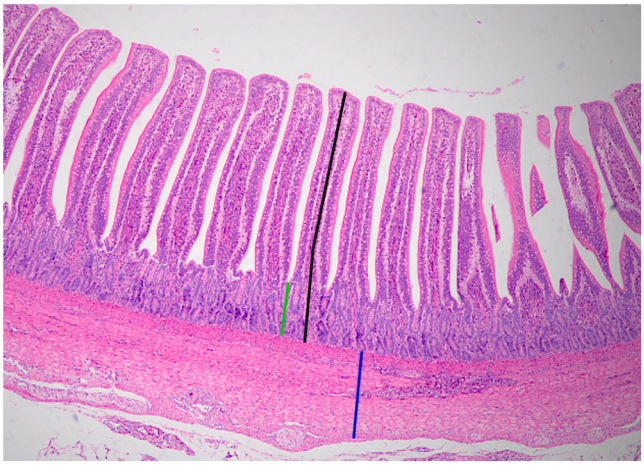

Fig. 1. Morphometric measurements of villus height (black bar), crypt depth (green bar), and muscular thickness (blue bars) on an ileum from a meat duck (hematoxylin and eosin staining, 100 ×).

Table 1
Dietary formulation and composition (as fed basis).

Item	Basal diet	Resistant starch diet
Ingredients, %		
Corn	59.82	44.85
Soybean meal	33.22	35.91
Raw potato starch	0.00	12.00
Soybean oil	0.50	1.50
Calcium Carbonate	1.10	1.04
Dicalcium phosphate	1.75	1.82
L-Lysine-HCL	0.12	0.07
DL-Methionine	0.16	0.17
L-Threonine	0.02	0.02
Bentonite	2.33	1.64
Sodium chloride	0.30	0.30
Choline chloride	0.15	0.15
Vitamin premix[1]	0.03	0.03
Mineral premix[2]	0.50	0.50
Total	100.0	100.0
Calculated analysis, %		
Apparent metabolism energy, kcal	2800	2800
Crude protein	19.50	19.51
Calcium	0.90	0.90
Non-phytate phosphorus	0.42	0.42
Digestibility Lysine	1.00	0.98
Digestibility Methionine	0.43	0.43
Nutrient analysis (%)		
Crude protein	19.45	19.32
Calcium	0.91	0.88
Total phosphorus	0.70	0.73
Crude Fiber	3.52	3.57
Resistant starch	3.73	7.66

[1] Provided per kilogram of diet: Cu ($CuSO_4 \bullet 5H_2O$), 8 mg; Fe ($FeSO_4 \bullet 7H_2O$), 80 mg; Zn ($ZnSO_4 \bullet 7H_2O$), 90 mg; Mn ($MnSO_4 \bullet H_2O$), 70 mg; Se ($NaSeO_3$), 0.3 mg; I (KI), 0.4 mg.

[2] Provided per kilogram of diet: retinol, 2.06 mg; cholecalciferol, 0.04 mg; vitamin E, 30.01 mg; thiamine, 1 mg; riboflavin, 3.9 mg; pyridoxine, 3.375 mg; vitamin B12, 0.01 mg; calcium pantothenate, 8.85 mg; folate, 0.5 mg; biotin, 0.1 mg; niacin, 49.25 mg.

junction, and preserved in 4 % paraformaldehyde for morphological examination. Additionally, ileal mucosa and cecal digesta samples were collected in sterile frozen storage tubes and stored at −80°C for subsequent analysis.

16S rRNA sequencing and data analysis

Microbial DNA was obtained from cecal digesta using the DNA stool mini kit (Qiagen, Valencia, CA, United States). DNA concentration was quantified using the PicoGreen dsDNA kit (Solarbio, Beijing, China), while the integrity was verified by 2 % agarose gel electrophoresis. The V4 region of 16S rRNA was amplified using primers 515F/806R. Libraries were constructed with the NEBNext Ultra II Kit (NEB#E7645L, NEW ENGLAND BioLabs) and sequenced on the Illumina HiSeq platform (PE250) at Rhonin Biosciences Co., Ltd (Chengdu, China). Raw sequences were processed using FLASH (v1.2.11) to merge paired-end reads. Low-quality reads were filtered ($Q < 30$ and sequence length <200 bp) and potential chimeric sequences removal with USEARCH (v7.0.1090). Taxonomic classification was performed using the SILVA database (Release 138) with a Naïve Bayes classifier integrated in QIIME2 (2020.2). The final reads were clustered as operational taxonomic units (**OTUs**) with a 97 % similarity threshold. alpha- and beta-diversity were calculated using R Vegan package (v4.0.5), Principal coordinates analysis (**PCoA**) was performed by R package mixOmics. The linear discriminant analysis (**LDA**) effect size (**LEfSe**) method was applied to differentiate taxa with significantly different genera. BioProject ID: PRJNA1248144.

Intestinal morphology

Approximately 1 cm of the ileum was fixed in 4 % paraformaldehyde. Tissue pieces were sectioned at 4-μm and stained with hematoxylin and eosin (**H&E**) for intestinal architecture. The villus height, crypt depth and muscular thickness of ileum were determined using an Olympus BX43 System Microscope (Olympus Corporation, Tokyo, Japan) from at least 10 well-oriented villi unit. The corresponding average value of villus height, crypt depth and muscular thickness, according to Fig. 1. The ratio of villus height to crypt depth for each bird were then calculated.

Cytokine concentrations analysis

Tumor necrosis factor (**TNF**)-α, interferon (**IFN**)-γ, interleukin (**IL**)-1β, and IL-6 concentrations in serum were measured using enzyme linked immunosorbent assay (ELISA) as described by the manufacturer's instructions. All duck-specific commercial kits (TNF-α: MM-32774O1; IFN-γ: MM-0073O1; IL-1β: MM-32771O1; IL-6: MM-91624O1) were obtained from MEIMIAN Biotechnology Co., Ltd (Jiangsu, China).

Quantitative real-time PCR

The ileal mucosa was ground in liquid nitrogen using a pre-chilled mortar and pestle to ensure RNA integrity and mixed with TRIzol reagent (Takara Bio, Inc., Dalian, China), and RNA was extracted following the manufacturer's instructions. The quality and concentration of the RNA were assessed before cDNA synthesis using the PrimeScript™ RT Reagent Kit (Takara Bio, Inc.). Quantitative real-time PCR was performed using TB Green™ Premix Ex Taq™ II (Takara Bio, Inc.) and primers designed with Primer 3.0 (Table 2) in an QuantStudio™ 6 Flex system (Applied Biosystems, USA). Melting curve analysis was conducted for all reactions to ensure primer specificity. Relative mRNA expression abundances were normalized using the geometric mean of two reference genes (*β-actin* and glyceraldehyde-3-phosphate dehydrogenase (***GAPDH***)), and fold changes were calculated for each target gene.

Statistical analysis

All data were analyzed using JMP software (SAS Institute, Inc., Cary, NC, USA). A statistical power of at least 80 % was achieved (α=0.05 as the significance level, effect size=1.0). Data normality and homogeneity of variance were assessed using the Shapiro-Wilk and Levene's tests, respectively. Survival analysis was conducted using the Kaplan-Meier plotter. An unpaired two-tailed *t*-test was used to compare differences between the CON and AB groups. One-way analysis of variance (ANOVA), followed by Tukey's test for multiple comparisons, was performed to identify potential differences among the CON, Post AB, and Post AB_RS groups. A Mantel test network heatmap analysis was constructed to examine correlations between microbial genera and inflammatory cytokines. All statistical results are presented as mean ± standard error. A *P*-value of less than 0.05 was considered statistically significant, while $0.05 < P < 0.10$ was interpreted as a trend.

Results

Antibiotics treatment induced dysbiosis in cecal microbiota

As illustrated in Fig. 2, the cecal microbial composition in the CON group was distinct from that in the AB group (Fig. 2B), indicating that antibiotic treatment significantly altered microbial structure. After 7 consecutive days of antibiotic administration, alpha diversity was notably reduced, as evidenced by significant decreases in both Chao1 richness and Shannon diversity indices ($P < 0.05$, Fig. 2C, D). At the phylum level, antibiotic treatment reduced the relative abundance of

Table 2
The primers for quantitative real-time PCR.

Gene	Gene ID	Primer	Sequence (5′–3′)	Size (bp)
EGFR	XM_027451316.2	Forward	acggactcgagctaccagaa	89
		Reverse	actgttttagccagcggaga	
GLP1R	XM_013103405.4	Forward	ttccaggaatccctcatctg	95
		Reverse	aggtacatgccttccaccag	
GLP2R	XM_013094791.2	Forward	gtttgctggccttactcacc	85
		Reverse	ttcctgcacttccatttttcc	
ZO-1	XM_013104939.1	Forward	tacgcctgtgaagaatgcag	86
		Reverse	ggagtggtggtgtttgcttt	
Occludin	XM_013109403.1	Forward	caggatgtggcagaggaatacaa	160
		Reverse	ccttgtcgtagtcgctcaccat	
Claudin 1	XM_013108556.1	Forward	tcatggtatggcaacagagtgg	179
		Reverse	cgggtgggtggataggaagt	
Mucin-2	XM_005024513.3	Forward	actagcacgagggaagtgga	108
		Reverse	tgggatgttgcaatgagtgt	
Bcl-2	XM_027451677.1	Forward	tgaggcctttgttcgatttc	89
		Reverse	ataagcgccaagagtgatgc	
Caspase3	XM_021279218.2	Forward	ggggtgacaagtgcagaagt	97
		Reverse	ctgtctgcctcaacaccaga	
Caspase8	XM_027461031.1	Forward	ggaaagcagtgccagaactc	96
		Reverse	taaaatgaagggtgccgaag	
Caspase9	XM_027443437.1	Forward	gggtaagcaacgtccagta	105
		Reverse	gggctgaaggtgatgttgtt	
NFκB	XM_027455993.1	Forward	gagcgttttcaagaggttgc	123
		Reverse	agggatcttctcctgccatt	
TLR4	NM_001310413.1	Forward	cagctgagtgtcctgttgga	141
		Reverse	cagcaggtcctccttttctg	
MyD88	NM_001310832.1	Forward	tgaagtgcaaagccatgaag	111
		Reverse	atttggccagtcttgtccag	
TNF-α	EU375296.1	Forward	agatgggaagggaatgaacc	51
		Reverse	gttggcataggctgtcctgt	
IFN-γ	XM_038175160.1	Forward	actggcttgaaaatccaacg	101
		Reverse	ggagactggctccttttcct	
IL-1β	DQ393268.1	Forward	gcatcaagggctacaagctc	131
		Reverse	caggcggtagaagatgaagc	
IL-6	AB191038.1	Forward	atctggcaacgacgataagg	87
		Reverse	ttgtgaggagggatttctgg	
IL-17	EU366165.1	Forward	atgcctgacccaaaaagatg	145
		Reverse	gtggtcctcatcgatcctgt	
IL-22	XM_038167538.1	Forward	caggaatcgcacctacacct	109
		Reverse	gcggttgttttccttgatgt	
GAPDH	XM_038180584.1	Reverse	gcagatgctggtgctgaata	106
		Forward	cggagatgatgacacgctta	
β-actin	NM_001310421.1	Forward	ccagccatctttcttgggta	105
		Reverse	gtgttggcgtacaggtcctt	

EGFR, Epidermal growth factor receptor; GLP-1/2R, Glucagon-like peptide-1/2 receptor; ZO-1, Zonula occludens 1; Bcl-2, B-cell lymphoma 2; TLR4, toll-like receptor 4; MyD88, myeloid differential protein 88; NFκB, Nuclear Factor-κ B; TNF-α, Tumor necrosis factor alpha; IFN-γ, Interferon gamma; IL, Interleukin; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase.

Firmicutes, Tenericutes, Actinobacteria, and Epsilonbacteraeota, while increasing the abundance of Bacteroidetes and Proteobacteria, thereby lowering the Firmicutes to Bacteroidetes ratio (Fig. 2E and F). Furthermore, linear discriminant analysis (**LDA**) revealed that ducks treated with antibiotics exhibited significantly higher proportions of Bacteroides, Alistipes, Escherichia-Shigella, Flavonifractor, and Anaerostipes compared to the CON group (Fig. 2G). In contrast, several beneficial bacteria, including Faecalibacterium, Butyricicoccus, and Ruminococcaceae, were enriched in the control group (Fig. 2G).

Antibiotics-induced microbiota dysbiosis associated with intestinal barrier disorder

Although there was no significant difference in body weight between the CON and AB groups at 14 d of age (Fig. 3A), antibiotic treatment significantly decreased ($P < 0.05$) the expression of genes related to intestinal integrity, including *Occludin, Claudin-1*, and *mucin-2*, compared to the CON group (Fig. 3B). Consequently, the mRNA levels of pro-inflammatory cytokines *TNF-α, IL-1β*, and *IL-6* were upregulated following antibiotic administration (Fig. 3C). In response to serum analysis, antibiotic-treated birds exhibited increased concentrations of IFN-γ ($P = 0.071$) and IL-1β ($P < 0.05$) relative to the CON ducks (Fig. 3D and E).

Dietary RS improved intestinal morphology and development of post-antibiotics-treated ducks

At 21 d, post-antibiotic-treated ducks that received either the basal diet or the RS diet exhibited a significant increase in body weight compared to the CON birds ($P < 0.05$; Fig. 4A). Meanwhile, neither the AB intervention nor dietary supplementation had an obvious effect on survival rate throughout the experimental period ($P = 0.819$; Fig. 4B). Despite a similar relative ileal weight across the three groups (Fig. 4C), ducks fed the basal diet after antibiotic treatment exhibited a notably shorter ($P < 0.05$) relative ileal length, which tended to recover ($P = 0.067$) with dietary RS supplementation (Fig. 4D). H&E staining revealed that ducks in the post-antibiotic basal diet group had sparser intestinal villi and reduced muscular thickness compared to the other groups (Fig. 4E and F). However, dietary RS supplementation restored villus density and led to an increase in villus height ($P = 0.058$) and muscular thickness ($P < 0.05$) in the ileum (Fig. 4E and F). Furthermore, post-antibiotic treatment significantly suppressed ($P < 0.05$) the expression of intestinal development markers, including glucagon-like peptide receptor 1 (*GLP-1R*), *GLP-2R*, epidermal growth factor receptor (*EGFR*), and the anti-apoptotic gene B-cell lymphoma-2 (*Bcl-2*), compared to the CON group (Fig. 4G and H). Dietary RS inclusion reversed ($P < 0.05$) the downregulation of *GLP-1R, GLP-2R* and *Bcl-2*, while remarkably inhibiting ($P < 0.05$) the mRNA expression of *caspase-3, caspase-8*, and *caspase-9* in the ileum (Fig. 3G and H). Regarding tight junction proteins (TJPs), post-antibiotic treatment notably reduced ($P < 0.05$) the expression of zonula occludens-1 (*ZO-1*) and *mucin-2* (Fig. 3I). However, dietary RS supplementation did not significantly restore the antibiotic-induced reduction in TJPs expression and even further suppressed ($P < 0.05$) *mucin-2* mRNA levels compared to the CON group (Fig. 4I).

Dietary RS reshaped antibiotics-induced disruption of cecal microbiota

Based on the PCoA plot, the samples in the CON group did not form a distinct cluster from those in the Post AB_RS group but were clearly separated from those in the Post AB group (Fig. 5A). Post-antibiotic treatment significantly reduced both Chao1 richness and Shannon diversity indices ($P < 0.05$), whereas dietary RS supplementation in post-antibiotic-treated ducks notably increased the Shannon diversity index ($P < 0.05$) (Fig. 5B and C). At the phylum level, the antibiotic-induced decrease in Firmicutes and Proteobacteria was partially reversed by dietary RS supplementation, whereas the relative abundance of Bacteroidetes decreased in the Post AB_RS group (Fig. 5D and E). Consequently, the Firmicutes to Bacteroidetes ratio, which was significantly reduced following post-antibiotic treatment, showed a trend toward reversal with dietary RS supplementation (Fig. 5E). According to data of LDA analysis, the most differentially enriched genera was revealed in the Post AB_RS group, including *Faecalibacterium, Coprobacter, Odoribacter, Ruminococcus torques, Ruminiclostridium 9, Barnesiella, Faecalitalea, Collinsella, Adlercreutzia* and *Megamona*, whereas the relative abundance of *Escherichia_Shigella, Phascolarctobacterium* and *Sphingomonas* increased in the Post AB group (Fig. 5F).

Dietary RS supplementation reduced the antibiotics-induced inflammation

As shown in Fig. 6, the ducks fed basal diet after antibiotics treatment had no significant effects on intestinal inflammatory response, except for having significantly higher *IL-22* mRNA levels in ileum ($P < 0.05$), while the RS diet supplementation notably downregulated the gene expressions of pro-inflammatory cytokines, including *TNF-α, IFN-γ, IL-1β* and

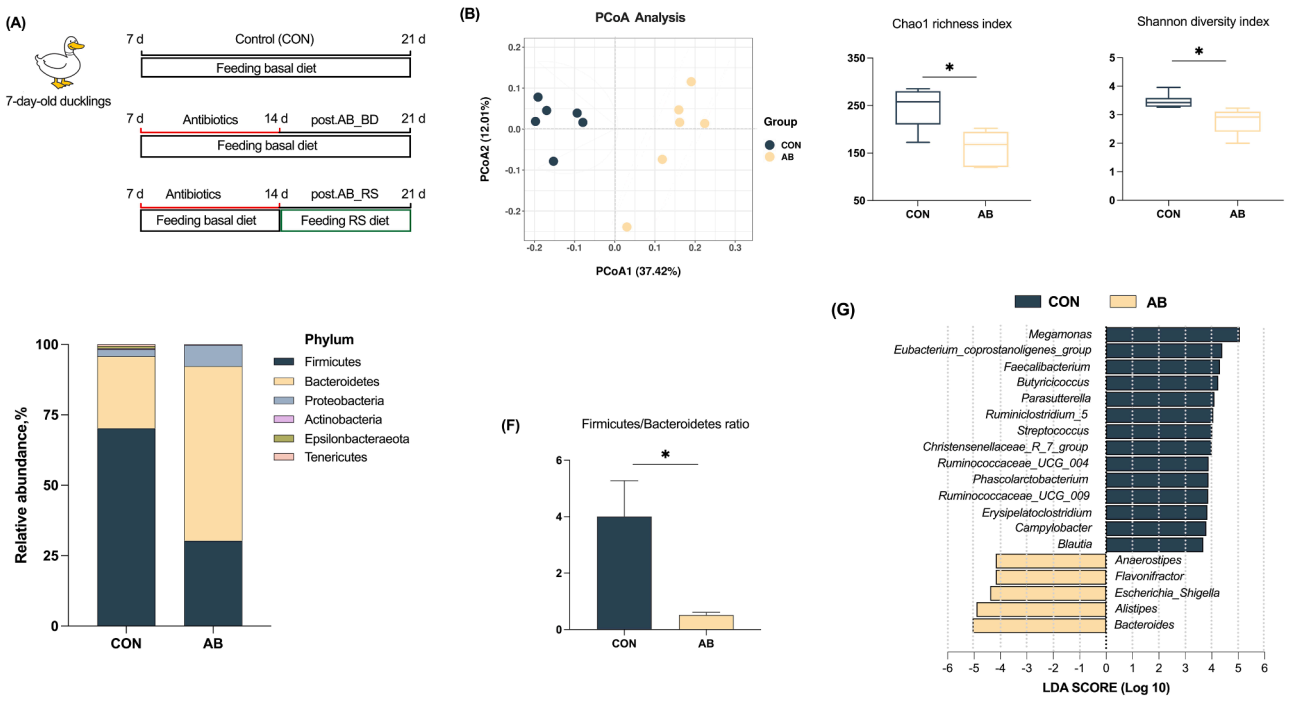

Fig. 2. Schematic presentation of the experimental design (A) and gut microbial composition in cecal digesta are altered in antibiotic (**AB**)-treated duck at 14 d (B-G). Effect of AB on the principal co-ordinates analysis (**PCoA**) analysis (B), Chao1 richness (C) and Shannon diversity (D) indexes, and composition profiles of gut microbiota at phyla level (E), the ratio of the relative abundance of Firmicutes to Bacteroidetes (F), and the linear discriminant analysis (LDA) showing the bacterial taxa that were significantly different in abundance (G) of 14-d-old ducks. Data represent means with standard deviation represented by vertical bars ($n = 6$). *Letter on bars means a significant difference at $P < 0.05$.

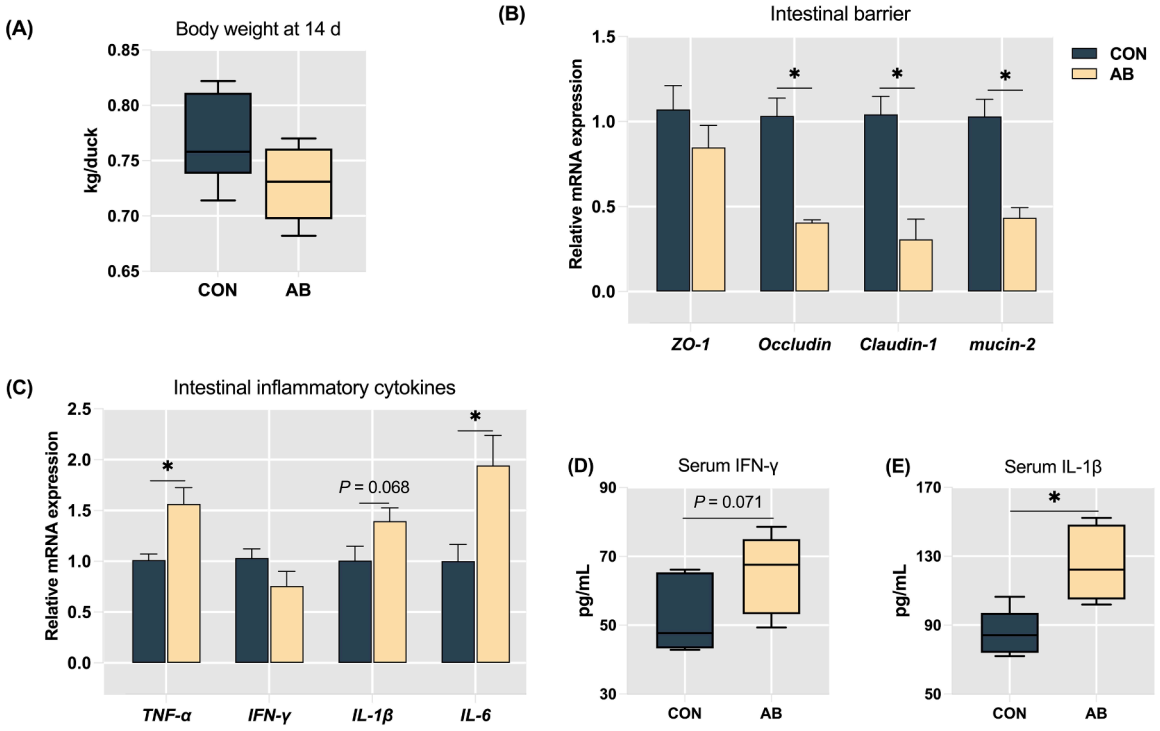

Fig. 3. Effect of antibiotics (**AB**) on body weight (A), the gene expression of intestinal barrier-related (B) and pro-inflammatory cytokines in ileum (C), and the concentration of serum IFN-γ (E) and IL-1β in serum (D) of 14-d-old ducks. Data represent means with standard deviation represented by vertical bars ($n = 6$). *Letter on bars means a significant difference at $P < 0.05$.

IL-6, as well as the transcription of nuclear factor kappa-B (*NF-κB*), myeloid differentiation primary response 88 (*MyD88*), and toll-like receptor 4 (*TLR4*) (Fig. 6A and B). In the serum of ducks, post-antibiotics treatment notably increased ($P < 0.05$) the concentrations of TNF-α, IFN-γ, IL-1β and IL-6 compared to the CON group. However, dietary RS supplementation reduced ($P < 0.05$) serum TNF-α and IL-1β levels in

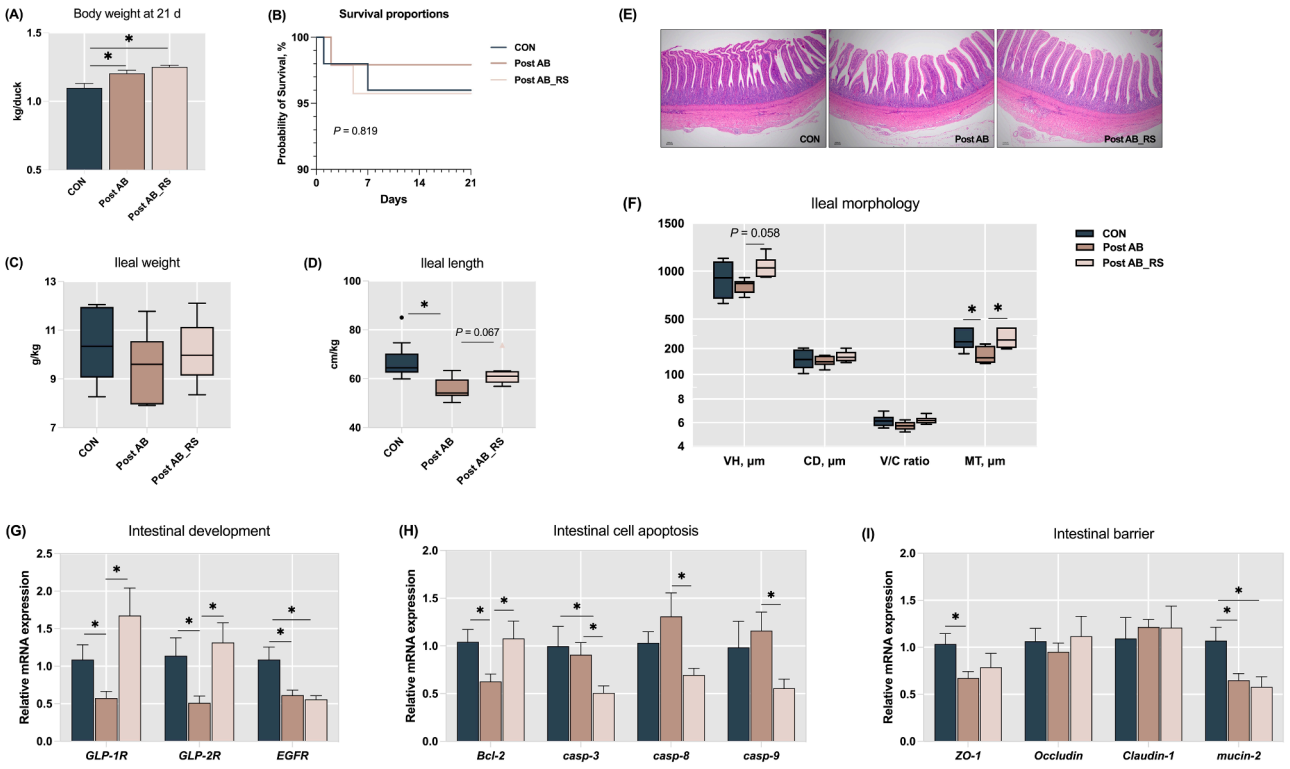

Fig. 4. Effect of dietary resistant starch (**RS**) supplementation on body weight (A), survival proportions (B) and intestinal development of ileum (C-I): weight (C), length (D), H&E staining (40 ×; E), morphological indicators (F), and the gene expression of intestinal development-related genes (G), cell apoptosis (H) and intestinal barrier-related genes (I) in antibiotics (**AB**)-treated duck at 21 d. Data represent means with standard deviation represented by vertical bars ($n = 8$). *Letter on bars means a significant difference at $P < 0.05$. VH: villus height; CD: crypt depth; V/C ratio: villus height/ crypt depth ratio; MT: muscular thickness.

post-antibiotics-treated ducks (Fig. 6C). Furthermore, from mantel test analysis (Fig. 6D), strong correlations ($P < 0.05$) were observed between serum inflammatory cytokines levels and the relative abundance of bacteria. In detail, TNF-α showed a strongly positive correlation with *Ruminococcus 2*. Meanwhile, IL-1β and IL-6 exhibited a strong positive correlation with *Blautia*. On the contrary, TNF-α was negatively correlated with *Barnesiella* and *Ruminiclostridium 9*, IFN-γ was negatively correlated with *Barnesiella, Phascolarctobacterium* and *Anaerofilum*; Serum levels of IL-1β was negatively correlated with *Megamonas, Faecalitalea, Adlercreutzia, Coprobacter, Collinsella, Phascolarctobacterium* and *Anaerofilum*; IL-6 was negatively correlated with *Ruminiclostridium 9*.

Discussion

The gut microbiota plays a crucial role in maintaining the intestinal barrier integrity of both humans and animals, ensuring dynamic stability and diversity throughout life (Adak and Khan, 2019). It has been noticed that ducklings are sensitive to exogenous interfering substances due to their underdeveloped digestive systems, making them prone to developmental anomalies and inflammation. (Lilburn and Loeffler, 2015). Therefore, using an antibiotic-induced dysbiosis model, the current study evaluated the protective role of RS against intestinal dysfunction in 21-d-old meat ducks. The results of 16S rRNA gene sequencing revealed that antibiotics treatment reduced bacterial diversity and altered the microbial composition of cecal contents, significantly lowering the ratio of Firmicutes to Bacteroidetes and increasing the abundance of the pathogenic *Escherichia-Shigella*. These changes were accompanied by intestinal barrier disruption and a systemic inflammatory response. Furthermore, following antibiotics administration, this study demonstrated that RS diet supplementation could restore microbial diversity, improve intestinal morphology in ducks suffering from antibiotic-induced dysbiosis, and further alleviate systemic inflammation.

Studies have suggested that short-term antibiotic exposure can alter the composition and function of the microbiota, shifting it into a long-term dysbiosis state. This disruption may have lasting deleterious effects on the host, increasing the risk of pathogen infections and disease (Becattini, et al., 2016; Lange, et al., 2016). Consistent with previous findings, the present study observed that early-life antibiotic exposure led to persistent alterations in the microbial community, characterized by a sustained reduction in microbial diversity and a decreased Firmicutes-to-Bacteroidetes ratio in the cecum one week after antibiotic administration. Moreover, the sustained enrichment of *Escherichia--Shigella* in post-antibiotic-treated ducks supports the notion that once the cecal microbiota becomes imbalanced, restoring homeostasis without external intervention is highly challenging. To address critiques of 'dysbiosis' conceptual ambiguity (Brussow, 2020), the present study operationalizes the term through the co-occurrence of sustained alpha-diversity decline (Chao1 and Shannon indices) and increased *Escherichia-Shigella* abundance, thereby distinguishing pathological microbial states from transient ecological fluctuations. A previous study also reported that a single early-life antibiotic exposure for five days significantly suppressed microbial diversity and altered community structure in both cecal and fecal samples of non-obese diabetic mice, with these effects persisting for weeks after the exposure ended (Zhang, et al., 2018). In this context, supplementing a 12 % RPS diet to post-antibiotic ducks in this study revealed that dietary RS effectively reshaped antibiotic-induced cecal microbiota disruptions, suggesting its potential to improve intestinal microbial composition in meat ducks (Qin, et al., 2020, 2023). Specifically, RS supplementation enriched the abundance of *Odoribacter, Faecalibacterium, Ruminiclostridium 9* and *Barnesiella* in ducks recovering from antibiotic treatment. Notably, *Odoribacter* has been shown to mitigate colitis through cellular and metabolic effectors by mediating IL-10, which promotes the differentiation of induced regulatory T cells (**iTregs**) and restrains inflammatory T

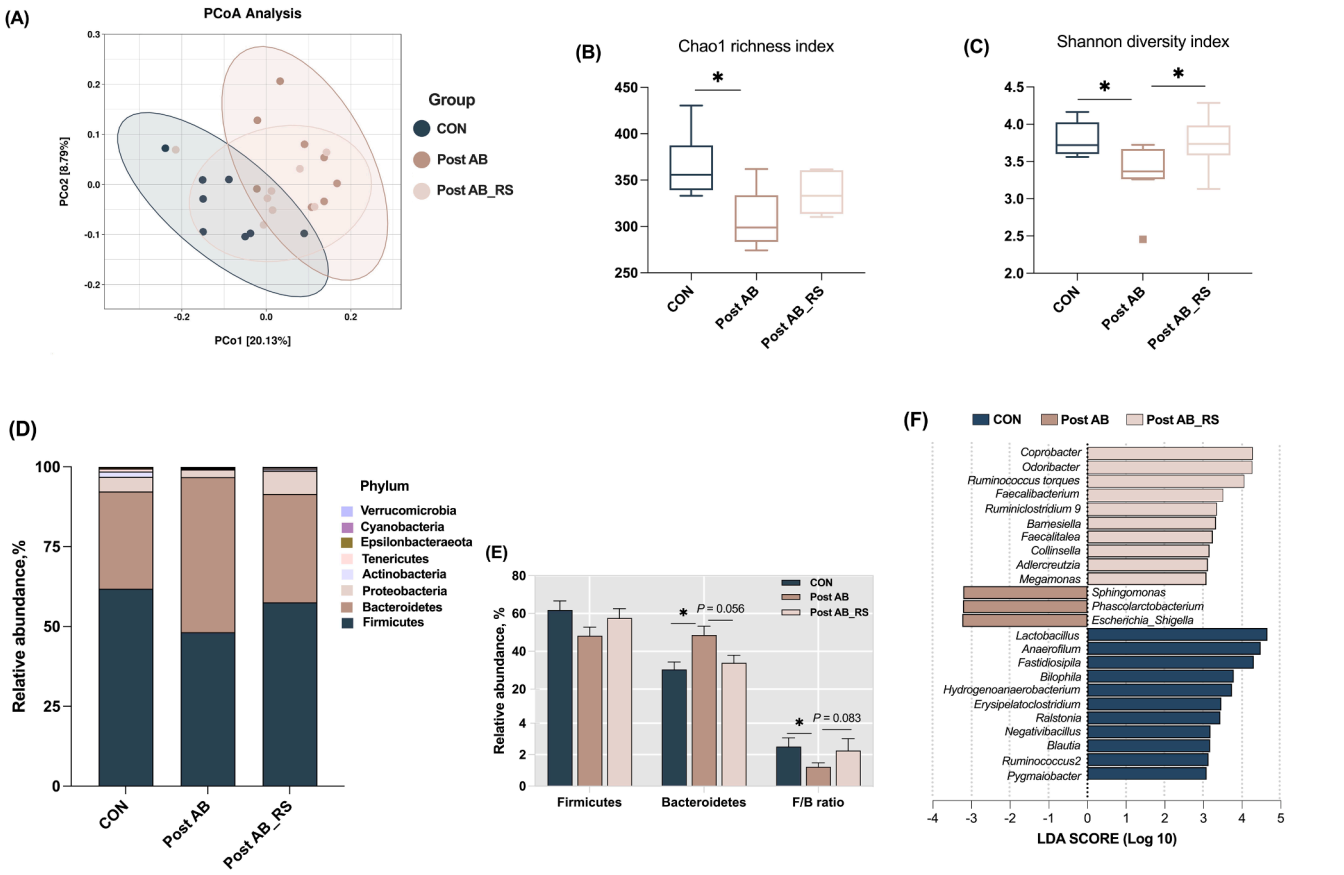

Fig. 5. Gut microbiota to dietary resistant starch (**RS**) supplementation in antibiotics (**AB**)-treated duck at 21 d: principal co-ordinates analysis (**PCoA**) analysis (A), Chao1 richness (B) and Shannon diversity (C) indexes, composition profiles of gut microbiota at phyla level (D), the relative abundance of Firmicutes and Bacteroidetes, and the ratio of the Firmicutes to Bacteroidetes (**F/B ratio**) (E), the linear discriminant analysis (LDA) showing the bacterial taxa that were significantly different in abundance (F). Data represent means with standard deviation represented by vertical bars ($n = 8$). *Letter on bars means a significant difference at $P < 0.05$.

cells in the lamina propria (Lima, et al., 2022). Similarly, *Barnesiella* has been associated with several immunomodulatory effects, contributing to a gut environment less prone to inflammation (Daillère, et al., 2016; Weiss, et al., 2014). The increased abundance of *Faecalibacterium* and the cellobiose-degrading bacterium *Ruminiclostridium,* both beneficial Firmicutes, may indicate a shift in the Firmicutes to Bacteroidetes ratio toward a more balanced state following RS supplementation after antibiotic exposure. Reflecting on the overall microbial community structure, the partial recovery of Shannon diversity (reflecting community evenness) but not Chao1 richness (indicating species loss) may implies that dietary RS fostered a functionally resilient microbiota with enriched these bacteria. This observation aligns with ecological frameworks positing that functional diversity can compensate for taxonomic simplification (Lozupone and Knight, 2008; Magurran, 2021). The RS-driven enrichment of immunomodulatory bacteria (such as *Odoribacter* and *Barnesiella*) and metabolic bacteria (such as *Ruminiclostridium*) further reflects a hierarchical restoration process (Hooks and O'Malley, 2017). Moreover, the integration of alpha diversity (community richness/evenness) and beta diversity (compositional shifts) is essential to comprehensively interpret ecological dimensions (Magurran, 2021). A framework particularly relevant was found in our study, the beta diversity analysis showed that the Post AB_RS group clustered between the Post AB and CON groups, suggesting that RS-fed ducks were gradually recovering from microbiota disruption toward a normal state. Collectively, these findings highlight a direct relationship between dietary RS supplementation and the restoration of microbial structure in ducks.

The gut microbiota of farm animals is highly sensitive to antibiotics, even when historically administered as growth promoters. Such perturbations can trigger dysbiosis and impair intestinal barrier integrity (Fishbein, et al., 2023), challenging the rationale for antibiotic abuse in livestock production. In present study, a significant increase in body weight was observed at 21 d but not at 14 d following antibiotic treatment, suggesting a time-dependent effect. A previous study found that mice treated with antibiotics for four weeks exhibited lower body weight compared to untreated counterparts (Ge, et al., 2017), and the growth-promoting effects of antibiotics did not become apparent until 19 d after administration in another mouse study (Zarrinpar, et al., 2018). These findings indicate that the impact of antibiotics on body weight may vary depending on duration, dosage, and host-specific responses. What is noteworthy is that this study highlights that post-antibiotic treatment impaired the ileal structure of ducks, as evidenced by reduced villus density, a thinner intestinal wall, and down-regulated expression of *ZO-1* and *mucin-2*. To explore the underlying mechanisms linking antibiotics treatment and intestinal morphology, we further examined genes related to enterocyte proliferation and apoptosis. It is well established that activation of GLP-1R signaling promotes intestinal growth and proliferation, as demonstrated by several studies (Koehler, et al., 2015; Zarrinpar, et al., 2018). Similarly, GLP-2 exerts its effects through its receptor, GLP-2R, which plays a crucial role in intestinal healing following injury (Kissow, 2015). Conversely, caspases are widely recognized for their role in regulating cell death. Specifically, caspase 8 and caspase 9 act as initiator caspases, triggering apoptosis by activating executioner caspases, such as caspase 3, ultimately leading to cell death (McIlwain, et al., 2013), in which anti-apoptotic gene Bcl-2, an essential regulator of programmed cell

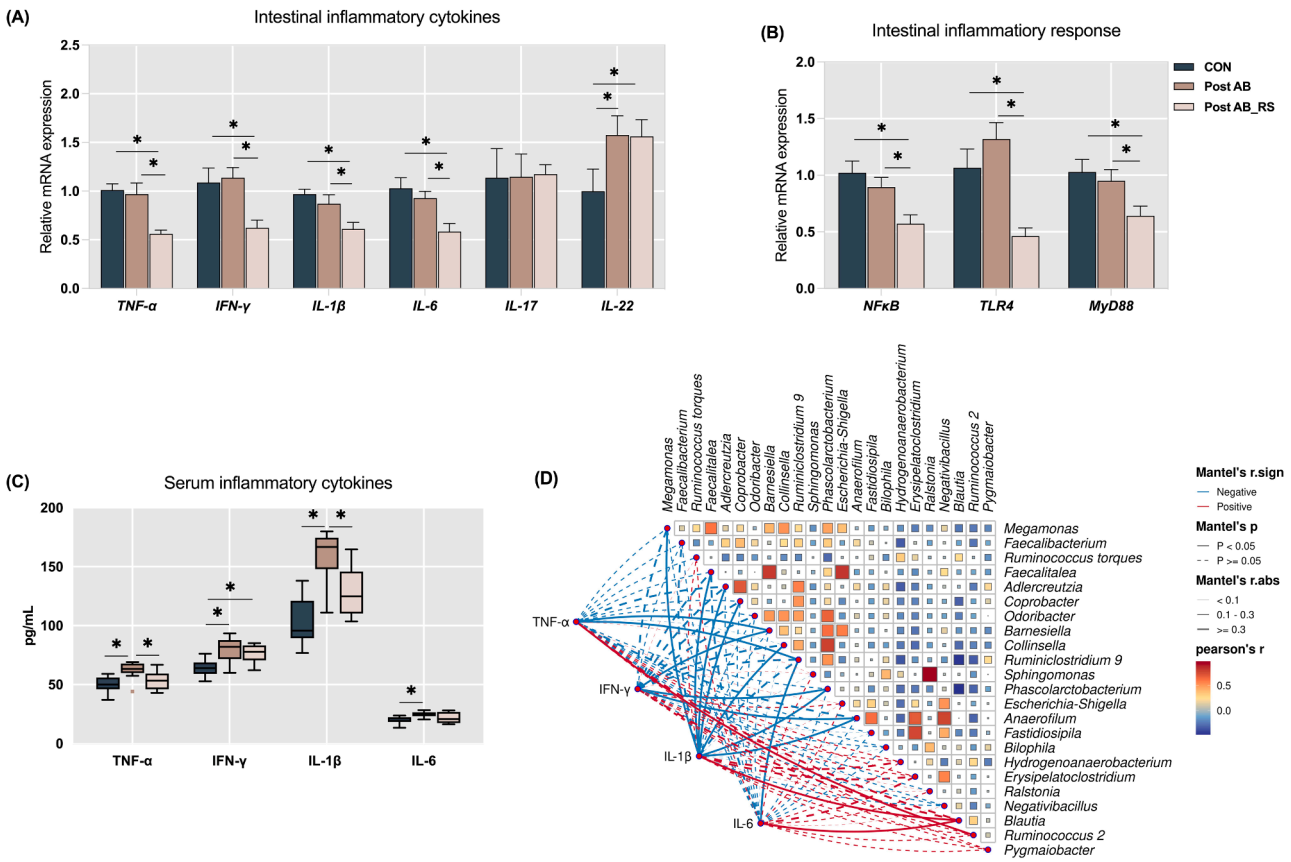

Fig. 6. Inflammatory response to dietary resistant starch (**RS**) supplementation in antibiotics (**AB**)-treated duck at 21 d: the gene expression of inflammatory cytokines (A) and TLR4/MyD88/NFκB signaling (B) in ileum, pro-inflammatory cytokines levels in serum (C), and the mantel test network heat map analysis between microbial genera and serum inflammatory cytokines (D). Data represent means with standard deviation represented by vertical bars ($n = 8$). *Letter on bars means a significant difference at $P < 0.05$.

death, is involved in the activation and function of the caspase cascade system (Ruvolo, et al., 2001). In this study, the significant suppression of *GLP-1R, GLP-2R, EGFR*, and *Bcl-2* expression following antibiotic treatment suggests that intestinal damage may result from inhibited proliferation and increased apoptosis of intestinal epithelial cells. More importantly, dietary RS supplementation not only increased villus height and muscular thickness but also reduced the transcription of *caspase 3, caspase 8*, and *caspase 9*. Additionally, it regulated the mRNA levels of *GLP-1R, GLP-2R*, and *Bcl-2* in the ileum of meat ducks. These findings suggest that dietary RS supplementation may facilitate intestinal development by promoting cell proliferation and reducing cell death in meat ducks following antibiotics treatment.

The concurrence of microbial disruption and barrier injury following antibiotic treatment appears to provide evidence of an antibiotic-induced inflammatory response. It is well known that the loss of intestinal homeostasis triggered by antibiotics often creates a pro-inflammatory environment, characterized by increased levels of cytokines such as TNF-α, IFN-γ, IL-1β, and IL-6 (Becattini, et al., 2016; Brennan and Garrett, 2016). In our study, the expression of pro-inflammatory cytokines *TNF-α, IL-1β*, and *IL-6* in the ileum was notably elevated at the end of antibiotics treatment but returned to baseline levels within a week after cessation, showing no difference from untreated ducks. However, *IL-22* expression in the ileum remained exceptionally elevated in antibiotic-treated birds (Post AB and Post AB_RS groups). IL-22 plays a pivotal role in mediating epithelial regeneration and reinforcing antimicrobial defenses during mucosal repair, as demonstrated in intestinal inflammation injury models (Dean, et al., 2024). Therefore, the persistence of IL-22 could indicate ongoing reparative processes, suggesting a transitional phase in restoration of barrier integrity post-antibiotic challenge. Meanwhile, our results confirm that antibiotic-induced intestinal and systemic inflammation persists for a short period post-treatment. In contrast, serum pro-inflammatory cytokine levels remained consistently higher in post-antibiotic-treated ducks compared to untreated ducks, suggesting that antibiotics induce a prolonged systemic inflammatory state. Notably, dietary RS supplementation reduced the gene expression of pro-inflammatory cytokines (*TNF-α, IFN-γ, IL-1β*, and *IL-6*) in the ileum and lowered their persistently elevated serum concentrations (TNF-α and IL-1β). This suggests that RS may contribute to the acceleration of recovery from inflammation. Consistent with our findings, previous studies have reported similar anti-inflammatory effects of dietary RS (Qin, et al., 2022; Wen, et al., 2022). In this study, dietary RS supplementation was sufficient to rapidly alleviate antibiotic-induced intestinal inflammation after treatment cessation. Additionally, the activation of the NF-κB pathway, triggered by TLR4 via the MyD88 adapter, is known to drive pro-inflammatory responses (Barton and Kagan, 2009). To further evaluate the inflammatory response, we examined the TLR4/MyD88/NF-κB signaling pathway and found that dietary RS inhibited the expression of *TLR4, MyD88*, and *NF-κB*. This inhibition may contribute to reduced intestinal inflammation by improving cecal microbial composition and modulating inflammatory cytokine expression, ultimately suppressing NF-κB activation. Given that many beneficial bacteria enriched by RS supplementation, such as *Faecalibacterium*, have demonstrated anti-inflammatory effects (Martin, et al., 2023), our research suggests that dietary RS may reduce inflammatory responses by optimizing the cecal microbial environment. Correlation analysis further indicated that the abundances of *Barnesiella, Ruminiclostridium 9, Megamonas, Faecalitalea, Adlercreutzia, Coprobacter* and *Collinsella* were

negatively correlated with serum inflammatory cytokine levels. Although the precise mechanisms underlying cecal microbiota alterations due to antibiotics treatment and RS intervention require further investigation, our findings provide valuable insights into their potential relationships in meat ducks.

In conclusion, this study confirmed that antibiotic-induced microbial disruption triggered both intestinal and systemic inflammation in ducks. Dietary supplementation with RS helped restore microbial diversity and reshape microbial composition, counteracting the loss of diversity and structural disruptions induced by antibiotic treatment. Consequently, the inclusion of dietary RS supported intestinal development and alleviated systemic inflammation. These findings suggest that cecal microbiota may directly mediate the protective effects of dietary RS in the ileum of ducks following antibiotic-induced dysbiosis. This study broadens our understanding of microbiota-targeted nutritional strategies for mitigating intestinal inflammation in poultry production.

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.

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