

Article

Purified Zearalenone at the Regulatory Limit Exhibits No Overt Toxicity in Broilers

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Abstract

Zearalenone (ZEA) is a prevalent non-steroidal estrogenic mycotoxin in feed and feedstuffs. This study investigated the effects of graded dietary purified ZEA standard (0, 0.2, 0.5, 1, 2, and 4 mg/kg) on growth performance, blood biochemistry, oxidative stress, immune response, intestinal morphology, histopathology, and gut microbiota in broilers. The use of purified ZEA standard eliminates confounding effects from co-occurring contaminants and the reduced nutritional quality of naturally contaminated feed, allowing an accurate assessment of ZEA-specific effects. A total of 216 one-day-old Arbor Acres male broilers were randomly allocated into six treatment groups, each with six replicates of six birds, for a 42-day trial. At the regulatory limit (0.5 mg/kg) and below, no overt toxic effects were observed on growth performance, hematology, or serum biochemistry. Although alterations in oxidative stress markers, specifically decreased liver superoxide dismutase (SOD) activity and reduced ileal glutathione peroxidase (GSH-Px) activity, and in immune markers, including increased interleukin-2 (IL-2) levels in the jejunum and ileum and decreased ileal interleukin-10 (IL-10) levels, were observed at 0.2–0.5 mg/kg, these changes did not cause tissue damage or functional impairment. Toxicological alterations emerged only at higher doses (1–4 mg/kg), comprising impaired jejunal morphology and moderate lung secretory cell metaplasia. The highest dose (4 mg/kg) further induced severe renal tubular degeneration and necrosis, accompanied by significant disruption of the jejunal microbiota. In conclusion, these findings indicate that purified ZEA at the regulatory limit exhibits no overt toxicity in broilers, although higher contamination levels pose clear risks to intestinal, pulmonary, and renal health.

Keywords: zearalenone; broilers; oxidative stress; immune response; gut microbiota

Key Contribution: This study accurately characterizes the dose–response relationship for purified ZEA in broilers. It confirms the safety of dietary ZEA at the current regulatory limit (0.5 mg/kg) and demonstrates clear multi-organ toxicity at higher doses (≥ 1 mg/kg), primarily affecting the intestine, lung, and kidney. These findings provide a definitive reference for refining feed safety standards and highlight the importance of monitoring sensitive health endpoints.



Received: 11 January 2026

Revised: 14 February 2026

Accepted: 16 February 2026

Published: 18 February 2026

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1. Introduction

Zearalenone (ZEA), a non-steroidal estrogenic mycotoxin produced by *Fusarium* species, is a globally prevalent contaminant in feed ingredients, particularly in cereals such as corn, wheat, sorghum, and their by-products [1]. Its contamination can lead to severe health issues in livestock and result in substantial economic losses. Following ingestion, ZEA can be biotransformed into a variety of metabolites by plants, microorganisms, animals, and humans, and the primary derivatives include zearalanone (ZAN), α -zearalanol (α -ZAL), β -zearalanol (β -ZAL), α -zearalenol (α -ZEL), and β -zearalenol (β -ZEL) [1,2]. Surveillance data underscore the severity of contamination. In China, extensive monitoring data highlight the scale of this issue, with reports indicating average ZEA contamination levels in feed ranging from 48.1 to 326.8 $\mu\text{g/kg}$ (0.0481 to 0.3268 mg/kg) between 2018 and 2020, and individual samples containing concentrations as high as 1599 $\mu\text{g/kg}$ [3]. Earlier surveys from 2013 to 2015 similarly documented a maximum ZEA content of 1518.2 $\mu\text{g/kg}$ in corn [4]. Critically, these real-world contamination levels pose a direct challenge to current regulatory standards, as they nearly reach and even exceed the maximum permitted levels established in China for ZEA in poultry feed [5]. Furthermore, the co-contamination of multiple mycotoxins in feed is prevalent, and their interactions may lead to additive or synergistic detrimental effects on animal health [6]. This context highlights the urgency of precisely defining the subclinical toxicity of ZEA alone under chronic exposure, as it is pivotal to ensuring the scientific validity and accuracy of our health risk assessment in real-world contamination scenarios.

Historically, poultry have been considered relatively resistant to ZEA, a conclusion largely drawn from studies employing high-dose acute exposure models. Early evidence came from research involving single oral doses as high as 15.0 g/kg (15,000 mg/kg) in laying hens or dietary levels up to 800 mg/kg over three weeks in broilers, which reported no significant adverse effects on mortality, growth performance, or major organ weights, with observations limited to minor alterations in reproductive organ size [7,8]. This historical evidence fostered a perception of broad tolerance in avian species. However, recent studies demonstrate that chronic exposure to ZEA within a range of relevant doses can induce a spectrum of subclinical and clinical adverse effects. Specifically, exposure to dietary ZEA at 7.9 mg/kg can cause significant physiological disruptions, including increased plasma γ -glutamyltransferase activity and elevated glutathione peroxidase activity in renal and duodenal tissues, suggesting the induction of oxidative stress [9]. Furthermore, exposure to a concentration as low as 2 mg/kg suppresses growth performance and impairs bone development in broilers [10]. These findings highlight a complex dose–response relationship for the toxicity of ZEA in poultry. They indicate that adverse effects can occur across a range of exposure levels and that total growth performance is an insufficiently sensitive endpoint for comprehensive risk assessment. Notably, the inconsistencies observed across studies may stem from the use of naturally contaminated feed in some experiments, where the effects of ZEA are confounded by its various derivatives, other co-occurring contaminants, and the reduced nutritional quality of mold-damaged feedstuffs. Consequently, safety evaluations must consider a broader range of physiological and biochemical endpoints, and more importantly, employ purified ZEA under controlled conditions, to accurately characterize the risk posed by chronic ZEA contamination in modern poultry production.

The toxicity of ZEA is mediated through complex and interconnected pathways. The primary action is its competitive binding to estrogen receptors (ERs) [11]. This binding activates transcription via estrogen response elements, leading to the disruption of endocrine homeostasis and hormonal balance [12]. Beyond the classical ER pathway, ZEA can also activate other nuclear receptors, thereby altering the metabolic kinetics, tissue

distribution, and ultimate toxicity of ZEA itself [13,14]. Oxidative stress is another critically important mechanism in ZEA toxicity. ZEA induces excessive generation and accumulation of reactive oxygen species, ultimately compromising cellular function and survival [15]. Concurrently, ZEA impairs the antioxidant defense system, thus weakening cellular antioxidant capacity and exacerbating oxidative damage [16,17]. ZEA can also exacerbate its overall toxicity by disrupting immune function, which includes aggravating inflammatory reactions through altered cytokine secretion and disturbing immune homeostasis [18,19]. Despite an advanced understanding of its toxic mechanisms, a systematic elucidation of the multi-organ toxicity profile and dose–response relationship of ZEA at real-world exposure levels is still lacking.

Based on this critical knowledge gap, the present study was designed to systematically evaluate the comprehensive health impacts of chronic ZEA exposure in broilers under experimental conditions that closely simulate real-world contamination scenarios. Specifically, we aimed to investigate the effects of graded dietary ZEA concentrations (0, 0.2, 0.5, 1, 2, and 4 mg/kg) on key physiological and health parameters. This gradient was strategically chosen to encompass the common contamination levels, including the regulatory limit of 0.5 mg/kg, and to reflect extreme contamination values detected in poultry feed. Importantly, purified ZEA was used in the study to precisely attribute any toxic effects to this specific mycotoxin, thereby excluding confounding factors from other coexisting contaminants or from the reduced nutritional quality of naturally mold-damaged feed. Our assessment included growth performance, hematology and serum biochemistry, markers of oxidative stress and cytokine production in serum and multiple tissues, intestinal morphology and histopathology of major organs, as well as alterations in the gut microbial community. By integrating these multifaceted endpoints, this study aims to establish a clear dose–response relationship for ZEA in broilers and elucidate potential subclinical damage that may not be reflected in conventional growth performance indices. The findings are expected to provide a more robust scientific basis for refining feed safety standards and developing targeted mitigation strategies to safeguard poultry health and productivity in the face of mycotoxin contamination.

2. Results

2.1. Effects of ZEA on Growth Performance of Broilers

As shown in Table 1, dietary ZEA contamination at levels of 0.2, 0.5, 1, 2, and 4 mg/kg did not affect the body weight (BW), average daily gain (ADG), average daily feed intake (ADFI), or feed-to-gain ratio (F:G) during the 0–21, 22–42, and overall 0–42 day periods ($p > 0.05$).

Table 1. Effects of dietary ZEA exposure on growth performance of broilers.

Items	Concentration of ZEA (mg/kg)						SEM	<i>p</i> -Values
	0	0.2	0.5	1	2	4		
Initial BW (g)	47.65	47.46	47.64	48.12	47.60	47.33	0.23	0.294
21 d BW (kg)	0.50	0.50	0.53	0.53	0.51	0.51	0.02	0.558
42 d BW (kg)	2.03	1.93	1.98	1.93	1.92	1.91	0.07	0.761
0–21 d								
ADG (g)	22.49	22.43	24.34	23.93	23.01	23.14	0.88	0.581
ADFI (g)	34.81	34.62	35.68	35.24	35.64	37.20	1.22	0.725
F:G	1.55	1.56	1.47	1.48	1.56	1.63	0.08	0.696
22–42 d								
ADG (g)	73.14	68.18	67.89	66.46	67.59	66.50	2.45	0.423
ADFI (g)	122.61	107.64	114.16	111.16	109.63	116.58	5.88	0.523
F:G	1.68	1.57	1.68	1.67	1.63	1.75	0.07	0.511

Table 1. Cont.

Items	Concentration of ZEA (mg/kg)						SEM	<i>p</i> -Values
	0	0.2	0.5	1	2	4		
0–42 d								
ADG (g)	48.43	45.87	46.65	45.71	45.84	45.35	1.59	0.776
ADFI (g)	78.71	71.13	74.92	73.20	72.64	76.89	3.30	0.603
F:G	1.63	1.54	1.61	1.60	1.59	1.70	0.06	0.590

Note: BW, body weight; ADG, average daily gain; ADFI, average daily feed intake; F:G, feed to gain ratio. SEM, standard error of the mean. *n* = 6.

2.2. Effects of ZEA on Hematological Parameters of Broilers

As detailed in Table 2, dietary ZEA contamination at 0.2–4 mg/kg did not induce any significant changes in the hematological parameters of broilers (*p* > 0.05).

Table 2. Effects of dietary ZEA exposure on hematological parameters of broilers.

Items	Concentration of ZEA (mg/kg)						SEM	<i>p</i> -Values
	0	0.2	0.5	1	2	4		
WBC (10 ⁹ /L)	118.88	115.32	115.69	116.82	116.13	119.85	1.18	0.059
RBC (10 ¹² /L)	2.61	2.81	2.79	3.05	2.99	2.88	0.14	0.313
HGB (g/L)	64.50	58.17	55.17	67.67	64.33	61.83	6.97	0.836
HCT (L/L)	0.25	0.28	0.27	0.30	0.29	0.29	0.01	0.234
MCV (fL)	95.98	98.03	96.38	98.10	97.92	98.98	1.10	0.386
MCH (pg)	22.55	20.58	19.60	21.75	21.07	20.75	1.37	0.863
MCHC (g/L)	218.00	199.17	193.00	172.53	204.17	198.33	17.57	0.761
PLT (10 ⁹ /L)	23.75	21.20	21.50	23.80	24.00	18.40	2.74	0.558
PCT (L/L)	0.03	0.02	0.02	0.03	0.03	0.02	0.003	0.623
MPV (fL)	11.00	11.12	11.15	11.18	11.10	11.16	0.13	0.936
PDW (%)	74.53	81.32	80.88	62.48	75.57	61.48	7.13	0.148

Note: WBC, white blood cell count; RBC, red blood cell count; HGB, hemoglobin; HCT, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; PLT, platelet count; PCT, plateletcrit; MPV, mean platelet volume; PDW, platelet distribution width. SEM, standard error of the mean. *n* = 6.

2.3. Effects of ZEA on Serum Levels of Biochemical Parameters, Oxidative Stress Markers, and Immune Cytokines of Broilers

The effects of dietary ZEA on serum biochemistry of broilers are summarized in Table 3. Dietary ZEA exposure significantly increased serum alkaline phosphatase (ALP) activity at 4 mg/kg (*p* < 0.01), but did not affect any other biochemical parameter across the 0.2–4 mg/kg dose range (*p* > 0.05).

Serum oxidative stress responses of 42-day-old broilers are shown in Figure 1. Dietary ZEA exposure at 0.2–4 mg/kg did not affect serum malondialdehyde (MDA) content (*p* > 0.05). In contrast, superoxide dismutase (SOD) activity was increased by ZEA at 1, 2, and 4 mg/kg (*p* < 0.05). Notably, serum glutathione peroxidase (GSH-Px) activity was elevated at 0.2 and 1 mg/kg ZEA but suppressed at 4 mg/kg ZEA (*p* < 0.05).

Serum cytokine profiles are presented in Figure 2. The levels of tumor necrosis factor- α (TNF- α) and interleukin-1 β (IL-1 β) did not differ among the treatment groups (*p* > 0.05). In contrast, interleukin-2 (IL-2) levels were increased in broilers exposed to 0.5–4 mg/kg ZEA (*p* < 0.05). Meanwhile, interleukin-4 (IL-4) levels were reduced by dietary ZEA at 1–4 mg/kg (*p* < 0.05). Furthermore, interleukin-10 (IL-10) level was decreased specifically at the highest dose of 4 mg/kg ZEA (*p* < 0.05).

Table 3. Effects of dietary ZEA exposure on serum biochemical parameters of broilers.

Items	Concentration of ZEA (mg/kg)						SEM	<i>p</i> -Values
	0	0.2	0.5	1	2	4		
ALT (U/L)	13.71	13.86	13.93	13.78	13.05	11.90	0.58	0.136
AST (U/L)	371.07	437.63	329.67	425.43	437.83	512.38	43.33	0.093
ALP (U/L)	1183.5 ^b	1077.6 ^b	1334.4 ^b	860.4 ^b	1311.9 ^b	2016.9 ^a	158.56	0.001
TP (g/L)	25.57	23.73	22.89	27.61	22.78	25.11	1.23	0.073
ALB (g/L)	11.85	11.32	10.48	12.35	10.77	11.90	0.52	0.117
TC (mmol/L)	3.11	2.69	2.72	3.21	2.81	3.05	0.17	0.179
TG (mmol/L)	0.38	0.47	0.44	0.43	0.42	0.40	0.05	0.832
GLU (mmol/L)	11.24	9.71	10.11	10.66	9.49	10.13	0.48	0.148
UREA (mmol/L)	0.42	0.44	0.45	0.51	0.47	0.53	0.04	0.355
TBIL (μmol/L)	9.16	7.67	7.93	8.31	7.22	8.95	0.71	0.368
CREA (μmol/L)	9.50	8.01	6.88	8.27	7.98	9.62	0.89	0.300

Note: ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; TP, total protein; ALB, albumin; TC, total cholesterol; TG, triglycerides; GLU, glucose; UREA, urea; TBIL, total bilirubin; CREA, creatinine. SEM, standard error of the mean. $n = 6$. ^{a,b} Means with different superscripts within a row differ ($p < 0.05$).

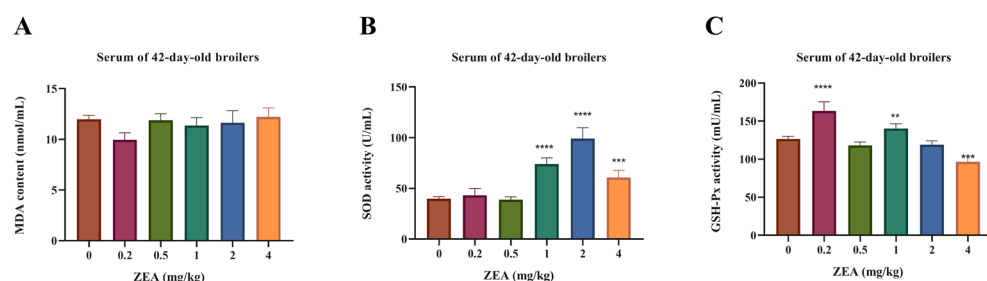


Figure 1. Effects of dietary ZEA exposure on serum oxidative stress indices of broilers. (A) Malondialdehyde (MDA) content, (B) superoxide dismutase (SOD) activity and (C) glutathione peroxidase (GSH-Px) activity. Data are presented as means $\pm$ SD, $n = 6$. Significant differences versus the control group (0 mg/kg ZEA), determined by one-way analysis of variance (ANOVA) followed by Duncan's multiple range test, are indicated as follows: ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

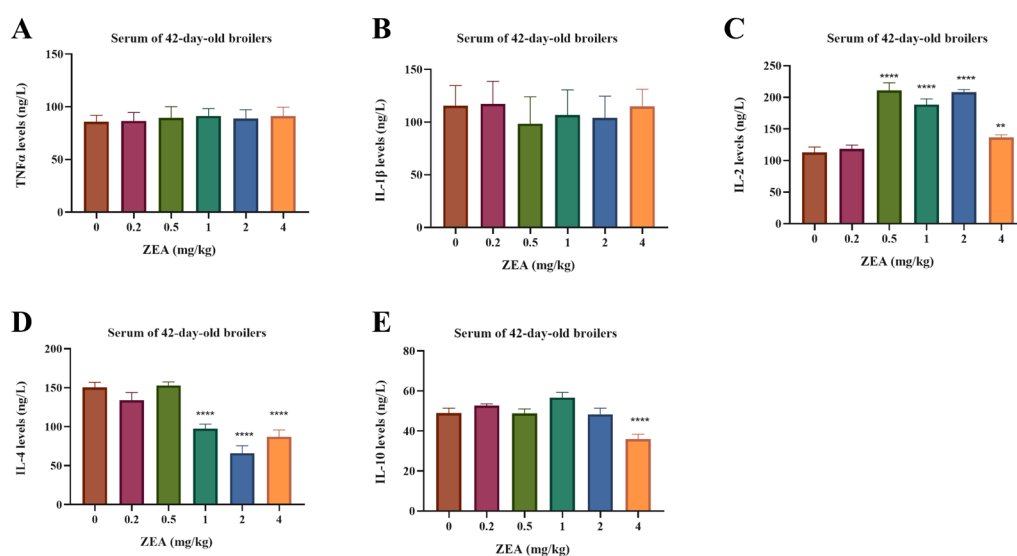


Figure 2. Effects of dietary ZEA exposure on serum immune cytokine levels of broilers. (A) Tumor necrosis factor- α (TNF- α), (B) interleukin-1 β (IL-1 β), (C) interleukin-2 (IL-2), (D) interleukin-4 (IL-4), and (E) interleukin-10 (IL-10). Data are presented as means $\pm$ SD, $n = 6$. Significant differences versus the control group (0 mg/kg ZEA), determined by one-way ANOVA followed by Duncan's multiple range test, are indicated as follows: ** $p < 0.01$, **** $p < 0.0001$.

2.4. Effects of ZEA on Liver Oxidative Stress Markers and Immune Cytokines of Broilers

Liver oxidative stress responses of broilers are shown in Figure S1. Dietary ZEA exposure at 0.2–4 mg/kg did not affect liver MDA content or GSH-Px activity ($p > 0.05$). However, SOD activity was reduced in the 0.2, 0.5 and 2 mg/kg ZEA groups ($p < 0.05$), and the total antioxidant capacity (T-AOC) was also lower in the 0.5 mg/kg group ($p < 0.05$). Regarding immune cytokine levels in the liver, no significant differences were observed among the groups for TNF- α , IL-1 β , IL-2, IL-4, or IL-10 levels ($p > 0.05$; Figure S2).

2.5. Effects of ZEA on Intestinal Oxidative Stress Markers and Immune Cytokines of Broilers

Immune cytokine levels in the jejunal mucosa are shown in Figure S3. No significant differences were observed in TNF- α levels among the groups ($p > 0.05$). IL-2 levels were increased by dietary ZEA exposure at 0.2, 0.5, and 4 mg/kg, and IL-4 levels were elevated at 0.2–4 mg/kg ($p < 0.05$). Additionally, IL-10 levels were higher following exposure to 0.2, 1, and 2 mg/kg ZEA ($p < 0.05$).

Oxidative stress indices in the ileal mucosa are presented in Figure 3. MDA content was increased by 0.5 and 1 mg/kg ZEA ($p < 0.05$), and SOD activity was elevated at 1–4 mg/kg ZEA ($p < 0.05$). In contrast, GSH-Px activity was reduced across all ZEA doses ($p < 0.05$). Immune cytokine profiles in the ileal mucosa are shown in Figure 4. Levels of TNF- α and IL-1 β were unaffected by ZEA treatment ($p > 0.05$). IL-2 levels were increased at 0.2, 0.5, 1, and 4 mg/kg ZEA ($p < 0.05$). IL-4 levels were elevated at 0.2 mg/kg but suppressed at 0.5–4 mg/kg ZEA ($p < 0.05$). Furthermore, IL-10 levels were reduced by ZEA at 0.2–2 mg/kg ($p < 0.05$).

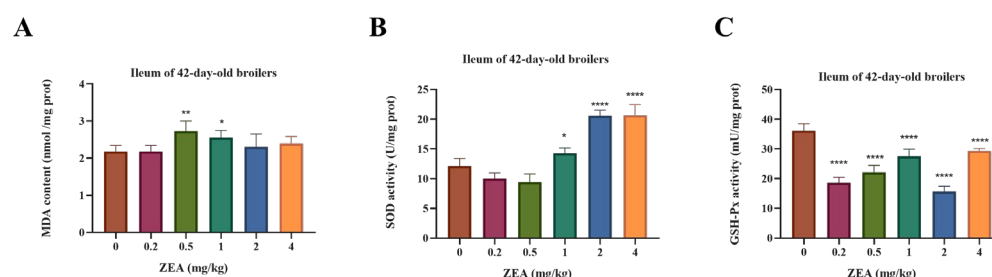


Figure 3. Effects of dietary ZEA exposure on ileal mucosal oxidative stress indices of broilers. (A) MDA content, (B) SOD activity and (C) GSH-Px activity. Data are presented as means $\pm$ SD, $n = 6$. Significant differences versus the control group (0 mg/kg ZEA), determined by one-way ANOVA followed by Duncan's multiple range test, are indicated as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

2.6. Intestinal Morphology and Histopathology Analysis

The effects of dietary ZEA exposure on intestinal morphology of broilers are summarized in Table 4. Compared with the control, villus height (VH) in the jejunum was reduced by dietary ZEA at 2 and 4 mg/kg ($p < 0.05$). Moreover, crypt depth (CD) was increased and the villus height-to-crypt depth ratio (VH:CD) was consequently decreased in the jejunum by ZEA at doses of 1, 2, and 4 mg/kg ($p < 0.05$). In the ileum, VH was not influenced by dietary ZEA ($p > 0.05$). Although CD did not differ between any ZEA group and the control (0 mg/kg ZEA), significant differences were observed among ZEA doses ($p < 0.05$). Specifically, CD in the 4 mg/kg group was lower than in the 0.2, 0.5, and 2 mg/kg groups, and that in the 1 mg/kg group was lower than in the 0.5 mg/kg group. Additionally, the VH:CD ratio was reduced in the 0.2 mg/kg ZEA group compared to the control ($p < 0.05$). Further histopathological examination revealed exfoliation of mucosal epithelial cells in the jejunum and ileum. In the jejunum, moderate exfoliation was observed in 4/6 birds at 2 mg/kg ZEA, and severe exfoliation in 3/6 birds at 4 mg/kg ZEA (Figure 5E,F). In the

ileum, severe exfoliation was observed in 2/6 birds at 1 mg/kg and 4/6 birds at 2 mg/kg ZEA (Figure 5J,K).

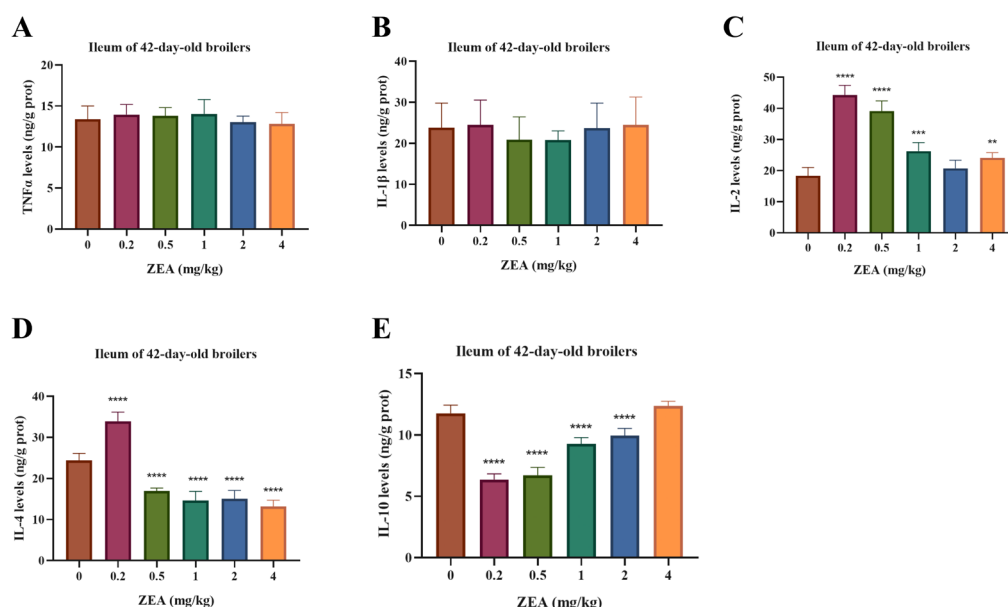


Figure 4. Effects of dietary ZEA exposure on ileal mucosal immune cytokine levels of broilers. (A) TNF- α , (B) IL-1 β , (C) IL-2, (D) IL-4, and (E) IL-10 levels. Data are presented as means $\pm$ SD, $n = 6$. Significant differences versus the control group (0 mg/kg ZEA), determined by one-way ANOVA followed by Duncan's multiple range test, are indicated as follows: ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Table 4. Effects of dietary ZEA exposure on intestinal morphology of broilers.

Items	Concentration of ZEA (mg/kg)						SEM	<i>p</i> -Values
	0	0.2	0.5	1	2	4		
Jejunum								
VH (μ m)	1125.83 ^a	1224.93 ^a	1052.56 ^{ab}	1176.81 ^a	919.41 ^b	881.04 ^b	58.35	0.001
CD (μ m)	155.28 ^c	173.19 ^c	191.80 ^{bc}	260.09 ^a	234.71 ^{ab}	228.83 ^{ab}	15.06	<0.001
VH/CD	7.36 ^a	7.51 ^a	6.00 ^{ab}	4.63 ^{bc}	4.00 ^c	3.95 ^c	0.50	<0.001
Ileum								
VH (μ m)	619.87	462.18	534.00	532.53	593.49	576.99	54.37	0.400
CD (μ m)	99.48 ^{abc}	112.10 ^{ab}	124.22 ^a	94.93 ^{bc}	114.41 ^{ab}	81.90 ^c	8.22	0.014
VH/CD	6.24 ^{ab}	4.29 ^c	4.37 ^{bc}	5.55 ^{abc}	5.37 ^{abc}	7.26 ^a	0.60	0.013

Note: VH, villus height; CD, crypt depth; VH/CD, villus height/crypt depth. SEM, standard error of the mean. $n = 6$. ^{a-c} Means with different superscripts within a row differ ($p < 0.05$).

2.7. Effects of ZEA on Histopathology of Major Organs in Broilers

Histopathology analysis results of the heart, liver, and spleen in broilers are shown in Figure S4. In all groups, cardiac histological architecture remained intact, with clearly defined endocardium, myocardium, and epicardium. Myocardial fibers were orderly arranged, and cardiomyocyte morphology was normal (Figure S4A–F). In liver tissue, hepatic lobules, central veins, and sinusoids maintained normal structure across all groups. Hepatocytes and biliary epithelial cells exhibited normal morphology and size. Mild periductal lymphocytic infiltration was observed in all groups, with no difference in severity compared to the control (Figure S4G–L). In the spleen, the proportion and architecture of red and white pulp were normal. Lymphocytes within the white pulp displayed normal morphology, size, and density. Germinal centers were observed in less than 5% of splenic follicles, with no significant differences among groups (Figure S4M–R).

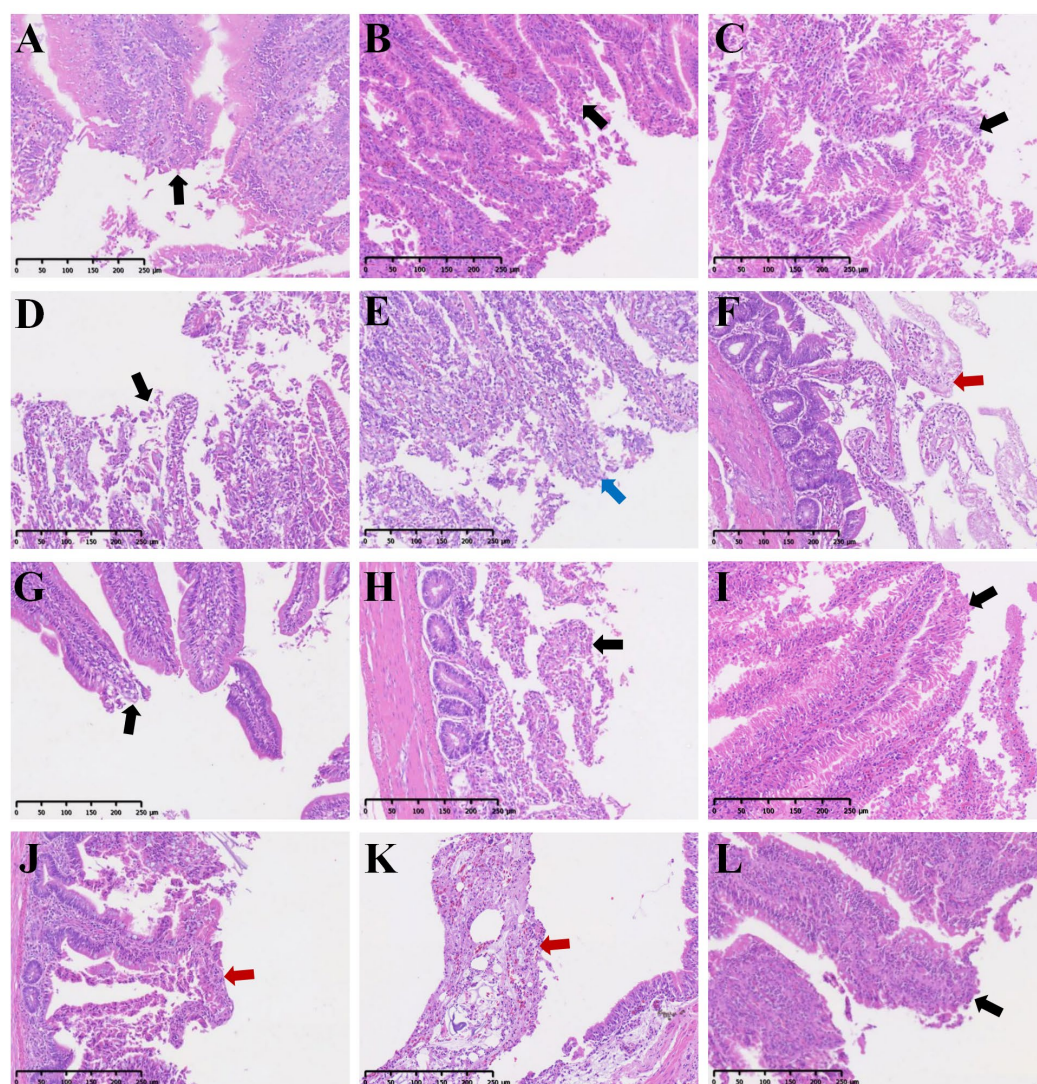


Figure 5. Intestinal histopathological changes in broilers following dietary ZEA exposure. (A–F) Representative jejunum sections and (G–L) representative ileum sections from broilers at dietary ZEA exposure of 0, 0.2, 0.5, 1, 2 and 4 mg/kg, respectively. Black, blue, and red arrows indicate mild, moderate, and severe exfoliation of mucosal epithelial cells, respectively. All specimens were examined at 100× magnification. Scale bar = 250 μ m.

Histopathological examination of lung and kidney tissues are presented in Figure 6. The pulmonary lobular structures remained normal with occasional mild inflammatory cell infiltration in the control group and the 0.2 and 0.5 mg/kg ZEA-treated groups, and no significant differences were observed between these groups (Figure 6A–C). However, moderate secretory cell metaplasia and mucus plug formation were noted in the 1, 2, and 4 mg/kg groups, with an identical incidence of 3/6, 4/6, and 4/6 birds for both lesions, respectively (Figure 6D–F). Regarding the kidney tissues, the glomerular and tubular structures were largely normal in the control group and the 0.2–2 mg/kg treatment groups, with mild basophilic changes observed in renal tubules and no marked intergroup differences (Figure 6G–K). In contrast, the 4 mg/kg dose group exhibited severe and diffuse degeneration and necrosis of renal tubular epithelial cells (6/6 birds), along with mild cast formation (6/6 birds) (Figure 6L).

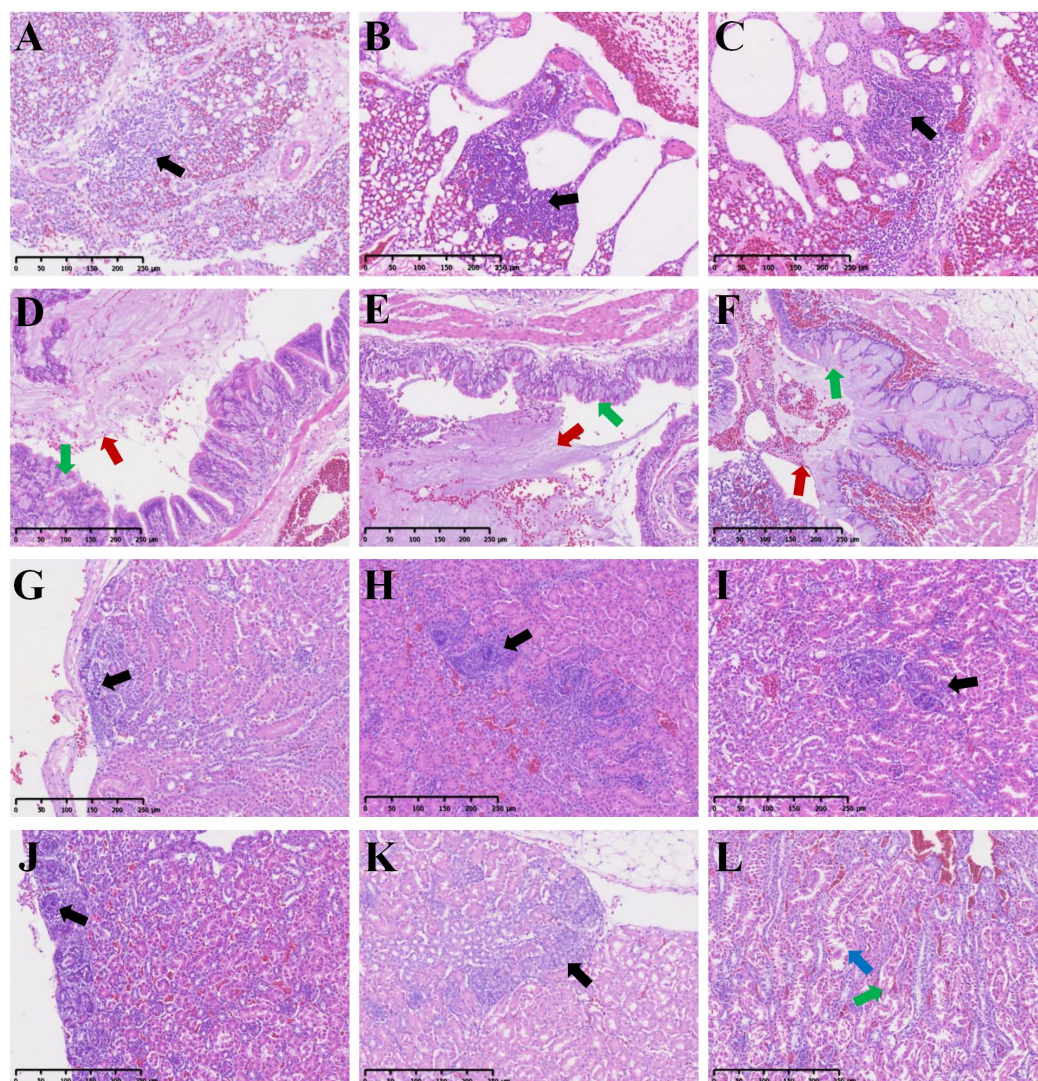


Figure 6. Histopathological changes of the lung and kidney in broilers following dietary ZEA exposure. (A–F) Representative lung sections and (G–L) representative kidney sections from broilers at dietary ZEA exposure of 0, 0.2, 0.5, 1, 2 and 4 mg/kg, respectively. Black, green, and red arrows in lung sections indicate mild inflammatory cell infiltration, moderate secretory cell metaplasia, and mucus plugs, respectively. Black, blue, and green arrows in kidney sections indicate mild tubular basophilia, severe and diffuse degeneration and necrosis of renal tubular epithelial cells, and mild tubular cast formation. All specimens were examined at 100× magnification. Scale bar = 250 μm.

2.8. Effects of ZEA on Jejunal Microbiota of Broilers

To investigate the impact of 4 mg/kg ZEA-contaminated diet on the jejunal microbiota of broilers, 16S rRNA sequencing was performed on jejunal content samples from the control and 4 mg/kg ZEA-treated group. As shown in Figure 7A, a total of 1691 and 324 OTUs were identified in the jejunal microbiota of the control and ZEA-treated broilers, respectively, with 269 OTUs shared between the two groups. Significant differences in alpha-diversity indices were observed between the groups (Figure 7B–E). The Chao and ACE indices estimate species richness. The Shannon and Simpson indices both reflect species diversity, where a higher Shannon value indicates greater diversity, while a higher Simpson value indicates lower diversity. Specifically, the Chao, ACE, and Shannon indices were lower, while the Simpson index was higher in the ZEA-treated group ($p < 0.05$). Principal coordinate analysis (PCoA) based on Bray–Curtis distances further revealed a

significant separation in jejunal microbial community structure between the two groups (Figure 7F; $p = 0.005$, $R = 0.3444$).

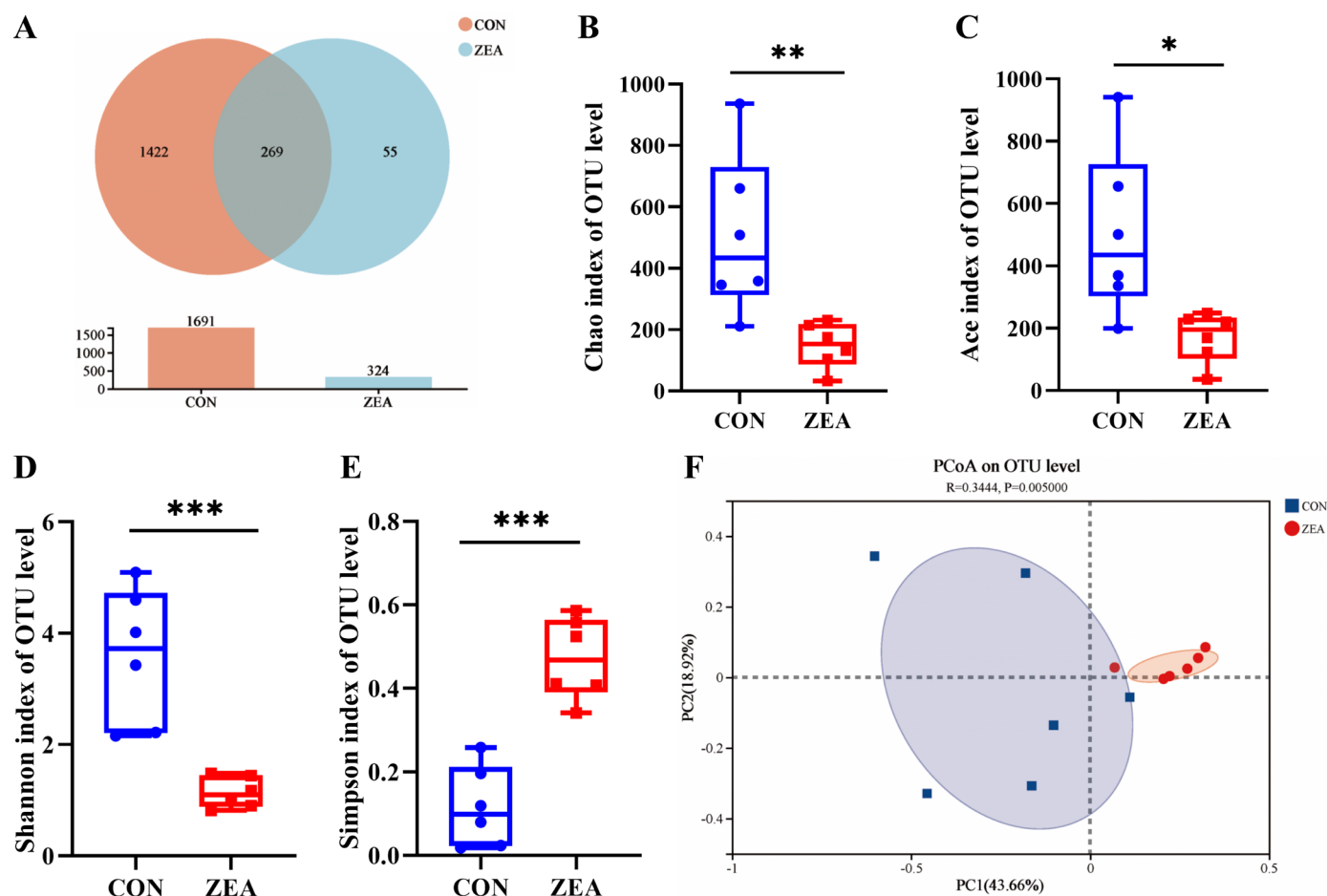


Figure 7. Effects of dietary ZEA (4 mg/kg) exposure on jejunal microbial diversity and community structure in broilers. (A) Venn diagram of OTUs. (B–E) α -Diversity indices (Chao, ACE, Shannon, and Simpson). (F) Principal coordinate analysis (PCoA) plot showing differences in microbial community structure based on Bray–Curtis distances. $n = 6$. Significant differences between the control group (CON) and ZEA group (4 mg/kg) are indicated, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

The composition of the jejunal microbiota is shown in Figure 8. Firmicutes, Lactobacillaceae, and *Lactobacillus* were the dominant phylum, family, and genus, respectively (Figure 8A–C). Differential abundance analysis revealed significant alterations in specific taxa. At the phylum level, the ZEA group exhibited a higher relative abundance of Firmicutes but lower abundances of Proteobacteria, Bacteroidota, and Cyanobacteria compared to the control group (Figure 8D, $p < 0.05$). At the family level, ZEA-treated broilers exhibited a significant increase in the abundance of Lactobacillaceae ($p < 0.05$). Conversely, significant decreases were observed in several families, such as Peptostreptococcaceae, Ruminococcaceae, Christensenellaceae, Pseudomonadaceae, and Enterococcaceae (Figure 8E, $p < 0.05$). At the genus level, the ZEA group showed a significant increase in *Lactobacillus* and decreases in several taxa including *Romboutsia* and Christensenellaceae_R-7_group (Figure 8F, $p < 0.05$). Furthermore, Linear discriminant analysis Effect Size (LEfSe) identified five taxa that were significantly enriched in the ZEA group (LDA score > 5), with f_Lactobacillaceae and g_Lactobacillus being among the most prominent biomarkers (Figure 8G).

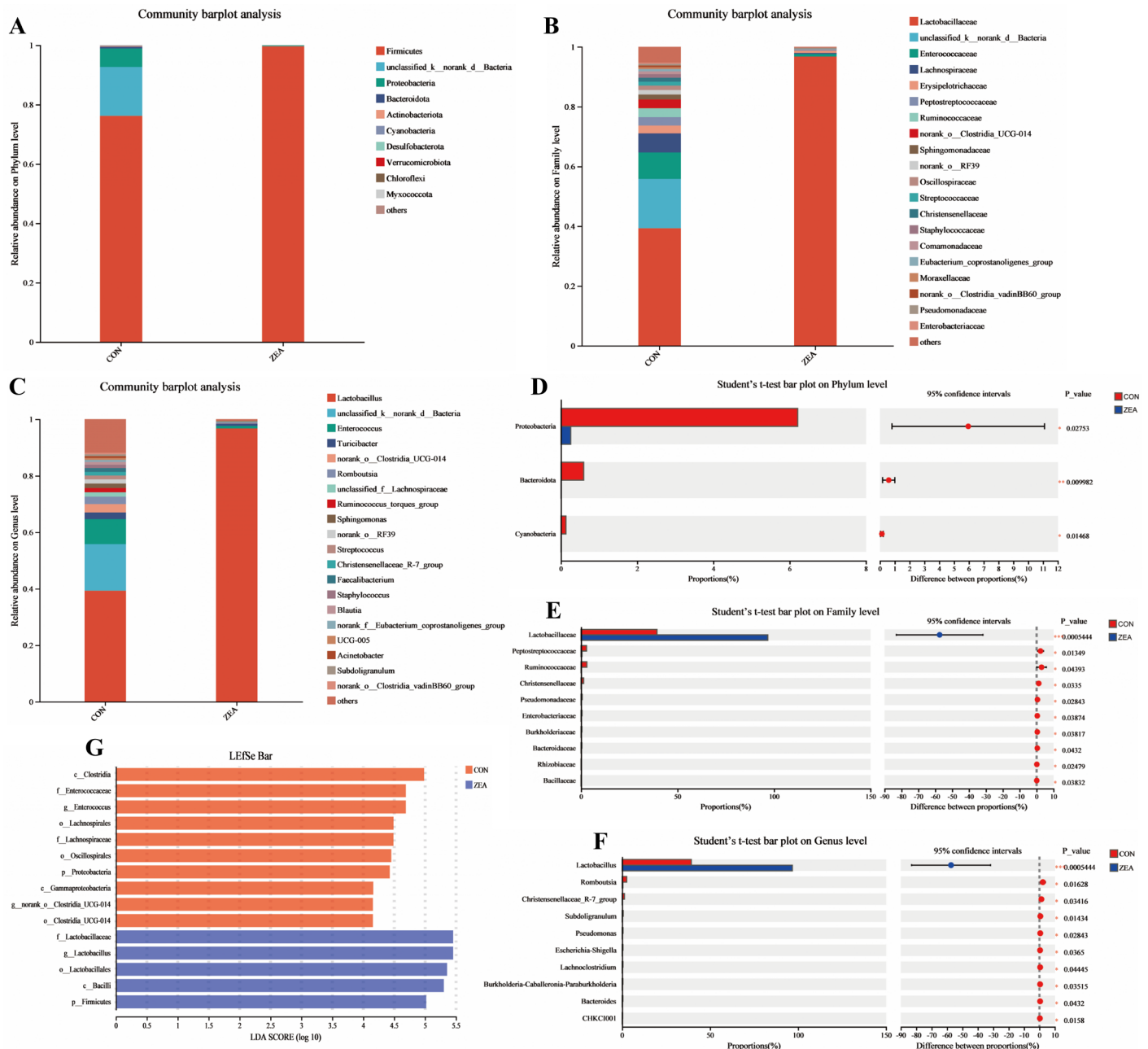


Figure 8. Effects of dietary ZEA (4 mg/kg) exposure on jejunal microbial composition in broilers. (A–C) Relative abundance bar plots of the microbiota at the phylum, family, and genus levels, respectively. (D–F) Differential abundance analysis showing taxa with significant changes ($p < 0.05$) at the phylum, family, and genus levels, respectively. (G) The LEfSe analysis of microbial community composition from phylum to genus level. $n = 6$. Significant differences between the control group (CON) and ZEA group (4 mg/kg) are indicated, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3. Discussion

The present study demonstrates that broilers possess considerable metabolic tolerance to ZEA, as chronic dietary exposure to feed contamination levels (0.2–4 mg/kg) throughout the 42-day production cycle did not induce statistically significant alterations in growth performance. This finding aligns with the established understanding of avian resistance to this mycotoxin. Notably, Allen et al. [8] observed in a short-term trial that exposure to a substantially higher ZEA concentration (up to 800 mg/kg) induced abnormal development

of reproductive organs in male broilers yet did not compromise fundamental growth parameters. Similarly, Zou et al. [20] reported no changes in the growth performance of female broilers fed a diet spiked with 1.0 mg/kg purified ZEA over a 15 d experiment. Collectively, these consistent observations across different management conditions and exposure durations and a broad dosage spectrum appear to suggest that a conserved and effective detoxification or adaptive mechanism may exist in poultry, which may buffer against the growth-impairing effects of ZEA.

This apparent tolerance was further corroborated by our assessment of key health biomarkers. Hematological analysis revealed no significant effects on blood routine indices across the tested dosage range (0.2–4 mg/kg), indicating that ZEA did not disrupt hematopoietic function or peripheral blood cell homeostasis at these feed contamination levels. This hematological stability finds support in a study with ducks, where ZEA administered at 500 µg/kg also did not alter hematology of heterocytes, lymphocytes and monocytes [21]. In the serum biochemical profile, however, a significant increase in ALP activity was observed specifically at the highest dose of 4 mg/kg. This elevation suggests a targeted hepatobiliary response at this threshold. Notably, our prior study demonstrated that serum ALP or other key liver enzymes (AST, ALT) were not affected in broilers at the regulatory limit of 500 µg/kg [22], and this result is also consistent with findings in duck serum biochemical indicators administered ZEA at the same dose [21]. This may suggest a dose-dependent hepatobiliary alteration, which becomes detectable only when exposure substantially exceeds the regulatory level. Collectively, these results indicate that the hematopoietic system maintains stability across the tested range, while specific hepatic biochemistry markers exhibit sensitivity specifically at 4 mg/kg, serving as more precise indicators of higher-intensity ZEA exposure.

We next evaluated the potential of ZEA to disrupt systemic oxidative homeostasis by measuring key oxidative stress indicators in serum, liver, and ileal mucosa. The present study revealed that the impact of ZEA contamination on oxidative stress indicators in broiler serum and tissues exhibited distinct dose–response relationships and tissue specificity. Serum results demonstrated that SOD activity was significantly increased following dietary ZEA exposure at a higher dose of 1–4 mg/kg, suggesting an activated systemic antioxidant enzyme response to counteract oxidative damage. Notably, a biphasic regulatory effect of ZEA on GSH-Px activity was observed. ZEA exposure at lower doses of 0.2–1 mg/kg significantly enhanced GSH-Px activity, whereas exposure at 4 mg/kg suppressed it. This phenomenon may be related to the compensatory threshold of the GSH-Px enzymatic system [23]. Importantly, the pronounced increase in serum SOD activity, alongside the adaptive modulation of GSH-Px, was not accompanied by a significant elevation in MDA levels. This indicates that the induced upregulation of these antioxidant enzymes collectively constituted an effective compensatory response, which successfully neutralized reactive oxygen species and prevented measurable systemic lipid peroxidation during the experimental period. Hepatic tissue analysis revealed tissue-specific differences in oxidative stress responses to ZEA exposure. Although doses of 0.2–0.5 mg/kg significantly reduced SOD activity, and T-AOC was lower specifically at 0.5 mg/kg, GSH-Px activity and MDA content did not change in any of the ZEA-treated groups. This may be attributed to the efficient clearance of lipid peroxidation products by the liver's unique phase II metabolic enzyme systems [24]. In the ileal mucosa, a significant decrease in GSH-Px activity was observed across all ZEA-treated groups, while SOD activity and MDA content increased in medium- to high-dose groups. This pattern, characterized by an imbalance in antioxidant enzyme activities and exacerbated lipid peroxidation, likely stemmed from the direct damaging effect of ZEA on intestinal mucosal epithelial cells [25]. These findings indicate that while low-dose exposure induces adaptive or compensatory

changes, the resulting oxidative damage may exceed the compensatory capacity primarily at higher doses.

Beyond the observed oxidative stress, our investigation also revealed tissue-specific immunomodulatory effects of purified ZEA in broilers and suggests a potential disruption of host immune homeostasis via the Th1/Th2 balance axis. In serum, ZEA increased levels of the Th1-type cytokine IL-2 at 0.5–4 mg/kg while decreasing Th2-type anti-inflammatory cytokines IL-4 (at 1–4 mg/kg) and IL-10 (specifically at 4 mg/kg). This shift, particularly evident at higher doses, indicates a promotion of pro-inflammatory Th1 responses and a concurrent suppression of Th2-associated anti-inflammatory functions, potentially elevating the risk of excessive systemic inflammation. In contrast, hepatic immune cytokine levels were not affected, likely buffered by the organ's robust metabolic detoxification capacity [26]. Notably, the pro- or anti-inflammatory effect of ZEA exhibits clear organ dependence, consistent with findings by Ben Salah-Abbès et al. [27,28]. Their study reported that ZEA exposure (40 mg/kg b.w.) significantly reduced plasma levels of TNF- α , IL-1 β , and IL-12 in mice [27]. In a separate study, they demonstrated that exposure to ZEA at the same concentration induced elevated renal levels of TNF- α , IL-6, and IL-10 in mice [28]. This tissue-specific modulation suggests that ZEA may influence local immune microenvironments through differential cellular signaling pathways. The effects of ZEA exposure in the intestinal mucosa were more complex. In the jejunal mucosa, levels of IL-2, IL-4, and IL-10 were generally increased, implying that low-to-medium ZEA doses may activate local immunoregulatory mechanisms to maintain gut immune homeostasis. The ileal mucosa, however, displayed a distinct pattern characterized by elevated IL-2 levels at 0.2–4 mg/kg, with IL-4 being significantly suppressed at 0.5–4 mg/kg and IL-10 consistently reduced at 0.2–2 mg/kg. This indicates a potential disruption of immune balance in the ileum, predisposing it to inflammatory responses and corroborating the overall suppression of Th2 immunity observed in serum. Such dysregulation of mucosal immunity may compromise intestinal barrier function, thereby creating a favorable niche for opportunistic pathogens [29]. Therefore, we speculate that dietary ZEA contamination may disrupt the avian immune system via a dual mechanism that involves systemically skewing the Th1/Th2 balance and locally altering the immune microenvironment to weaken mucosal defenses. These findings elucidate another dimension of ZEA's subclinical changes, occurring even at exposure levels that do not compromise overt growth performance.

Our investigation further demonstrated that dietary purified ZEA contamination induces differential and segment-specific structural damage to the intestinal morphology of broilers. Jejunal morphology was significantly affected by ZEA at 1–4 mg/kg, manifesting as reduced VH, increased CD, and a consequently decreased VH/CD ratio. This villus shortening compromises intestinal barrier integrity by increasing permeability and reducing the absorptive surface area, potentially leading to nutrient malabsorption and weakened disease resistance [30]. Histopathological observations in the jejunum revealed pronounced epithelial cell exfoliation and architectural disarray in high-dose groups, suggesting that ZEA may impair epithelial regeneration by disrupting the homeostasis of crypt stem cell proliferation and differentiation [31]. In contrast, ileal morphology exhibited greater tolerance. No statistically significant alterations in VH or CD were detected in the ileum across ZEA-treated groups compared with the control. However, at high doses, the ileal mucosa also showed signs of compromised epithelial integrity. This different sensitivity between the jejunum and ileum to ZEA exposure further emphasizes the intrinsic physiological and functional heterogeneity along the intestinal tract [32]. These alterations in morphology and histopathology collectively threaten intestinal barrier function and homeostasis, providing a plausible mechanistic basis for the increased susceptibility to opportunistic infections.

Moreover, our findings revealed significant differential histopathological responses among the major organs of broilers following dietary purified ZEA exposure. Histopathological assessment indicated that the heart, liver, and spleen possessed substantial tolerance to ZEA, as no notable histological alterations attributable to the mycotoxin were observed. In the heart, myocardial fibers maintained structural integrity without inflammatory changes, suggesting no direct cardiac toxicity. Hepatic architecture remained intact, with only mild lymphocyte infiltration comparable to the control group. Similarly, in the spleen, the observed features of occasional germinal centers were consistent with those in the control group, indicating no specific pathological impact. In contrast, the lungs and kidneys displayed discernible pathological changes under high-dose ZEA exposure. The lungs of broilers treated with 1–4 mg/kg ZEA presented moderate secretory cell metaplasia and mucus plug formation. This suggests that ZEA may stimulate abnormal hyperplasia of the respiratory mucosa, potentially compromising normal pulmonary function [33]. Histological alterations in kidneys were more pronounced. The 4 mg/kg ZEA group exhibited severe tubular epithelial cell degeneration, necrosis, and cast formation, indicating that high-dose ZEA likely impairs renal filtration and reabsorption functions through direct toxicity or oxidative stress mechanisms. However, only mild tubular basophilia was observed in the lower-dose groups (0.2–2 mg/kg), with no significant difference from the control, highlighting the clear dose-dependency of ZEA-induced nephrotoxicity [34]. Thus, the organ-specific toxicity of ZEA in broilers demonstrates that the heart, liver, and spleen possess robust resistance to damage, whereas the lungs and kidneys are more susceptible at higher doses. These results indicate that in broilers, dietary purified ZEA at or below the regulatory limit (0.5 mg/kg) induced no notable histopathological alterations in any major organ examined. In contrast, chronic exposure to higher doses (≥ 1 mg/kg) could pose potential risks to the respiratory and urinary systems, as evidenced by lung metaplasia and severe renal tubular damage.

Finally, our analysis explored the intestinal microbial ecosystem, which plays a crucial role in nutrient metabolism and barrier defense. Dietary purified ZEA exposure at 4 mg/kg significantly altered the jejunal microbial diversity of broilers. Alpha-diversity analysis showed significant reductions in the Chao, Ace, and Shannon indices, alongside an increase in the Simpson index following dietary ZEA exposure, indicating diminished species richness and community evenness. PCoA analysis confirmed a significant separation between the two groups at the OTU level, and this restructuring of the microbial community likely originated from the selective pressure exerted by ZEA on specific bacterial taxa. Moreover, the jejunal microbiota composition was significantly reshaped by ZEA contamination. Specifically, the relative abundance of Firmicutes was increased, while that of Proteobacteria, Bacteroidota, and Cyanobacteria was decreased. This result differs from previous reports associating mycotoxin exposure with increased Bacteroidota [35]. The difference may involve a combination of host species specificity, toxin action sites, and dose-dependent effects. The observed increase in the Firmicutes-to-Bacteroidota ratio in this study may reflect a ZEA-induced transition in the gut metabolic mode from a fiber-degrading (Bacteroidota-dominated) profile toward one favoring rapid carbohydrate fermentation (Firmicutes-dominated). This adaptive change might be linked to the host's energy reallocation strategy in response to toxin-induced stress. At the family and genus levels, the abundance of Lactobacillaceae and its subordinate genus *Lactobacillus* significantly increased in the ZEA-treated group. It is noteworthy that although *Lactobacilli* are generally considered beneficial, their over-proliferation could exacerbate niche competition, potentially leading to dysbiosis and affecting intestinal motility [36]. Concurrently, this study observed a decrease in the abundance of short-chain fatty acid-producing families such as *Peptostreptococcaceae* and *Ruminococcaceae*, which may result from a disrupted com-

pensatory adjustment in maintaining microbiota homeostasis. Furthermore, the observed reduction of opportunistic pathogens like *Enterococcus* and the overall suppression of *Proteobacteria* lead us to hypothesize that ZEA may indirectly and selectively inhibit the colonization of aerobic pathogens by compromising the intestinal epithelial barrier [37,38]. It should be noted that the microbiota analysis was conducted only at the highest dose (4 mg/kg). Therefore, the observed dysbiosis specifically reflects a high-dose effect, and extrapolation to lower exposure levels would require further investigation.

Taken together, our results delineate a clear dose–response relationship for purified ZEA in broilers. This raises a key question regarding the interpretation of the statistically significant alterations detected at the regulatory limit (0.5 mg/kg) and at a lower dose (0.2 mg/kg), including reduced ileal GSH-Px activity, modulated cytokine profiles (IL-2, IL-4, and IL-10), and decreased hepatic T-AOC. We interpret these changes primarily as a physiological adaptive response, whereby the organism mobilizes its biochemical and immune defenses to maintain homeostasis against a low-level ZEA exposure. This interpretation is supported by the fact that no histopathological lesions were observed, and that growth performance, hematology, and clinical biochemistry remained normal at these doses. Consequently, these findings support the conclusion that purified ZEA at the current regulatory limit does not induce overt toxicity in broilers. However, we acknowledge that under the complex and multifaceted stress conditions of commercial poultry production, such subclinical perturbations could diminish metabolic resilience and serve as early warning indicators of compromised poultry health. Therefore, while the regulatory limit appears adequate for preventing overt toxicity, monitoring these sensitive biomarkers could be valuable for early warning in high-risk scenarios.

4. Conclusions

In conclusion, this study indicates that purified ZEA at the current regulatory limit (0.5 mg/kg) exhibits no overt toxicity, as alterations in oxidative stress and immune markers at 0.2–0.5 mg/kg did not cause tissue damage or functional impairment, with no adverse effects on growth performance, hematology, serum biochemistry, or histopathology. However, a clear toxicity response was identified at higher exposure levels. Jejunal morphological impairment and mild lung secretory cell metaplasia occurred at 1–4 mg/kg. Severe renal tubular epithelial degeneration and necrosis as well as significant jejunal microbiota dysbiosis were observed exclusively at 4 mg/kg. These findings strengthen the scientific basis for the current regulatory limit and highlight the organ-specific risks of higher-level ZEA contamination, thus providing critical data for risk assessment and a definitive reference for refining feed safety standards.

5. Materials and Methods

5.1. Chemicals, Animals, and Ethics

The Zearalenone (ZEA) standard with a purity of $\geq 99\%$ was purchased from Qingdao IniKem BioPharmaTech Co., Ltd. (Qingdao, China). A total of 216 one-day-old Arbor Acres (AA) male broilers were obtained from the Hebei Kangyu Poultry Breeding Co., Ltd. (Langfang, China). The animal trial protocol was reviewed and approved by the China Agricultural University Laboratory Animal Welfare and Animal Experimental Ethical Inspection Committee (Approval No. AW91903202–1–3), and all procedures were performed in accordance with the relevant guidelines.

5.2. Experimental Design, Diets, and Management

A total of 216 one-day-old male AA broilers were randomly divided into six treatment groups, with six replicates per group and six birds per replicate. The six groups were

fed a corn–soybean meal basal diet contaminated with 0, 0.2, 0.5, 1, 2, and 4 mg/kg ZEA, respectively, and the trial lasted for 42 days. The basal diet was formulated according to the NRC (1994) nutritional requirements of broilers [39]. To prepare the ZEA contaminated diets, different quantities of ZEA standard were dissolved in 10 mL of dimethyl sulfoxide (DMSO). The control diet (0 mg/kg ZEA) was prepared identically with the same volume of DMSO (10 mL) to serve as a true vehicle control. The DMSO solution was thoroughly mixed with a premix and then blended with other feed ingredients via stepwise gradient dilution. The diets for each treatment group were prepared in batches of 50 kg for the 0–21 d period and 150 kg for the 22–42 d period, resulting in final DMSO concentrations of approximately 0.2 mL/kg and 0.067 mL/kg feed, respectively. These concentrations are negligible and unlikely to exert biological effects. The composition and nutrient levels of the basal diet are presented in Table S1. High-quality corn was selected to ensure minimal background mycotoxin contamination. The major mycotoxins of ZEA, Deoxynivalenol (DON), aflatoxin B₁ (AFB₁), ochratoxin A (OTA), T-2 toxin, and fumonisin B₁ (FB₁) in corn were analyzed and not detected using a previously published liquid chromatography–tandem mass spectrometry (LC-MS/MS) method based on immunoaffinity column cleanup and isotope dilution [40]. This method provided limits of detection (LOD) and quantification (LOQ) for the six mycotoxins of 0.075–1.5 µg/kg and 0.25–5 µg/kg, respectively. Specifically for ZEA, the LOD and LOQ were 0.375 µg/kg and 1.25 µg/kg, and its recovery from spiked chicken feed was validated to be 97.6–116.5% [40]. The measured values of ZEA in the six treatment groups are provided in Supplementary Table S2.

The experiment was conducted in the Animal Digestion and Metabolism Laboratory of the College of Animal Science and Technology, China Agricultural University. Broilers were housed in wire-floored cages (0.9 × 0.6 × 0.4 m) under controlled environmental conditions. A 24 h lighting schedule was provided for the first 3 days, followed by a 23 h light to 1 h dark cycle until day 42. The room temperature was maintained at 34–35 °C initially and then gradually reduced by 2 °C per week, stabilizing at 24–26 °C for the remainder of the study. Relative humidity was kept at 45–55%. Birds had free access to feed and water throughout the experimental period. Routine immunization and all other husbandry practices followed standard broiler production protocols.

5.3. Sample Collection and Preparation

On day 42, six broilers (one broiler per replicate) were randomly selected from each treatment group, and blood samples were collected from the wing veins after a 12 h fast. For hematology analysis, 2 mL of blood was transferred to anticoagulant tubes and maintained at 4 °C. For serum preparation, another 5 mL of blood was collected in plain tubes. The samples were allowed to clot at room temperature for 30 min and subsequently centrifuged at 3000× g for 10 min. The obtained serum was stored at −20 °C for further analysis.

After blood collection, the broilers were euthanized via exsanguination through the jugular vein. Samples of the heart, liver, spleen, lungs, kidneys, jejunum, and ileum were immediately collected. For histopathological examination, sections of each organ were rinsed with 0.9% physiological saline and fixed in 4% paraformaldehyde buffer. Fixed intestinal segments were also used for morphology analysis. Separately, for tissue biochemical assays, liver tissue samples were collected, and mucosal scrapings from the jejunum and ileum were obtained using glass slides. Additionally, the jejunal contents were aseptically collected for microbial analysis. All samples for tissue biochemical and microbial assays were immediately snap-frozen in liquid nitrogen and stored at −80 °C until further analysis.

5.4. Growth Performance

The body weight (BW) and feed intake were recorded on a per-replicate basis at the start and on days 21 and 42. Average daily gain (ADG), average daily feed intake (ADFI), and the feed-to-gain ratio (F:G) were calculated for the periods of 0–21, 22–42, and 0–42 days.

5.5. Blood Sample Analysis

For whole blood samples, hematological parameters were analyzed using a fully automated hematology analyzer (TEK-II, Shenzhen Tekang Biotech Co., Ltd., Shenzhen, China), including white blood cell count (WBC), red blood cell count (RBC), hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelet count (PLT), plateletcrit (PCT), mean platelet volume (MPV), and platelet distribution width (PDW).

For serum samples, biochemical parameters, including alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), total protein (TP), albumin (ALB), total cholesterol (TC), triglycerides (TG), glucose (GLU), urea (UREA), total bilirubin (TBIL), and creatinine (CREA), were measured using a fully automated biochemical analyzer (BS-420, Shenzhen Mindray Bio-Medical Electronics Co., Ltd., Shenzhen, China). Additionally, oxidative stress markers, including malondialdehyde (MDA), superoxide dismutase (SOD), and glutathione peroxidase (GSH-Px), were measured using commercial colorimetric assay kits (Shanghai Hengyuan Biological Technology Co., Ltd., Shanghai, China) following the manufacturer's instructions. Immune parameters, including tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β), interleukin-2 (IL-2), interleukin-4 (IL-4), and interleukin-10 (IL-10), were quantified using commercial enzyme-linked immunosorbent assay (ELISA) kits (Shanghai Hengyuan Biological Technology Co., Ltd., Shanghai, China).

5.6. Tissue Oxidative Stress Marker and Inflammatory Cytokine Assays

Approximately 0.2 g of liver, jejunal, and ileal mucosal tissue was homogenized in nine volumes of cold physiological saline to prepare a 10% (*w/v*) homogenate. The protein concentration of the homogenate was determined using a BCA protein assay kit (Beyotime Biotechnology, Shanghai, China). In liver tissue, the homogenates were analyzed for oxidative stress markers, including MDA, SOD, GSH-Px, and total antioxidant capacity (T-AOC), using the same commercial colorimetric assay kits as described for serum analyses in Section 5.5. Inflammatory cytokines, including TNF- α , IL-1 β , IL-2, IL-4, and IL-10, were measured using the same commercial ELISA kits as described for serum analyses in Section 5.5. In jejunal mucosa, the homogenates were analyzed for inflammatory cytokines (TNF- α , IL-2, IL-4, and IL-10) using the same ELISA kits. In ileal mucosa, the homogenates were analyzed for MDA, SOD, and GSH-Px using the same colorimetric assay kits. Inflammatory cytokines (TNF- α , IL-1 β , IL-2, IL-4, and IL-10) were measured using the same ELISA kits. All tissue-based results were normalized to the protein concentration.

5.7. Tissue Histopathological and Intestinal Morphology Analysis

The fixed organ (heart, liver, spleen, lungs, kidneys) and intestinal (jejunum and ileum) samples were processed through standard dehydration, clearing, paraffin embedding, sectioning, and hematoxylin–eosin (H&E) staining. Tissue histopathological analysis was performed by examining HE-stained sections under an upright microscope (Primo Star, Carl Zeiss, Jena, Germany). All histopathological evaluations were performed blinded to the treatment groups. For intestinal morphology analysis, well-oriented villi and crypts in the jejunum and ileum were selected. Villus height (VH, from the villus tip to the crypt-villus junction) and crypt depth (CD, from the junction to the crypt base) were measured using

image analysis software (Hamamatsu NDP. view2), and then the villus height-to-crypt depth ratio (VH/CD) was calculated.

5.8. 16S rRNA Sequencing

Microbial genomic DNA was extracted from the jejunal content samples (from the 0 and 4 mg/kg ZEA groups) using the FastPure Stool DNA Isolation Kit (Shanghai Majorbio Bio-pharm Technology Co., Ltd., Shanghai, China). The V3-V4 hyper-variable region of the bacterial 16S rRNA gene was amplified with primers 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'). The amplicons were purified, quantified, and used to construct sequencing libraries with the NEXTFLEX Rapid DNA-Seq Kit (Bioo Scientific, Austin, TX, USA). Paired-end sequencing (2 × 300 bp) was conducted on the Illumina NextSeq 2000 platform (Illumina Inc., San Diego, CA, USA) according to the standard protocols by Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China). Raw sequences were quality-filtered by fastp (v0.19.6) and merged by FLASH (v1.2.11) with the following criteria: (i) The reads were truncated at any site receiving an average quality score of <20 over a 10 bp sliding window, and the truncated reads shorter than 50 bp were discarded; reads containing ambiguous characters were also discarded. (ii) Only overlapping sequences longer than 10 bp were assembled based on their overlap, with a maximum mismatch ratio of 0.2; reads that could not be assembled were discarded. (iii) Samples were distinguished according to the barcode and primers, and the sequence direction was adjusted, with exact barcode matching and 2 nucleotide mismatch in primer matching. Operational Taxonomic Units (OTUs) were clustered at a 97% similarity threshold using UPARSE (v11.0.667). To minimize the effects of sequencing depth on alpha and beta diversity measures, samples were rarefied to the smallest library size among all samples. Subsequent bioinformatic processing and statistical analysis of the sequencing data were conducted using the analysis pipelines available on the Majorbio Cloud Platform.

5.9. Statistical Analysis

All data except for microbiota data were analyzed by one-way analysis of variance (ANOVA) using SPSS software (v21.0). Significant differences among treatment means were compared using Duncan's multiple range test, with $p < 0.05$ considered statistically significant. For microbiota data, Alpha diversity indices were compared using the Wilcoxon rank-sum test. Beta diversity based on Bray–Curtis distance was visualized by principal coordinate analysis (PCoA), and the significance of community structure separation was assessed using permutational multivariate analysis of variance (PERMANOVA). For differential abundance analysis between the control and 4 mg/kg ZEA groups, Student's t-test was used to compare the relative abundances of each taxon. p -values were adjusted for multiple comparisons using the Benjamini–Hochberg false discovery rate (FDR) procedure, with significance set at $p < 0.05$. The data from a replicate pen was calculated as the experimental unit for the growth performance evaluation. The broiler individual from each replicate was regarded as the basic unit for other measured parameters.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/toxins18020102/s1>, Table S1: Ingredients composition and nutrient levels of the basal diet (%; as-fed basis); Table S2: The determination of zearalenone in treatment diets (mg/kg); Figure S1: Effects of dietary ZEA exposure on liver oxidative stress indices of broilers; Figure S2: Effects of dietary ZEA exposure on liver immune cytokine levels of broilers; Figure S3: Effects of dietary ZEA exposure on jejunal mucosal immune cytokine levels of broilers; Figure S4: Histopathological analysis of the heart, liver, and spleen in broilers following dietary ZEA exposure.

Author Contributions: Conceptualization, Y.L. and W.Z.; methodology, Y.L. and W.Z.; formal analysis, Y.L., W.Z., Q.D. and S.L.; investigation, Y.L. and W.Z.; resources, W.N., W.Y. and Y.C.; data curation, Y.L. and W.Z.; writing—original draft preparation, Y.L.; writing—review and editing, W.Z., Q.D., S.L., W.H., W.N., W.Y. and Y.C.; supervision, Y.C.; project administration, Y.C.; funding acquisition, W.Y. and Y.C. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the National Key Research and Development Programs of China, grant number 2023YFD1301002.

Institutional Review Board Statement: The animal study protocol was approved by the China Agricultural University Laboratory Animal Welfare and Animal Experimental Ethical Inspection Committee (Approval No. AW91903202–1–3).

Informed Consent Statement: Not applicable.

Data Availability Statement: The original contributions presented in this study are included in the article/Supplementary Materials. Further inquiries can be directed to the corresponding author.

Conflicts of Interest: The authors declare no conflicts of interest.

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