

Article

Ameliorative Impacts of an Essential Oil Blend on Immune Function and Intestinal Health in Broilers Challenged with a High-Dose Coccidial Vaccine

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Abstract

Background: The emergence of coccidial drug resistance has intensified the search for sustainable, residue-free solutions to control poultry coccidiosis. This challenge has positioned plant essential oils as promising candidates among the priority research areas. The study aimed to investigate the efficacy of essential oils in improving immune function and intestinal health in white-feathered broilers challenged with a high-dose coccidial vaccine. **Methods:** A total of 480 one-day-old broilers were randomly assigned to five treatments: uninfected (CON), infected (EC), infected + 500 g/t narasin (AT), and infected + 200 or 400 g/t essential oil blend (EOB200 or EOB400). There were 6 replicates per treatment and 16 broilers per replicate. All infected treatments received a 30-fold coccidial vaccine on d 14. The CON group was administered an equal volume of sterile normal saline on d 14 of the experiment. One bird per replicate was sampled on d 22, and the remainder were raised until d 42. **Results:** Results showed that the challenge increased the fecal oocyst counts on d 21 and 28 and elevated intestinal lesion scores and serum interleukin-6 (IL-6) levels on d 21, while it decreased IL-10 levels on d 21 and reduced the villus height-to-crypt depth (V:C) ratio in the duodenum and jejunum ($p < 0.05$). Additionally, compared with the EC group, the AT, EOB200, and EOB400 groups reduced oocyst excretion and serum superoxide dismutase (SOD) ($p < 0.05$). The EOB200 group also showed the highest serum transforming growth factor- β (TGF- β) concentration, along with the lowest immunoglobulin G (IgG) level ($p < 0.05$). Moreover, within the infected groups, the EOB200 group exhibited the highest duodenal villus height and V:C ratio ($p < 0.05$). **Conclusions:** These findings indicated that the EOB200 and EOB400 groups alleviated intestinal damage caused by the coccidial vaccine with an efficacy comparable to that of antibiotics, and the EOB200 group exhibited a superior effect. The results confirm that the essential oil blend holds great application potential as an alternative to antibiotics.

Keywords: white-feathered broiler; coccidial vaccine; essential oil blend; intestinal health



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1. Introduction

Coccidiosis, caused by the massive proliferation of *Eimeria* parasites within intestinal epithelial cells, remains a prevalent and economically significant disease in poultry production [1]. The intracellular replication of the parasite damages the intestinal mucosa, impairing nutrient digestion and absorption, which consequently suppresses growth performance [2–4]. Current control strategies primarily rely on the use of anticoccidial drugs and live vaccines, often employed in rotation or combination [5]. Conventional approaches to coccidiosis control that depend on anticoccidial drugs, while demonstrating effectiveness in practice, have aroused increasing worries related to drug resistance and chemical residue accumulation, thus boosting the research interest in natural and safe substitute solutions [6–8].

Plant essential oils (PEO) enhance poultry production performance via improved nutrient digestion and absorption, as well as through enhanced antioxidant status and immune responses [9,10]. Commonly used essential oils in broiler diets include anise, oregano, cinnamon, garlic, thyme, and turmeric [11]. It has been found that the inclusion of oregano essential oil, fennel essential oil, or a blend of thyme, mint, and eucalyptus essential oils in the diet increased villus height and width and reduced crypt depth [12–14]. Additionally, the anticoccidial potential of numerous plant extracts and compounds has been documented extensively. Several natural products, such as extracts from *Melaleuca toosendan*, maslinic acid, and *Ulmus macrocarpa*, have demonstrated potential against coccidia [15,16]. It is also considered that combinations of plant natural products may have even better anticoccidial effects. For example, a blend of thymol, carvacrol, and saponins has been shown to exhibit in vitro anti-coccidial activity against *Eimeria* in chickens [17]. In summary, essential oils and their blends offer a potent natural means to enhance intestinal health and serve as effective alternatives to anticoccidial drugs.

Therefore, the study aimed to investigate the efficacy of essential oils in improving immune function and intestinal health in white-feathered broilers challenged with a high-dose coccidial vaccine. This will provide a scientific basis for utilizing essential oils as a viable, natural strategy for integrated coccidiosis control in poultry production.

2. Materials and Methods

2.1. Essential Oil Blend and Drugs

The essential oil blend was obtained from Centree Bio-Tech (Wuhan) Co., Ltd. (Wuhan, China). The active components of essential oil blend were 3% cinnamaldehyde, 1.8% carvacrol, 2% thymol, 10% gallnut, and the carrier was silicon dioxide. The trivalent live coccidiosis vaccine (containing *Eimeria tenella*, *Eimeria maxima*, and *Eimeria acervulina*) was produced by Foshan Zhengdian Biotechnology Co., Ltd. (Foshan, China). Each vial contained 5 mL of the vaccine with a total of 800,000 sporulated oocysts, which is sufficient for the normal immunization of 2000 chickens. The standard immunization dose per chicken contained 400 sporulated oocysts. For the 30-fold dose challenge, 28 mL of distilled water was added to each vial. Subsequently, 0.5 mL of the diluted vaccine was administered to each chicken via oral gavage.

2.2. Animal Ethics Statement

The experiment was conducted at the Liyuan Experimental Base of Anyou Biotechnology Technology Group Co., Ltd. (Shanghai, China). All animal procedures were approved by the Animal Care Committee of the Anyou Biotechnology Technology Group Co., Ltd. (Approval No: ANS-CEUA-PJT/PL/202310/069).

2.3. Experimental Design

A total of 480 one-day-old white-feathered broilers were randomly assigned to five groups: (1) uninfected (CON), (2) infected (EC), (3) infected + 500 g/t narasin (AT), and 4/5) infected + 200/400 g/t essential oil blend (EOB200/EOB400). There were 6 replicates per group and 16 broilers per replicate. On d 14, all birds in the infected groups (EC, AT, EOB200, and EOB400) received an oral inoculation of a 30-fold coccidial vaccine (1.2×10^4 oocysts/bird). The CON group was administered an equal volume of sterile normal saline on d 14 of the experiment. On d 22, one broiler with a body weight closest to the mean of its respective treatment group was selected from each replicate and slaughtered for sample collection. The remaining broilers were continuously raised until the end of the experiment on d 42.

2.4. Experimental Diet and Animal Management

The dietary composition and nutrient levels are presented in Table 1. Broilers were housed in an environmentally controlled, floor-rearing facility with ad libitum access to feed and water and under 24 h continuous lighting [18]. The ambient temperature was maintained at 32–34 °C for the first week and then reduced by 2–3 °C each subsequent week. All management practices, including routine husbandry and immunization, were uniformly applied across all treatment groups. No anticoccidial drugs were administered during the experiment.

Table 1. Composition and nutrients levels of experimental diet (air-dry basis, %).

Items	1–21 D	22–42 D
Ingredients, g		
Corn	501.35	393.65
Soybean meal	254.00	206.00
Wheat	--	150.00
Wheat flour	80.00	--
Canola meal		30.00
Cottonseed meal	50.00	50.00
Dicalcium phosphate	11.70	10.00
Corn gluten meal	50.00	50.00
Limestone	13.50	13.40
Duck fat	11.00	70.00
NaCl	2.60	2.40
L-Lysine·HCl	2.85	1.75
DL-Methionine	3.00	2.80
Premix ¹	20.00	20.00
Total	1000.00	1000.00
Nutrient levels ²		
Metabolizable energy, Kcal/kg	2900.00	3200.00
Crude protein, %	23.00	22.00
Crude fat, %	3.80	8.83
Crude fiber, %	3.00	3.00
Calcium, %	0.90	0.88
Available phosphorus, %	0.58	0.56
Lysine, %	1.32	1.28

¹ The premix provides the following for per kg of diet: Vitamin A, 10 000 IU; Vitamin D₃, 2 500 IU; Vitamin E, 50 IU; Vitamin K₃, 3.5 mg; Vitamin B₁, 2.5 mg; Vitamin B₆, 5 mg; Vitamin B₁₂, 0.025 mg; Choline chloride, 350 mg; Nicotinic acid, 30 mg; Riboflavin, 8 mg; Pantothenate, 10 mg; Biotin, 0.15 mg; Folic acid, 1.2 mg; Fe (FeSO₄·H₂O), 85 mg; Mn (MnSO₄·H₂O), 100 mg; Zn (ZnSO₄·H₂O), 75 mg; Cu (CuSO₄·5H₂O), 8.0 mg; I (KI), 0.6 mg; Se (Na₂SeO₃), 0.3 mg. ² The nutrient levels were calculated.

2.5. Growth Performance

Body weight (BW) was recorded on d 1, 21, and 42 after a 12 h fast. Average daily gain (ADG) was calculated for the periods of d 1–21 and 22–42 based on the BW data. Feed intake was recorded daily on a per-pen basis, and the residual feed was weighed on d 22 and 42 to determine the average daily feed intake (ADFI). The feed conversion ratio (F:G) was calculated as ADFI divided by ADG for each phase. Health status and mortality were monitored daily throughout the trial. For any dead birds, the date of death, body weight, and pen number were recorded. The calculations were performed as follows:

$$\text{ADG} = (\text{final pen weight} - \text{initial pen weight} + \text{body weight of dead or removed birds}) / (\text{number of remaining birds} \times \text{trial days} + \text{trial days of dead or removed birds}) \quad (1)$$

$$\text{ADFI} = (\text{weight of first feed addition} + \text{weight of second feed addition} + \dots + \text{weight of last feed addition} - \text{weight of residual feed}) / (\text{number of remaining birds} \times \text{trial days} + \text{trial days of dead or removed birds}) \quad (2)$$

$$\text{F:G} = \text{ADFI} / \text{ADG} \quad (3)$$

2.6. Quantification of Coccidian Oocyst Shedding in Feces

On d 21 and d 28 of the experiment, all fresh fecal samples were collected from each replicate pen with continuous observation during the collection process to minimize fecal contact with the litter, and thoroughly mixed to ensure the representativeness of the samples. A 2 g aliquot of homogenized feces was mixed with 20 mL of saturated sodium chloride solution. The mixture was then diluted to a final volume of 60 mL with the same solution, filtered through a 60-mesh sieve, and thoroughly mixed. Subsequently, both chambers of a McMaster counting slide were filled with the fecal suspension. After a 5 min settling period to allow oocyst flotation, the oocysts in both chambers (each with a volume of 0.15 mL) were counted under an Eclipse Ci-L camera microscope (Nikon Corp., Tokyo, Japan) at 100× magnification. The number of oocysts per gram (OPG) of feces was calculated using the following formula: $\text{OPG} = \text{the number of oocysts in two chambers} \times \text{dilution factor} \times (\text{fecal sample weight} / \text{counting chamber volume})$.

2.7. Sample Collection

On the morning of d 21, after a 12 h fast, six birds per treatment with body weights close to the group average were selected for blood and tissue sampling. Blood was collected via cardiac puncture into anticoagulant tubes. Plasma was separated by centrifugation at $3500 \times g$ for 10 min at 4 °C and stored at −20 °C. Immediately thereafter, the birds were euthanized, and the abdominal cavity was opened to isolate the duodenum, jejunum, and cecum. The specific slaughter procedure was as follows: Broilers were gently captured to minimize stress, then the neck was rapidly twisted once for complete cervical dislocation to ensure immediate painless unconsciousness. Successful euthanasia was confirmed by the loss of corneal reflex and no voluntary movement before dissection and sample collection. The lumens of the duodenum and jejunum were gently flushed with ice-cold saline, and mucosal samples were collected by scraping with sterile glass slides. These samples were flash-frozen in liquid nitrogen and stored at −80 °C. Additionally, approximately 3 cm segments from the mid-duodenum and mid-jejunum were collected and fixed in 4% paraformaldehyde for morphological analysis.

2.8. Intestinal Lesion Score

On d 21 of the trial, the middle segments of the duodenum, jejunum and cecum were collected for lesion scores. Intestinal lesion scores were evaluated as described by Johnson

and Reid [19]. This procedure involved opening the intestinal tract and conducting a macroscopic assessment of both the mucosal lesions and the luminal contents.

2.9. Organ Index

Organ index was calculated on d 21. Six birds per treatment with body weights close to the group average were selected, weighed, euthanized, and dissected. The spleen, liver, bursa of fabricius, thymus, duodenum, jejunum, and ileum were then collected and weighed. The organ index was calculated as follows: Organ index (%) = [Organ weight (g)/Live body weight (g)] × 100.

2.10. Measurement of Serum Antioxidant Capacity

Key biomarkers of oxidative stress, including serum total antioxidant capacity (T-AOC), malondialdehyde (MDA), superoxide dismutase (SOD), and glutathione peroxidase (GSH-Px), were measured with specific commercial assay kits (Jiangsu Aidisheng Biotechnology Co., Ltd., Yancheng, China) according to the manufacturer's guidelines.

2.11. Serum Immunological Parameters

The serum concentrations of interleukin 1 (IL-1), interleukin 6 (IL-6), interleukin 10 (IL-10), transforming growth factor- β (TGF- β), and immunoglobulin G (IgG) were measured using commercial ELISA kits (Jiangsu Meimian Industry Co., Ltd., Yancheng, China) following the manufacturer's protocols.

2.12. Analysis of Intestinal Morphology

Following fixation in 4% paraformaldehyde, duodenum and jejunum samples were dehydrated through a graded ethanol series, embedded in paraffin, and sectioned at 5 μ m. The sections were stained with hematoxylin and eosin (H&E) and imaged under a Nikon Eclipse Ci-L microscope (Nikon Corp., Tokyo, Japan) at 40 $\times$ magnification. For morphometric assessment, five intact, well-oriented villi and their corresponding crypts were randomly selected per section. Villus height and crypt depth were measured using Image-Pro Plus 6.0 software, and the villus height-to-crypt depth ratio (V:C) was calculated.

2.13. Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from the mucosal tissues of the duodenum and jejunum using the RNAiso Plus (TaKaRa Biotechnology Co., Ltd., Dalian, China), and the concentration and purity of total RNA were assayed by a spectrophotometer (NanoDrop, Gene Co., Ltd., Guangzhou, China) at 260 and 280 nm according to the manufacturer's instructions. Reverse transcription was carried out using the Prime Script RT reagent kit (Yeastar Biotechnology Co., Ltd., Shanghai, China) according to the manufacturer's instructions.

Quantitative real-time PCR (qPCR) was performed to analyze the expression levels of *Occludin*, *Claudin-4*, *MUC-2*, and *ZO-1* using Hieff[®] qPCR SYBR Green Master Mix (Yeastar Biotechnology Co., Ltd., Shanghai, China) and the ABI ViiA 7 Real-Time PCR detection system (Applied Biosystems, Foster City, CA, USA). The experiment was carried out in a 10 μ L reaction mixture, which included 5 μ L of SYBR Green Master Mix (1 $\times$), 1 μ L each of reverse and forward primers, 1 μ L of RNase-free Water, and 2 μ L of cDNA template. The thermal cycling parameters were as follows: 95 $^{\circ}$ C for 5 min and 40 cycles of 95 $^{\circ}$ C for 10 s, 60 $^{\circ}$ C for 30 s. The primer sequences used for amplifying the target genes are listed in Table 2. The expression of the target genes, normalized to the housekeeping gene *GAPDH*, was calculated using the $2^{-\Delta\Delta C_t}$ method [20].

Table 2. Sequence of primers used for the real-time quantitative PCR analysis.

Genes ¹	Primer Sequences (5'-3') ²	Product (bp)
<i>GAPDH</i>	F: TGACCACTGTCCATGCCATC R: CTTTCCCCACAGCCTTAGCA	135
<i>Occludin</i>	F: ATCAACGACCGCCTCAATCA R: TCCGCTTCAGGTCTTTGAGC	112
<i>Claudin-4</i>	F: CATCGCCCTGTCCGTCATC R: AGTTCATCCACAGCCCTTCC	131
<i>Mucin-2</i>	F: TGTGTAAGTGCACCAAGGCT R: TCACACTCCCAATGCCAACA	154
<i>ZO-1</i>	F: GCCTACTGCTGCTCCTTACA R: GCGGTAAGGATGATGGCTCT	113

¹ ZO = Zonula occludens. ² F = forward primer; R = reverse primer.

2.14. Statistical Analysis

Statistical analyses were performed using IBM SPSS Statistics 27.0 (IBM Corp., Armonk, NY, USA). Data are presented as mean and SEM. Infected groups were assessed by one-way analysis of variance (ANOVA), and Duncan's test was used for post hoc comparisons following a significant main effect. An independent samples *T*-test was employed specifically to compare the CON and EC groups. Differences were considered statistically significant at $p < 0.05$, and values in the range of $0.05 < p \leq 0.10$ were considered indicative of a statistical trend.

3. Results

3.1. Growth Performance of Broilers

Table 3 summarizes the growth performance of broilers in different experimental groups during the trial. On d 1 to 21 of the trial, the 30-fold coccidial vaccine challenge on d 14 resulted in no effects on growth performance of broilers ($p > 0.05$). Compared with the CON group, the EC group exhibited a 1.04% reduction in BW21 and a 3.87% increase in the F:G ratio ($p > 0.05$). Relative to the EC group, the AT and EOB200 groups showed numerically higher BW21 (by 2.66% and 1.64%, respectively) and ADG (by 2.84% and 1.75%, respectively). The EOB200 group also had a numerically lower F:G ratio (by 2.48%). Nevertheless, none of these differences were statistically significant ($p > 0.05$). From 22 to 42 d, compared with the AT group, the EC, EOB200, and EOB400 groups had lower BW42 and ADG, and a higher F:G ratio ($p < 0.05$).

Table 3. Effects of essential oil blend the growth performance of white-feathered broilers challenged with coccidial vaccine.

Items ¹	CON	EC	AT	EOB200	EOB400	SEM	<i>P</i> ₁	<i>P</i> ₂
1 to 21 d								
BW1, g	40.91	40.86	40.95	40.86	40.85	0.034	0.654	0.751
BW21, g	631.88	625.29	641.93	635.52	608.05	7.196	0.798	0.446
ADFI, g	45.76	46.84 ^{ab}	48.68 ^a	46.29 ^{ab}	44.77 ^b	0.397	0.799	0.018
ADG, g	29.55	29.22	30.05	29.73	28.36	0.359	0.363	0.448
F:G	1.55	1.61	1.62	1.57	1.58	0.016	0.369	0.675
22 to 42 d								

Table 3. Cont.

Items ¹	CON	EC	AT	EOB200	EOB400	SEM	P ₁	P ₂
BW22, g	635.01	610.33	636.34	637.30	613.23	8.055	0.444	0.586
BW42, g	2261.14	2204.69 ^b	2381.68 ^a	2197.01 ^b	2155.83 ^b	23.831	0.496	0.001
ADFI, g	142.81	136.70	135.16	133.64	132.74	2.342	0.438	0.910
ADG, g	77.59	75.21 ^b	82.85 ^a	74.36 ^b	73.71 ^b	0.924	0.538	<0.001
F:G	1.86	1.80 ^a	1.63 ^b	1.80 ^a	1.81 ^a	0.356	0.718	0.055

¹ BW = body weight; ADFI = average daily feed intake; ADG = average daily weight gain; F:G = feed:gain; P₁: The *p*-value of *T*-test; P₂: The *p*-value of one-way analysis of variance; CON = control group; EC = coccidial vaccine challenge group; AT = narasin (500 g/t) and coccidial vaccine challenge group; EOB200 = essential oil blend (200 g/t) and coccidial vaccine challenge group; EOB400 = essential oil blend (400 g/t) and coccidial vaccine challenge group. In the same row, values with different letter superscripts mean significant difference among coccidia vaccine-challenged broilers (*p* < 0.05). The results are expressed as mean and SEM (*n* = 6).

3.2. Fecal Oocysts Count and Intestinal Lesion Scores

Compared with the CON group, the EC group had a higher number of fecal oocysts on d 21 and 28 (*p* < 0.05; Figure 1A,B). Relative to the EC group, the AT, EOB200, and EOB400 groups had lower fecal oocyst counts on d 21 (*p* < 0.05; Figure 1A). On d 28, the AT and EOB200 groups also exhibited reduced oocyst counts compared to the EC group (*p* < 0.05; Figure 1B). Meanwhile, the CON group had a small number of oocysts in feces, which was attributed to the natural environmental exposure of broilers during the breeding period (Figure 1A–C).

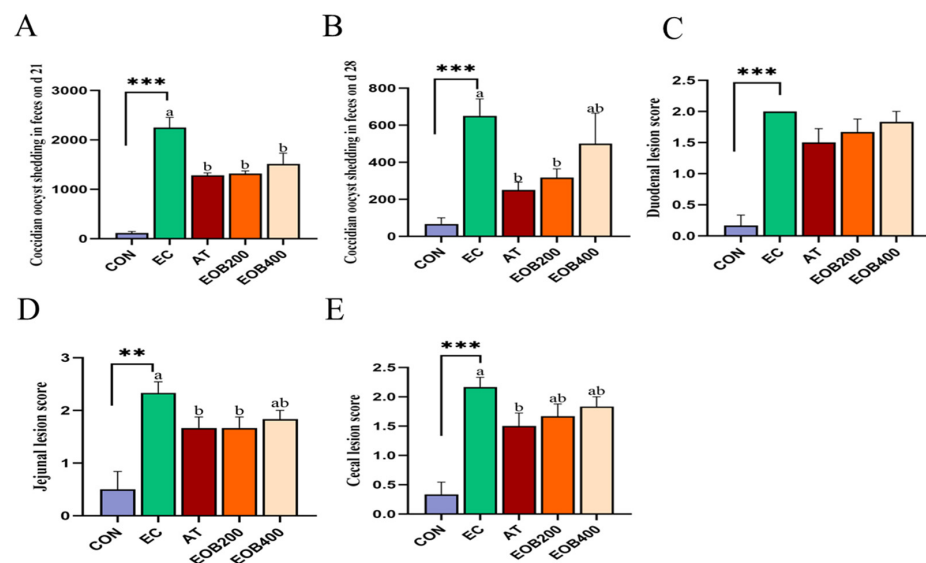


Figure 1. Effects of essential oil blend on the fecal oocyst count and intestinal lesion scores of white-feathered broilers challenged with coccidial vaccine. (A) Coccidian oocyst shedding in feces on d 21. (B) Coccidian oocyst shedding in feces on d 28. (C) Duodenal lesion score on d 21. (D) Jejunal lesion score on d 21. (E) Cecal lesion score on d 21. CON = control group, EC = coccidial vaccine challenge group, AT = narasin (500 g/t) and coccidial vaccine challenge group, EOB200 = essential oil blend (200 g/t) and coccidial vaccine challenge group, EOB400 = essential oil blend (400 g/t) and coccidial vaccine challenge group. * Significantly different between CON vs. EC, ** *p* < 0.01, *** *p* < 0.001. Letters above the bars (a, b) indicate significant differences (*p* < 0.05) among coccidia vaccine-challenged broilers. The results are expressed as mean and SEM (*n* = 6).

Intestinal lesion scores in the duodenum, jejunum, and cecum were higher in the EC group than in the CON group (*p* < 0.05; Figure 1C–E). However, these scores were reduced in the AT and EOB200 groups compared with the EC group in the jejunum, and in the AT group in the cecum (*p* < 0.05; Figure 1D,E).

3.3. Organ Index of Broilers

As shown in Table 4, compared with the CON group, coccidial vaccine challenge had no effect on the organ index of broilers ($p > 0.05$). Similarly, compared with the EC group, the AT, EOB200, and EOB400 groups also did not affect the organ index ($p > 0.05$).

Table 4. Effects of essential oil blend on organ index of white-feathered broilers challenged with coccidial vaccine (%).

Items ¹	CON	EC	AT	EOB200	EOB400	SEM	P_1	P_2
Liver	2.56	2.66	2.76	2.73	2.74	0.042	0.324	0.903
Spleen	0.12	0.12	0.12	0.12	0.12	0.002	0.617	0.923
Bursa of fabricius	0.27	0.28	0.32	0.30	0.29	0.009	0.898	0.588
Duodenum	1.64	1.66	1.45	1.69	1.65	0.037	0.945	0.246
Jejunum	2.64	2.50	2.51	2.51	2.88	0.070	0.516	0.248
Ileum	1.87	2.03	1.80	1.93	2.11	0.065	0.511	0.593
Thymus	0.27	0.23	0.25	0.23	0.27	0.013	0.341	0.822

¹ AT = narasin (500 g/t) and coccidial vaccine challenge group; CON = control group; EC = coccidial vaccine challenge group; EOB200 = essential oil blend (200 g/t) and coccidial vaccine challenge group; EOB400 = essential oil blend (400 g/t) and coccidial vaccine challenge group; P_1 : The p -value of T -test; P_2 : The p -value of one-way analysis of variance. The results are expressed as mean and SEM ($n = 6$).

3.4. Serum Antioxidant Capacity of Broilers

Compared with the CON group, the EC group showed elevated serum T-AOC and SOD levels ($p < 0.05$; Figure 2A,C). Meanwhile, relative to the EC group, the T-AOC content was reduced in the AT and EOB400 groups, and the SOD content was lower in the AT, EOB200, and EOB400 groups ($p < 0.05$; Figure 2A,C). However, no differences were observed in MDA and GSH-Px levels among all groups ($p > 0.05$; Figure 2B,D).

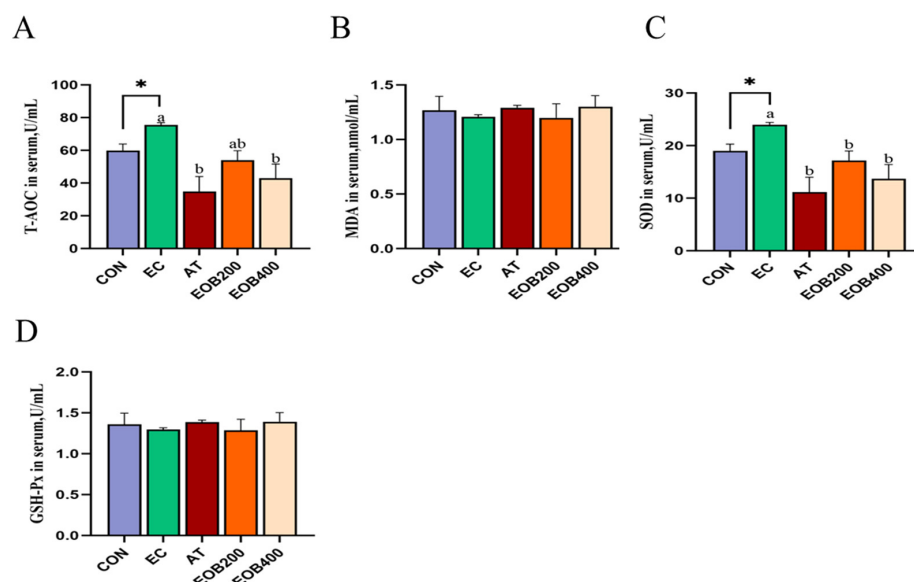


Figure 2. Effects of essential oil blend on serum antioxidant indices in white-feathered broilers challenged with coccidial vaccine. (A) Total antioxidant capacity (T-AOC) in serum. (B) Malondialdehyde (MDA) in serum. (C) Superoxide dismutase (SOD) in serum. (D) Glutathione peroxidase (GSH-Px) in serum. CON = control group, EC = coccidial vaccine challenge group, AT = narasin (500 g/t) and coccidial vaccine challenge group, EOB200 = essential oil blend (200 g/t) and coccidial vaccine challenge group, EOB400 = essential oil blend (400 g/t) and coccidial vaccine challenge group. * Significantly different between CON vs. EC, * $p < 0.05$. Letters above the bars (a, b) indicate significant differences ($p < 0.05$) among coccidia vaccine-challenged broilers. The results are expressed as mean and SEM ($n = 6$).

3.5. Serum Immunity of Broilers

The coccidial challenge induced a marked pro-inflammatory response, as indicated by an increase in IL-6 and a decrease in IL-10 in the EC group compared with the CON group ($p < 0.05$; Figure 3B,C). A tendency toward a decrease in IL-1 β ($p = 0.057$; Figure 3A) and an increase in IgG ($p = 0.087$; Figure 3D) was also observed in the EC group.

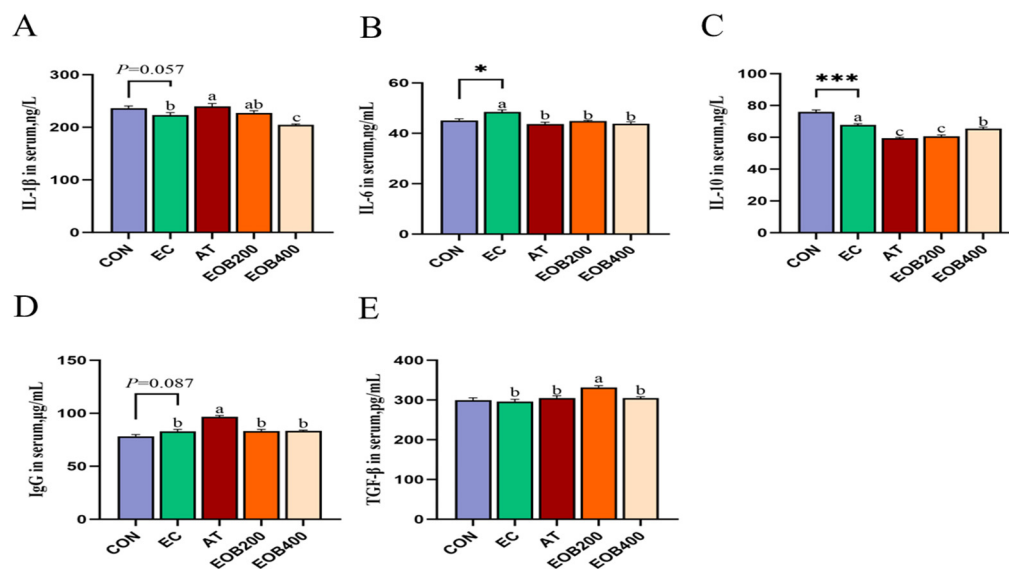


Figure 3. Effects of essential oil blend on serum immunity in white-feathered broilers challenged with coccidial vaccine. (A) Interleukin-1 β (IL-1 β) in serum. (B) Interleukin-6 (IL-6) in serum. (C) Interleukin-10 (IL-10) in serum. (D) Immunoglobulin G (IgG) in serum. (E) Transforming growth factor- β (TGF- β) in serum. CON = control group, EC = coccidial vaccine challenge group, AT = narasin (500 g/t) and coccidial vaccine challenge group, EOB200 = essential oil blend (200 g/t) and coccidial vaccine challenge group, EOB400 = essential oil blend (400 g/t) and coccidial vaccine challenge group. * Significantly different between CON vs. EC, * $p < 0.05$, *** $p < 0.001$. Letters above the bars (a, b, c) indicate significant differences ($p < 0.05$) among coccidia vaccine-challenged broilers. The results are expressed as mean and SEM ($n = 6$).

Dietary treatments effectively modulated these immune parameters. Compared with the EC group, IL-1 β levels were elevated in the AT group but reduced in the EOB400 group ($p < 0.05$; Figure 3A). Levels of IL-6 and IL-10 were lower in the AT, EOB200, and EOB400 groups ($p < 0.05$; Figure 3B,C). Furthermore, IgG was higher in the AT group, and TGF- β was higher in the EOB200 group ($p < 0.05$; Figure 3D,E).

3.6. Intestinal Health of Broilers in Duodenum and Jejunum

Intestinal morphology was assessed in duodenal sections by H&E staining (Figure 4A). The CON group exhibited intact morphology with long villi, though occasional villi showed thickening and blunting (green arrows). The EC group had shorter villi of uneven thickness, with a small number being thickened/blunt (green arrows) and a considerable amount of epithelial exfoliation (black arrows). The AT group presented long villi but with considerable epithelial exfoliation (black arrows). The EOB200 group displayed long, uneven villi, occasional thickening/blunting (green arrows), and occasional epithelial exfoliation (brown arrows). The EOB400 group demonstrated relatively long, uneven villi, a small number of thickened/blunt villi (green arrows), and considerable epithelial exfoliation (black arrows). The results demonstrate that the EOB200 group alleviated the intestinal injury caused by coccidial vaccine challenge to a certain degree. Additionally, in the duodenum, the EC group exhibited deeper crypt depth and lower V:C ratio than the CON group ($p < 0.05$;

Figure 4B). In contrast, the EOB200 group showed increased villus height and elevated V:C ratio compared to the EC group ($p < 0.05$; Figure 4B).

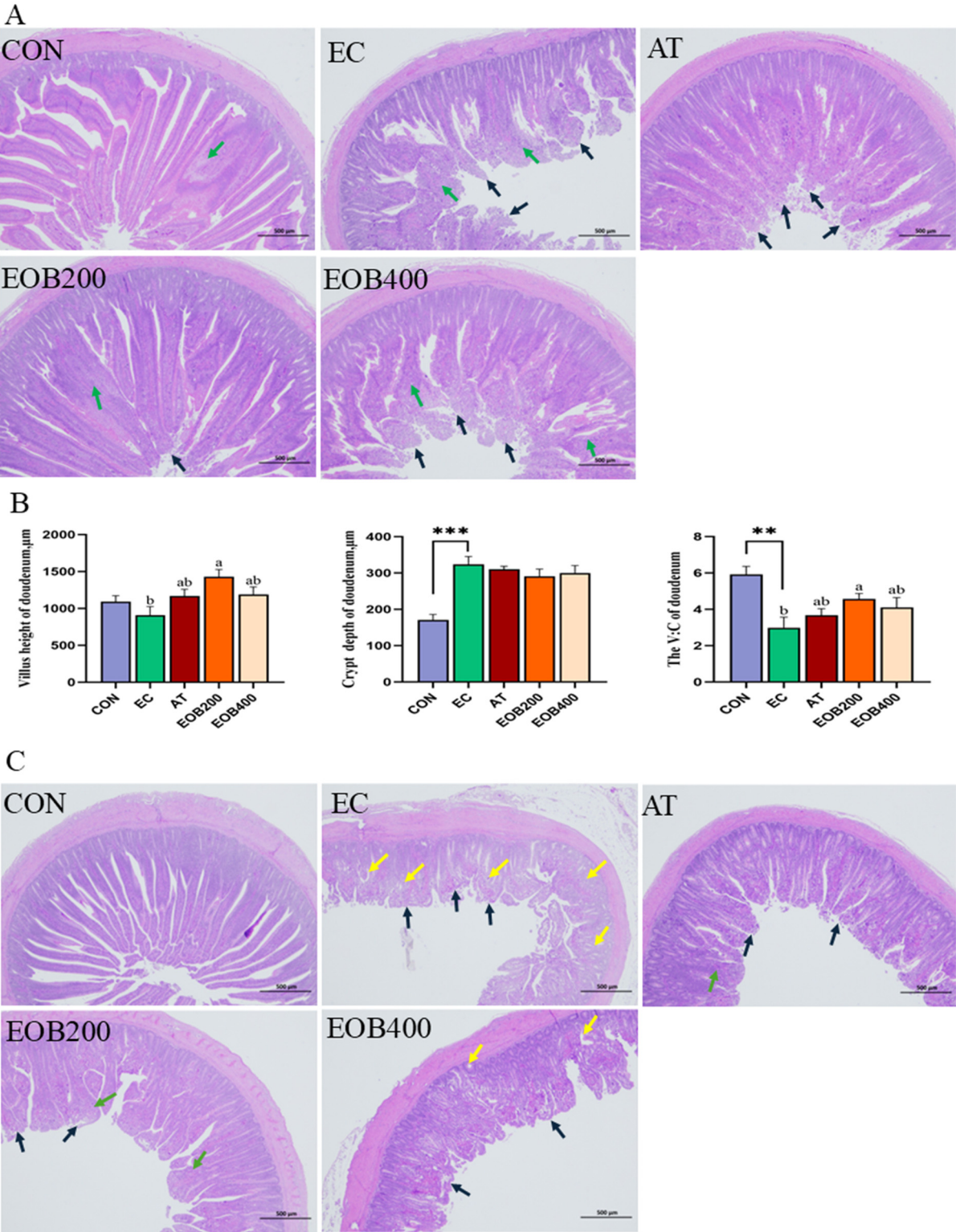


Figure 4. Cont.

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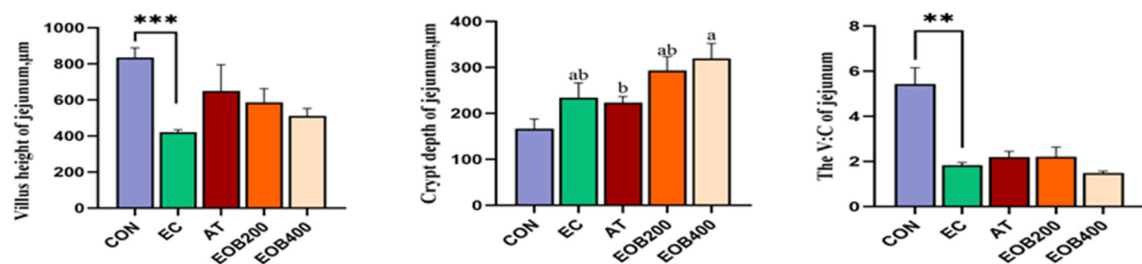


Figure 4. Effects of essential oil blend on intestinal morphology in white-feathered broilers challenged with coccidial vaccine. (A) Hematoxylin and eosin staining of duodenum on d 21. Scale bar = 500 μm. (B) Villus length, crypt depth, and villus-to crypt ratio (V:C) of duodenum in different treatment groups were measured. (C) Hematoxylin and eosin staining of jejunum on d 21. Scale bar = 500 μm. (D) Villus length, crypt depth, and villus-to crypt ratio (V:C) of jejunum in different treatment groups were measured. CON = control group, EC = coccidial vaccine challenge group, AT = narasin (500 g/t) and coccidial vaccine challenge group, EOB200 = essential oil blend (200 g/t) and coccidial vaccine challenge group, EOB400 = essential oil blend (400 g/t) and coccidial vaccine challenge group. * Significantly different between CON vs. EC, ** $p < 0.01$, *** $p < 0.001$. Black arrows: epithelial exfoliation of villi. Yellow arrows: dilated intestinal glands. Green arrows: thickened and blunted villi. Letters above the bars (a, b) indicate significant differences ($p < 0.05$) among coccidia vaccine-challenged broilers. The results are expressed as mean and SEM ($n = 6$).

Intestinal morphology was assessed in jejunum sections by H&E staining (Figure 4C). The CON group displayed intact morphology without significant infiltration or exfoliation. The EC group exhibited shorter villi with uneven thickness and irregular contours, considerable epithelial exfoliation (black arrows), locally disorganized and dilated intestinal glands (yellow arrows) containing luminal cellular clusters. The AT group showed shorter, uneven villi, occasional thickening/blunting (green arrows), and extensive epithelial exfoliation (black arrows). The EOB200 group presented shorter, uneven villi, limited villus blunting (green arrows) and epithelial exfoliation (black arrows), along with relatively regular glandular arrangement. The EOB400 group was characterized by villi of variable thickness and irregular shape, extensive epithelial exfoliation (brown arrows), and locally irregular gland arrangement with a few dilated glands (yellow arrows). The results demonstrate that AT and EOB200 groups alleviated the intestinal injury caused by coccidial vaccine challenge. In the jejunum, the EC group had reduced villus height and V:C ratio relative to the CON group ($p < 0.05$; Figure 4D). Meanwhile, compared with the AT group, crypt depth was greater in the EOB400 group ($p < 0.05$; Figure 4D).

The expression of intestinal tight junction-related genes is presented in Table 5. Coccidial vaccine challenge had no significant effect on the expression of tight junction-related genes in the duodenum and jejunum of broilers ($p > 0.05$). Dietary supplementation with the essential oil blend also exerted no significant effect on the expression of these genes in the duodenum and jejunum after coccidial challenge ($p > 0.05$).

Table 5. Effects of essential oil blend on intestinal tight junctions in white-feathered broilers challenged with coccidial vaccine.

Items ¹	CON	EC	AT	EOB200	EOB400	SEM	P_1	P_2
Duodenum								
<i>Occludin</i>	1.00	1.37	0.88	1.79	1.59	0.157	0.484	0.389
<i>ZO-1</i>	1.00	0.60	0.49	0.34	0.70	0.120	0.426	0.640
Jejunum								
<i>Occludin</i>	1.00	0.75	0.66	0.84	0.96	0.073	0.327	0.564

Table 5. Cont.

Items ¹	CON	EC	AT	EOB200	EOB400	SEM	P ₁	P ₂
ZO-1	1.00	1.28	1.47	0.95	1.53	0.150	0.324	0.708
Mucin-2	1.00	0.88	0.74	0.57	0.85	0.097	0.728	0.770
Claudin-4	1.00	0.97	1.46	0.70	1.13	0.135	0.919	0.469

¹ AT = narasin (500 g/t) and coccidial vaccine challenge group; CON = control group; EC = coccidial vaccine challenge group; EOB200 = essential oil blend (200 g/t) and coccidial vaccine challenge group; EOB400 = essential oil blend (400 g/t) and coccidial vaccine challenge group; P₁: The *p*-value of *T*-test; P₂: The *p*-value of one-way analysis of variance. The results are expressed as mean and SEM (*n* = 6).

4. Discussion

The invasion and proliferation of coccidia in intestinal epithelial cells lead to epithelial desquamation and villus damage, which compromises nutrient digestion and absorption and ultimately impairs broiler growth performance [3,21]. Mohiti and Ghanaatparast [22] reported that a severe challenge with a 50 × dose of live oocyst vaccine in 22-day-old Ross broilers reduced ADG during 22–28 and 29–35 d of age and increased the F:G ratio over the 22–42 and 1–42 d periods. Several natural feed additives have been documented to mitigate the adverse effects of coccidiosis. For example, dietary supplementation with 100 g/t of tannic acid was shown to ameliorate the negative impacts of an *Eimeria* challenge, improve the F:G ratio, and enhance host immunity [23]. Similarly, Bafundo et al. [24] reported that saponins (250 and 500 g/t) improved growth performance and reduced lesion scores in coccidia-vaccinated broilers. In line with these findings, our results indicated that although the essential oil blend did not induce statistically significant improvements in the growth performance of challenged broilers, it numerically reduced the F:G ratio. This promising trend suggests a potential for the formulation to positively influence feed efficiency.

The present study demonstrated that challenge with a coccidial vaccine increased oocyst excretion in feces on both d 21 and 28 and induced higher lesion scores in the duodenum, jejunum, and cecum, confirming the successful establishment of intestinal damage consistent with previous findings [22,25]. The dietary supplementation with the essential oil blend effectively mitigated these effects. Specifically, the AT, EOB200, and EOB400 groups exhibited reductions in oocyst output and lower jejunal and cecal lesion scores compared to the EC group. The observed anticoccidial efficacy aligns with the known properties of various plant-derived compounds. For instance, the compound essential oil—a blend of eucalyptus oil, apigenin, and eugenol essential oil—showed significant anticoccidial effects, as evidenced by an Anticoccidial Index (ACI) of 169.2 [26]. Qaid et al. [27] found that cinnamon oil at a high concentration exhibits moderate anticoccidial activity (ACI = 146). *Lycium barbarum* extract (10 g/kg) was reported to show good anticoccidial efficacy with an ACI of 162.56 [16]. Other studies have similarly documented the capacity of essential oils, such as oregano oil, to ameliorate lesions and reduce oocyst shedding [22]. Collectively, these results indicate that the essential oils possess anticoccidial efficacy comparable to that of conventional antibiotics, highlighting their potential as a viable natural alternative for integrated coccidiosis control.

The antioxidant system is a relatively complex defense system in the organism. The SOD, GSH-Px, T-AOC, and MDA are common indicators for assessing antioxidant capacity, and changes in their activities or contents can reflect the strength of the organism's antioxidant capacity to a certain extent. When the body's antioxidant defense system is activated, it upregulates enzyme activity in a compensatory manner to counteract oxidative stress [28]. Abdelhady et al. [29] found that 3-day-old Indian River broilers vaccinated with a 50-fold dose of coccidial oocyst vaccine exhibited elevated CAT and SOD activity. This is consistent with the results of our study. Bozkurt et al. [30] reported that supplementing

the diet of broilers with oregano essential oil at 12 and 24 mg/kg significantly reduced the serum antioxidant/oxidative stress indices NO, MDA and SOD, alleviated oxidative stress induced by *Eimeria* infection, and that the effects of oregano essential oil on these indices were not significantly different from those of monensin. The findings of this experiment indicate that the T-AOC and SOD levels in the AT, EOB200, and EOB400 groups were all lower than those in the EC group, which typically implies an alleviation of the oxidative stress state.

The coccidial challenge in this study successfully induced a systemic pro-inflammatory state, as evidenced by a significant increase in serum IL-6 and a concurrent decrease in IL-10 in the EC group compared to the CON group. The IL-6 is a key pro-inflammatory cytokine rapidly released upon pathogen invasion, whereas IL-10 acts as a crucial anti-inflammatory mediator to prevent excessive inflammation [31–33]. The reduction in IL-6 levels in the AT, EOB200, and EOB400 groups compared to the EC group indicates that both the antibiotic and the essential oil blend effectively contained the coccidia-induced inflammatory cascade. Interestingly, the EOB200 group exhibited an increase in serum TGF- β . This elevated TGF- β likely contributed to immunomodulation by curbing the overactivation of T cells and macrophages while enhancing regulatory T cell function, thereby facilitating pathogen clearance without excessive inflammation and promoting immune rebalancing [33,34]. Notably, serum IgG levels were lower in the EOB200 group than in the EC group. The IgG provides protection through pathogen neutralization, phagocytosis enhancement, and T-cell regulation [35]. Although coccidial infection typically induces specific IgG production as part of humoral immunity, the high level of TGF- β in the EOB200 group may have moderately suppressed effector B cell activation and antibody production. Collectively, these findings demonstrate that essential oil blend, particularly EOB200 group, effectively mitigate coccidia-induced inflammation in broilers.

The intestine, as the primary site for nutrient absorption, is fundamental to sustaining health and growth performance in broilers. Coccidial infection compromises this function by damaging the intestinal morphology. Our results confirm previous findings that essential oil blend can ameliorate such damage. Studies have shown that oregano essential oil and monensin improve intestinal morphology in coccidia-infected chickens, with enhancements in villus structure helping to compensate for impaired feed efficiency [30]. Similarly, essential oil supplementation has been reported to increase duodenal villus height and the villus height to crypt depth ratio in challenged broilers [36]. This promising trend, coupled with the significant morphological improvements, suggests that the essential oil blend helps protect intestinal integrity.

5. Conclusions

In summary, the experimental findings showed that the essential oil blend (200 g/t and 400 g/t) can serve as a feasible adjunctive alternative to traditional anticoccidial regimens for broilers under a high dose coccidial vaccine challenge stress. Notably, 200 g/t was the optimal supplementation level, exerting the most significant effects on improving intestinal mucosal health, regulating immune function, and alleviating coccidial vaccine-induced damage (Figure 5). These results confirm its potential to partially reduce reliance on traditional anticoccidial drugs under similar conditions, and provide an eco-friendly nutritional strategy for mitigating coccidial stress in broilers.

However, the immune response and lesion characteristics induced by a high dose of coccidial vaccine oocysts differ from those induced by wild-type virulent strains; thus, the use of a high dose of coccidial vaccine for challenge in this study has certain limitations.

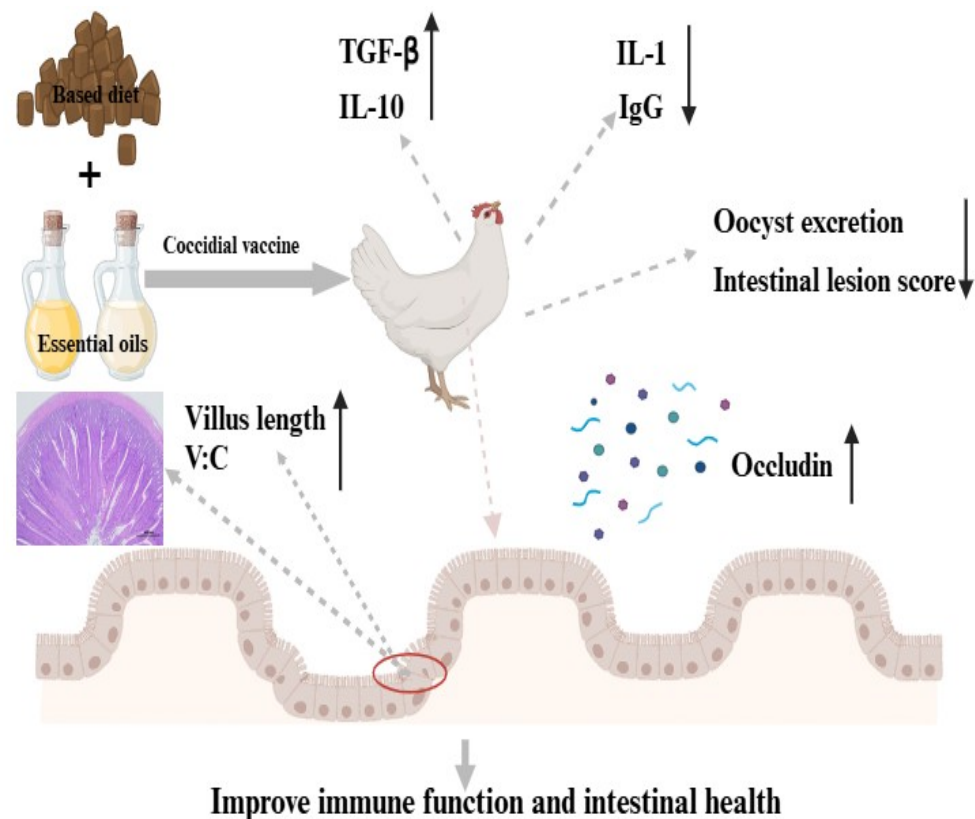


Figure 5. Ameliorative impacts of an essential oil blend on immune function and intestinal health in broilers following a high-dose coccidial challenge.

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Institutional Review Board Statement: Ethics approval and consent to participate. Our study was carried out in compliance with the ARRIVE guidelines (AVMA Guidelines for the Euthanasia of Animals: 2020 Edition). All experimental animal procedures complied with the laboratory animal management and welfare regulations approved by the Ethics Committee of Anyou Biotechnology Technology Group Co., Ltd., Suzhou, Jiangsu, China. All animal experiments were approved by the Animal Care Committee of Anyou Biotechnology Technology Group Co., Ltd. (approval number, ANS-CEUA-PJT/PL/202310/069).

Data Availability Statement: The raw data supporting the conclusions of this article will be made available by the authors on request.

Conflicts of Interest: Author Hongjun Yang is employed by the company Centree Bio-Tech (Wuhan) Co., Ltd. Authors Minmin Li, Chunxue Liu, Yifen Hung, Bo Shen, Shuaipeng Guo, Rui Xu, Tao Hu, Wenjing Geng, and Gaiqin Wang are employed by the company Anyou Biological Technology Group Co., Ltd. The company Anyou Biological Technology Group Co., Ltd. funded the study. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results. The research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Abbreviations

The following abbreviations are used in this manuscript:

V:C	villus height-to-crypt depth
IgG	immunoglobulin G
TGF- β	transforming growth factor- β
BW	body weight
ADG	average daily gain
ADFI	average daily feed intake
F:G	average daily feed intake: average daily gain
OPG	oocyst per gram
T-AOC	total antioxidant capacity
MDA	malondialdehyde
SOD	superoxide dismutase
GSH-Px	glutathione peroxidase
IL	interleukin
MUC	mucin
ZO-1	zonula occludens-1

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