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Efficacy of *Lactobacillus plantarum* 1582-Fermented Chive (*Allium schoenoprasum*) as a Natural Antibiotic Against *Eimeria acervulina* in Broiler Chicken

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ABSTRACT

This study evaluated the efficacy of chive (*Allium schoenoprasum*) fermented by *Lactobacillus plantarum* 1582 (FC) as an antibiotic alternative in controlling *Eimeria acervulina* infection in broiler chickens. A total of 250 J-Dabaco male chickens were divided into five treatment groups, each with five replicates (cages) of 10 chickens: PC - positive control, NC - negative control, FC1 - supplemented with 1% FC, FC3 - supplemented with 3% FC, and antibiotic treatment (AB). Chickens in the NC, FC1, FC3, and AB groups were experimentally infected with *E. acervulina* at 14 days of age and monitored until day 42. Assessed variables included growth performance (body weight gain (BWG), feed intake (FI), feed conversion ratio (FCR), survival rate (SR), production efficiency index (PEI), serum immunoglobulins (IgA, IgM, IgG), ileal mRNA expression of tight junction (ZO-1, Claudin-2) and immune-related genes (IL-4, TNF-α, IFN-γ), fecal oocyst counts, and intestinal lesion scores. The results showed that both FC3 and FC1 groups improved BWG, FI, FCR, SR, and PEI, with the FC3 group showing the best performance, equivalent to the AB group. Additionally, FC contributed to preserving the integrity of the intestinal epithelium by enhancing tight junction protein expression (ZO-1, Claudin-2) and reducing inflammatory responses (IFN-γ, TNF-α), as well as reinforcing the intestinal barrier by improving villus morphology and reducing intestinal mucosal damage scores. Moreover, a significant reduction in *Eimeria* oocyst counts in the excretion demonstrated effective parasite control. These findings suggest that FC, especially at 3% concentration, can be an effective alternative to antibiotics in broiler farming for controlling coccidiosis and improving the safety and sustainability of production.

Keywords: ***Allium schoenoprasum; antibiotic replacement; broiler chickens; Eimeria acervulina; Lactobacillus plantarum***

INTRODUCTION

Coccidiosis in chickens is a severe intestinal disease caused by the intracellular protozoan *Eimeria* spp. (Li *et al.*, 2022), and remains one of the most serious issues in the commercial poultry industry, leading to significant economic losses worldwide (Britez *et al.*, 2023). Recent strategies for controlling coccidiosis have primarily focused on using chemical medications and vaccines. However, the widespread and prolonged use of chemical drugs has led to the emergence of drug-resistant strains (Abou-Jaoudeh *et al.*, 2024). Furthermore, the presence of drug residues in poultry products is increasingly drawing attention (Bacanli & Başaran, 2019). Therefore, there is a need for safe and effective alternatives to coccidiostats.

Plant-based formulations have shown potential in controlling coccidiosis in poultry without negatively impacting productivity (Galli *et al.*, 2021), with fewer side effects and a lower likelihood of developing drug resistance (Memon *et al.*, 2021). The bioactive compounds in herbs may directly disrupt the coccidia lifecycle, alter oocyst formation, inhibit sporulation (Fatemi *et al.*, 2015), and kill sporozoites (Kim *et al.*, 2013). Additionally, the immunomodulatory, antioxidant, and anti-inflammatory properties of these compounds help preserve the intestinal ecosystem and health, thus enhancing the host's defense against coccidia (Irawan *et al.*, 2021). Chives (*Allium schoenoprasum*), a common herb in Vietnam, are rich in flavonoids (particularly quercetin) and organosulfur compounds, showing potential for improving animal health (Hai *et al.*, 2020), especially immunity in broilers (Hai & Hoa, 2020). However, the direct addition of chives to animal feed may be limited in effectiveness due to the plant cell wall, which hinders the absorption of these compounds (Charen & Harbord, 2020).

Fermentation of herbs can promote the growth and reproduction of probiotics while enhancing their ability to colonize the intestinal tract (Wang *et al.*, 2017). *Lactobacillus plantarum* is one of the most common probiotics, capable of adhering to the intestinal

mucosa, producing digestive enzymes, balancing the gut microbiota, and stimulating immunity (Li *et al.*, 2025). Despite its many benefits, the effectiveness of *L. plantarum* alone in disease prevention may be limited. Combining probiotics with fermented herbal medicines is considered a promising strategy to enhance disease prevention and improve animal health.

Our recent study (Hai *et al.*, 2024) successfully isolated *L. plantarum* 1582 from indigenous free-range chicken excretion, capable of fermenting chives and maintaining high survival rates in the chicken digestive system. Thus, the hypothesis tested was that *L. plantarum* 1582-fermented chives could improve growth, health, and eliminate the negative effects of disease in broiler chickens infected with *E. acervulina*. This study aimed to assess the efficacy of chives fermented by *L. plantarum* 1582 as a potential antibiotic alternative for controlling *E. acervulina* infection and enhancing productivity in broiler chickens.

MATERIALS AND METHODS

Ethical Statement

In this study, all procedures related to the experimental chicken's care, feeding, and slaughtering were conducted according to the standards and approved by the Animal Ethics Advisory Committee, Hue University, Vietnam (Approval Number: HUVNO39).

Fermented Chive Preparation

Chives (*A. schoenoprasum* - GenBank ID on NCBI: NC_057575.1, 4-5 months old, cultivated according to the Vietgap biosafety standards) were processed and finely ground to make the raw material for the preparation. The fermentation process was carried out following the description of Hai *et al.* (2024) with some modifications, summarized as follows: the raw chives were fermented with *L. plantarum* 1582 (GenBank ID on NCBI: MT597487.1) (10^8 cfu/mL) in a medium containing 5% NaCl and 3% glucose, incubated at 37 °C under anaerobic conditions with a shaking speed of 60 rpm for 72 hours. After fermentation, the product was mixed with cassava starch at a ratio of 3:7 (w/w) and dried at 50 °C (SS-5730HP freeze dryer, Vietnam) to a moisture content of about 3%, then ground into a fine powder using a TMND-A18 grinder (Vietnam). The number of *Lactobacillus* spores was quantified according to TCVN 8737:2011, with approximately 2.7-3 x 10^8 CFU. Bioactive compounds in chives and *Lactobacillus* fermented chives are presented in Table 1.

Preparation of Coccidia

The virulent strain of *E. acervulina* was isolated from the gastrointestinal tract of indigenous chickens and maintained at the Parasitology Laboratory, University of Agriculture and Forestry, Hue University, Vietnam. The oocyst isolation process was performed using the flotation method, followed by sporulation at 28 °C in a 2.5% potassium dichromate solution for 48 hours (approximately 95% of the oocysts were sporulated) and stored at 4 °C for up to 1 month before use.

Experimental Animals

Day-old J-Dabaco commercial chicks were obtained from Dabaco Company, Vietnam and raised throughout the experiment (1-42 days of age) in a well-ventilated house with a cross-ventilation system. During the first week, the chicks were kept together in a brooder with a floor bedded with microbial bedding made from rice husk. From 8 to 42 days of age, the chicks were housed in metal cages with dimensions of L × W × H (0.9 × 0.5 × 0.5 m). Continuous fluorescent lighting was provided throughout the experiment. The temperature of the barn was maintained at 35 °C for the first 2 weeks and gradually reduced to 25 °C until the end of the experiment. The diet was formulated using local ingredients, meeting the poultry feeding standards of the Ministry of Agriculture and Rural Development (10 TCN 661-2005) (Table 2). The experimental chickens had free access to feed and water without antibiotics throughout the experiment.

The raw materials and medicinal ingredients (if applicable) were mixed using an HM-150 mixer (Hai Minh Co., Ltd, Vietnam; 5.5 kW, 50 rpm, ~1500 kg/h). The mixed feed was then pelleted using an S270 pellet mill (Binh Quan Group, Vietnam; 11 kW, 1450 rpm, ~500 kg/h) with a die size of 3 mm for the starter phase and 5 mm for the finisher phase, maintaining a moisture content of approximately 3%.

Table 1. Bioactive compounds in chives and *Lactobacillus* fermented chives

Ingredients/Compounds	Fresh chives	Fermented chives	Determination method
Polyphenol (mg/g)	10-15	15-25	Folin-Ciocalteu
Saponin (mg/g)	0.1-0.2	0.1-0.2	Vanillin-sulphuric acid
Quercetin (mg/g)	2-4	3-6	UV-Vis
Sulfur compounds			
Thiosulfate (mg/g)	5-7	~2-3	GC
S-allyl cysteine (mg/g)	0.1-0.3	1-3	GC-MS
Organic acids			
Lactic acid (%)	negligibility	0.5-1.5	HPLC
Acetic acid (%)	negligibility	0.1-0.5	HPLC
Citric acid (%)	~0.2-0.5	~0.5-1	HPLC

Note: Samples were analyzed at the Institute of Biotechnology, Hue University, Vietnam in December 2024.

Table 2. Ingredients, composition, and nutritional composition of the diet for broiler chickens (as-fed basis)

Items	Starter phase (<19 days of age)	Finisher phase (≥19 days of age)
Ingredient composition (%)		
Yellow corn	47.1	58.4
Soybean meal (36.7% crude protein)	44.1	33.7
Fish meal	5.0	4.0
$CaCO_3$ (38%)	2.0	2.0
$CaHPO_4$	1.0	1.0
Sodium chloride	0.4	0.4
Choline chloride (50%)	0.02	0.02
DL-Methionine (99.5%)	0.2	0.2
Vitamin premix[1]	0.1	0.1
Mineral premix[2]	0.1	0.1
Calculated nutritional values (%)		
Crude protein	22.2	18.0
Crude fat	4.48	5.48
Crude fiber	5.05	5.05
Calcium	1.10	1.00
Phosphorus (Total P)	0.51	0.44
Lysine	1.32	1.04
Methionine	0.50	0.45
Methionine + Cystine	0.90	0.80
Metabolizable energy (kCal/kg)	3000.0	3200.0

Note: [1] Each kilogram of vitamin premix contained 10 mg nicotinamide, 0.02 mg cholecalciferol, 0.3 mg folic acid, 2 mg pyridoxine HCl, 1.8 mg all-trans-retinyl acetate, 8 mg cyanocobalamin, 2.2 mg menadione, 8.3 mg alpha-tocopherol acetate, 160 mg choline chloride, and 20 mg D-biotin. 2 Each kilogram of mineral premix contained 60 μg selenium (Se), 200 μg cobalt (Co) from $CoSO_4$, 800 μg iodine (I) from KI, 2 mg copper (Cu) from $CuSO_4 \cdot 5H_2O$, 24 mg zinc (Zn) from ZnO, 16 mg iron (Fe) from $FeSO_4 \cdot 7H_2O$, and 32 mg manganese (Mn) from $MnSO_4 \cdot H_2O$.

Experimental Design

Healthy one-day-old male chickens (250 chicks) were randomly assigned into 5 groups (5 replicates, 10 chicks per cage): positive control (PC, no supplementation, no *E. acervulina* infection), negative control (NC, no supplementation, *E. acervulina* infection), 1% FC supplementation (FC1), 3% FC supplementation (FC3), and antibiotic treatment with Sulcox (Anvet Pharma JSC, Vietnam) (AB). Groups 2-5 were orally administered 1 ml of distilled water containing 2.0×10^4 oocysts of *E. acervulina*, while group 1 received 1 ml of distilled water at 14 days of age (directly into the crop using a long pipette) (Kumar *et al.*, 2014).

On day 6 post-infection (DPI), two chickens from each cage were randomly selected for blood collection to determine serum immunoglobulin levels. Afterward, the chickens were humanely euthanized by cervical dislocation for intestinal damage scoring. On the last day of the experiment, three chickens from each cage were randomly selected for necropsy to examine immune organs, collect small intestine samples for immune gene expression analysis, and conduct histopathological examination (duodenum, jejunum, ileum).

Indicators and Research Methods

Evaluation of productive performance. Feed intake (FI), the daily feed intake of chicks in each treatment, was determined at 07:00 hours by weighing the feed provided and subtracting the residual feed using a bench scale (Tanita, model Tanita KD-192, 1 g precision). FI was expressed as grams per chick per day, averaged across the replicate (10 chicks per cage).

Body weight gain (BWG): The individual body weight (BW) of each chick was measured by using electronic balances (WMS, Model WMS-HAW, 0.1 g precision) at the start (day 1) and end (day 42) of the experiment. BWG = Final BW − Initial BW

Feed conversion ratio (FCR): FCR = FI/BWG

Survival rate (SR): The number of surviving chicks in each cage was recorded daily. SR (%) = (Number of surviving chicks/Initial number of chicks) x 100

Production efficiency index (PEI): PEI = [BWG (kg) × SR(%)] x 100/ [Number of experimental days (42 days) x FCR] (Martins *et al.*, 2016).

Immunoglobulin content. On 6 DPI, blood samples were collected from two randomly selected chicks per cage via venipuncture. Blood samples were centrifuged (3,000 rpm, 10 minutes) using a benchtop centrifuge (Thermo Fisher Scientific) to separate serum. The serum was stored at -20 °C in a laboratory freezer until analysis. Concentrations of IgA (catalog no. MBS705241), IgM (MBS706158), and IgG (MBS260043) were determined by ELISA (MyBioSource, USA). The assay was performed according to the manufacturer's instructions. Briefly, serum samples were diluted and added to pre-coated microplates. Following incubation and washing steps, enzyme-conjugated antibodies were introduced, and a substrate solution was then added to initiate a colorimetric reaction. Finally, optical density (OD) was measured at 450 nm using a microplate reader (BioTek ELx800, USA).

Immune gene expression. Ileal mucosa samples were collected from three chicks per cage on day 42 during the necropsy. Total RNA was extracted from the ileal mucosa using a Treezol kit (Invitrogen, USA) following the manufacturer's protocol. RNA quality and concentration were assessed using a Nanodrop Lite spectrophotometer (Thermo Fisher Scientific, USA) at 260/280 nm. RNA was reverse-transcribed into cDNA using the FIRE Script RT cDNA Synthesis Kit (Solid Biodyne, Estonia). The reaction included an RNA template, primers, and reverse transcriptase, incubated according to kit instructions. Primers for target genes (IL-4, IL-1β, TNF-α, IFN-γ, ZO-01, claudin-2, and occludin) and reference gene GAPDH were synthesized by Sangon Biotech (China) (Table 3). RT-qPCR was performed on a Quant Studio™ 5 system (Thermo Fisher Scientific, USA) with an amplification cycle consisting of 95 °C (1 minute), 40 cycles of 95 °C (15 seconds), and 60 °C (60 seconds). Gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak &

Table 3. Primer sequences used in RT-qPCR Primer sequences used in RT-qPCR for analyzing tight junction and immune-related gene expression in ileal mucosa samples of broiler chickens infected with *Eimeria acervulina* and supplemented with *Lactobacillus plantarum* 1582-fermented chive

Gene	Primer sequence (5′ to 3′)			GenBank ID
	Forward primer	Reverse primer	Size (bp)	
Tight-binding protein				
ZO-1	CTTCAGGTGTTTCTCTTCCTCCTC	CTGTGGTTTCATGGCTGGAT	121	XM_413773.4
Occludin	GCAGATGTCCAGCGGCCCCC	CGAAGAAGCAGATGAGGCAGAG	89	NM_205128.1
Claudin-2	CAFCCTCCTGGGTCTGGTTGGT	GACAGCCATCCGCATCTTCT	198	NM_001013611.2
Pre-inflammatory cytokines				
IL-4	GTGCCCACGCTGTGCFC3C1C	AGGAAACCTCTCCCTGGATGTC	82	GU119892.1
IL-1β	GCCCGAGCCAACCCCTGC	AGCAACGGGACGGTAATGAA	204	NM_204524.1
TNF-α	CTCAGGACAGCCFCTGCCAACA	CCACCACACGACAGCCAAGT	177	XM_015294125.2
IFN-γ	CCTCGCAACCTTCACCTCAC	CGCTGFC1ATCGTTG TCTTGGAG	76	FJ977575.1
GAPDH	AACTTTGGCATTGTGGAGGG	ACGCTGGGATGATGTTCTGG	130	NM_204305.1

Schmittgen, 2001) with GAPDH as the reference gene. Data were processed using Quant Studio™ software and exported to Excel 2023 for statistical analysis.

Oocyst output in excretion. From days 4 to 11 post-infection with *E. acervulina*, excretion samples were collected and assessed daily. Excretion from each cage was thoroughly mixed, and then 2 g samples were randomly selected and placed into three 15 mL centrifuge tubes. These samples were used to quantify oocysts using the flotation method with saturated sucrose solution, as described by Ho *et al.* (2021). Specifically, each fecal sample (2 g) was mixed with 10 mL of distilled water and centrifuged at 4,000 rpm for 10 minutes (Thermo Fisher Scientific ST8). After removing the supernatant, 10 mL of saturated sucrose solution was added, mixed, and centrifuged at the same speed. The floatable material was transferred to a new tube and mixed well, then 10 µL was placed on a glass slide, covered with a coverslip, and examined under a microscope. Oocysts were counted three times per tube, and the oocyst count per gram of excretion (OPG) was calculated using the formula OPG = n × 500, where n is the average number of oocysts counted.

Intestinal damage scoring. On 6 DPI, two chicks per cage were euthanized, and intestinal segments (duodenum, jejunum, ileum) were collected during necropsy. Lesion scores were assessed according to Conway and McKenzie (2007) on a scale from 0 (no significant damage) to 4 (severe damage). The scoring evaluated macroscopic lesions, such as mucosal hemorrhages, thickening, or necrosis, specific to *E. acervulina* infection. Scoring was performed by visual inspection under a stereomicroscope (model not specified) and confirmed using a Nikon Eclipse E200 microscope (Nikon Instruments Inc., USA) at low magnification (40× or 100×) to assess tissue integrity.

Histopathological examination. Intestinal samples (1 cm segments from the duodenum, jejunum, and ileum) were collected on day 42 during necropsy and fixed in 10% formalin saline for 24 hours and processed according to the method of Layton *et al.* (2019). The tissue was dehydrated through a graded ethanol series (70%, 90%, and 100%) for 2 hours each, cleaned with xylene (twice, 1 hour each), and infiltrated with molten paraffin wax (twice, 1 hour each) before embedding. The tissue samples were sectioned at 5 µm using a rotary microtome (Leica RT 25, UK), mounted on slides, dried, and stained with Hematoxylin & Eosin (H&E). Morphological features (villus height, crypt depth) were analyzed using a Nikon Eclipse E200 microscope (Nikon Instruments Inc., USA) and Image-Pro Plus 6.0 software.

Statistical Analysis

The study's data were processed using Excel 2019, followed by statistical analysis using SPSS 22.0. Results are presented as means (Mean) and standard error of the mean (SEM). One-way ANOVA with post-hoc Bonferroni test was used to assess statistical differences, and the χ^2 test was used to evaluate percentage differences at α= 0.05.

RESULTS

Productivity of Broiler Chickens Supplemented with Fermented Chive

The results from Table 4 indicate that the supplementation with FC, particularly at 3%, improved productivity significantly ($p<0.05$), as shown by higher body weight gain (BWG) and feed intake (FI) when compared to the control groups. All groups maintained a 100% survival rate, indicating that supplementation with FC preparations (FC3 or FC1) did not negatively affect the normal growth of the chickens (Figure 1).

After *E. acervulina* infection, the FC3 group exhibited the highest BWG (714.7 g/bird), significantly higher ($p<0.05$) than the NC group and equivalent to the AB group. The FI in the FC3 group was also the highest (1668.2 g/bird), indicating increased palatability or metabolic demand, supporting weight gain. Overall, both the FC3 and FC1 groups helped mitigate the negative impact of *E. acervulina* infection on production performance, with BWG improvement of 12.16% and 7.97%, respectively, and FI increased by 12.98% and 10.03%, with FC3 showing a statistically significant difference ($p<0.05$) compared to the PC

Table 4. Growth performance of broiler chickens supplemented with fermented chive

Variables	Treatments					SEM	p-value
	PC	NC	FC1	FC3	AB		
Pre-challenged period (1-7 days old)							
BWG (g/bird)	25.6	27	26.5	25.6	26.6	0.25	0.196
FI (g/bird)	45.6	45.6	45.5	45.6	45.6	0.3	0.343
FCR	1.8	1.7	1.7	1.8	1.7	0.2	0.167
Post-challenged period (8-42 days old)							
BWG (g/bird)	699.4^{a}	633.6^{b}	686.7^{ab}	714.7^{a}	699.4^{a}	33.3	<0.001
FI (g/bird)	1589.2^{b}	1471.4^{c}	1623.2^{ab}	1668.2^{a}	1630.8^{ab}	63.5	<0.001
FCR	2.3	2.3	2.4	2.3	2.3	0.1	0.071
Full time (1-42 days old)							
BWG (g/bird)	724.4^{ab}	659.4^{c}	711.9^{b}	739.5^{a}	725.0^{ab}	34.9	<0.001
FI (g/bird)	1634.4^{ab}	1516.5^{c}	1668.4^{ab}	1713.5^{a}	1576.2^{bc}	89.5	<0.001
FCR	2.3^{ab}	2.3^{ab}	2.3a	2.3^{a}	2.8^{b}	0.1	<0.001
PEI	74.9^{a}	53.3^{c}	59.3b	68.4^{a}	71.5^{a}	3.6	<0.001

Note: In the same row, values with different lower-case letters (a-c) indicate statistically significant differences (p<0.05). Weight gain (BWG), feed intake (FI), feed conversion ratio (FCR), production efficiency index (PEI). PC (positive control): no FC supplementation, no *Eimeria acervulina* infection; NC (negative control): no supplementation, *E. acervulina* infection; FC1: 1% FC supplementation; FC3: 3% FC supplementation; AB: antibiotic treatment with Sulcox.

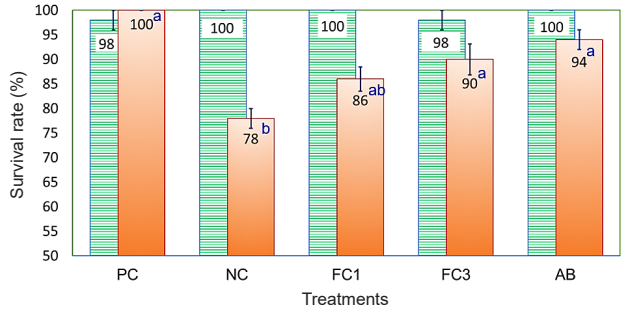

Figure 1. Survival rate of broiler chickens before and after challenge with *Eimeria acervulina.* Values with different letters (a, b) indicate statistically significant differences (p<0.05). PC (positive control): no FC supplementation, no *E. acervulina* infection; NC (negative control): no supplementation, *E. acervulina* infection; FC1: 1% FC supplementation; FC3: 3% FC supplementation; AB: antibiotic treatment with Sulcox. Pre-chalenge (■); Post-chalenge (■).

group. Regarding survival rate, the FC3 group achieved 90.0%, significantly higher than the NC group (78%) and similar to the AB group (93.9%). The production efficiency index (PEI) for the FC3 and FC1 groups was also significantly higher than the NC group (p<0.05) and equivalent to the AB and PC groups.

Immune Function of Broiler Chickens Supplemented with Fermented Chive

The results from Table 5 indicate that the weight of the bursa of Fabricius and spleen in the FC treatments with 2.4 g/kg and 2.3 g/kg supplementation significantly increased (p<0.05) compared to the control groups. Specifically, the weight of the bursa of Fabricius and spleen in the FC3 group was higher than that of the NC, PC, and AB groups. Meanwhile, the weight of the thymus in the experimental groups ranged from 3.4 g/kg to 3.9 g/kg, but there were no statistically significant differences (p= 0.261).

The immunoglobulin (IgA, IgM, IgG) levels in the serum of the experimental chickens at 42 days of age (27 days after infection) showed significant differences in IgA and IgM levels between the treatments (p<0.001). The *E. acervulina*-challenged group (NC) (2.18-2.19 g/L) had significantly lower values (p<0.001) compared to the PC and FC1 groups (3.22–3.23 and 3.22–3.62 g/L).

According to the results from Table 6, the FC3 group showed a significant improvement in the intestinal epithelial barrier with a ZO-1 level (3.01) that was 3.7 times higher (p= 0.019) compared to the NC group (0.81). Claudin-2 also significantly increased (2.75 vs 0.42; p= 0.018), while occludin showed no difference between the groups. For cytokines, IL-4 levels were high in the AB, FC3, and FC1 groups (3.11-3.37), significantly higher (p<0.05) compared to the NC and NC groups (1.84-2.32). In contrast, TNF-α and IFN-γ levels in the FC3 and AB groups decreased significantly (~42% and 21% compared to NC; p<0.05).

Oocyst Output in Excretion of Broiler Chickens Supplemented with Fermented Chive

Table 7 presents the number of oocysts per gram of excretion (OPG) in the experimental groups from day 4 to day 11 after *E. acervulina* infection. The results show that the negative control (NC) group had no detectable OPG throughout the experiment. In contrast, the negative control (NC) group had the highest OPG, peaking on day 7 (17,790.5) before gradually decreasing until day 11. The FC1 group had significantly lower OPG than the NC group at all time points, particularly on day 7 (10,040.0 vs 17,790.5). Meanwhile, the FC3 and AB groups showed a stronger trend of reducing OPG, with the AB group having the lowest OPG on day 11 (9.3). These results indicate that FC significantly reduces oocyst load in excretion, reflecting its inhibitory effect on *E. acervulina* growth.

Table 5. Immune organ index and immunoglobulin levels of broiler chickens supplemented with fermented chive

Treatments	Immune organ index (g/kg BW)			Serum immunoglobulin content (g/L)		
	Fabricius	Spleen	Thymus	IgA	IgM	IgG
PC	2.00^{b}	1.80^{b}	3.43	3.22^{b}	3.25^{b}	1.92
NC	2.03^{b}	1.84^{b}	3.81	2.19^{a}	2.18^{a}	1.89
FC1	1.97^{b}	1.77^{b}	3.73	3.26^{b}	3.62^{b}	2.01
FC3	2.43^{a}	2.26^{a}	3.90	2.90^{b}	3.32^{b}	1.95
AB	1.99^{b}	1.79b	3.50	2.46^{ab}	2.69^{ab}	1.97
SEM	0.01	0.01	0.05	0.03	0.03	0.02
P-value	<0.01	<0.01	0.261	<0.01	<0.01	0.566

Note: In the same column, values with different letters (a, b) indicate statistically significant differences (p<0.05). PC (positive control): no FC supplementation, no *Eimeria acervulina* infection; NC (negative control): no supplementation, *E. acervulina* infection; FC1: 1% FC supplementation; FC3: 3% FC supplementation; AB: antibiotic treatment with Sulcox.

Table 6. Immune gene expression levels of broiler chickens supplemented with fermented chive

Gene	Treatments					Pooled SEM	p-value
	PC	NC	FC1	FC3	AB		
Tight-binding protein							
ZO-1	0.92^{b}	0.81^{b}	0.94^{b}	3.01^{a}	0.95^{b}	0.12	<0.001
Occludin	1.11	1.15	0.96	1.07	1.19	0.12	0.145
Claudin-2	0.91^{bc}	0.42^{d}	1.23^{b}	2.75^{a}	1.15^{b}	0.13	<0.001
Pre-inflammatory cytokines							
IL-4	2.32^{b}	1.84^{b}	3.11^{a}	3.28^{a}	3.37^{a}	0.19	<0.001
IL-1β	1.23	1.26	1.36	1.33	1.33	0.23	0.948
TNF-α	1.13^{c}	2.00^{a}	1.61^{b}	1.21^{c}	1.18^{c}	0.06	<0.001
IFN-γ	1.83^{c}	3.59^{a}	2.87^{b}	2.13^{bc}	2.11^{bc}	0.13	<0.001

Note: Within the same row, values with different letters (a, b) indicate statistically significant differences (p<0.05). PC (positive control): no FC supplementation, no *Eimeria acervulina* infection; NC (negative control): no supplementation, *E. acervulina* infection; FC1: 1% FC supplementation; FC3: 3% FC supplementation; AB: antibiotic treatment with Sulcox.

Table 7. Oocyst output in broiler chickens excretion supplemented with fermented chive

Day-post infection	Treatments					Pooled SEM	p-value
	PC	NC	FC1	FC3	AB		
4	0	1425.^{3}	1304.8	1214.8	1164.8	252.5	0.013
5	0	3117.5^{a}	2548.6^{b}	2058.6^{bc}	1608.6^{c}	333.0	0.021
6	0	4856.2^{a}	2688.6^{b}	1898.7^{c}	1348.6^{cd}	280.3	<0.001
7	0	17790.5^{a}	10040.0^{b}	5650.0^{c}	3600.0^{d}	725.7	<0.001
8	0	9415.5^{a}	5100.5^{b}	1710.5^{c}	1260.5^{d}	419.5	<0.001
9	0	3595.0^{a}	1495.5^{b}	985.5^{cd}	535.5^{d}	361.0	0.018
10	0	1179.8^{a}	1030.3^{a}	520.0^{b}	270.0^{c}	61.8	0.022
11	0	516.8^{a}	209.3^{b}	59.3^{c}	9.3d	11.1	<0.001

Note: Within the same row, values with different letters (a-c) indicate statistically significant differences (p<0.05). PC (positive control): no FC supplementation, no *Eimeria acervulina* infection; NC (negative control): no supplementation, *E. acervulina* infection; FC1: 1% FC supplementation; FC3: 3% FC supplementation; AB: antibiotic treatment with Sulcox

Small Intestinal Mucosa of Broiler Chickens Supplemented with Fermented Chive

The scoring of intestinal damage was performed on the duodenum, ileum, and jejunum based on the different infection sites of *E. acervulina* as presented in Table 8 and illustrated in Figure 2. Chickens in the NC group had a score of 0 (100%) for all three intestinal sites assessed for damage, indicating no observed damage in this group and confirming that no cross-contamination occurred between the groups. Furthermore, for the three regions, the damage levels tended to decrease, with the NC group showing significantly higher intestinal damage scores (1.72-3.12) (p<0.001) compared to the other groups (0.92-1.95). The NC group exhibited the highest damage level, reflecting a high level of infection in the absence of intervention; the use of FC preparations in both the FC1 and FC3 groups reduced the damage levels in the duodenum, jejunum, and ileum by 57.4%, 34.7%, and 46.5% in the FC3 group and 37.5%, 42.9%, and 40.0% in the FC1 group, respectively, which were similar to the AB group.

The study results indicate that the supplementation of FC preparations, particularly in the FC3 group, had a negative effect on the duodenal mucosal morphology of chickens infected with *E. acervulina*. Overall, the duodenal mucosa of the NC group showed significant damage, with the villus height (VH) (1817.5 μm) and VH:CD (4.79) being significantly lower, and the crypt depth (CD) (379.6 μm) being significantly higher

Table 8. Lesion score and the intestinal histology (duodenum, jejunum, and ileum) of broiler chickens supplemented with fermented chive

Variables	Treatments					Pooled SEM	p-value
	PC	NC	FC1	FC3	AB		
Lesion score							
Duodenum	0^{d}	3.12^{a}	1.95^{b}	1.33^{bc}	1.44bc	0.47	<0.01
Jejunum	0^{d}	2.19^{a}	1.25^{b}	1.43^{b}	1.04bc	0.32	<0.01
Ileum	0^{c}	1.72^{a}	1.05^{b}	0.92^{b}	0.94b	0.27	<0.01
Intestinal histology							
Duodenum							
VH (μm)	2389.27^{a}	1817.52^{c}	2150.48^{b}	2190.63^{b}	2244.63^{ab}	150.12	0.015
CD (μm)	270.19^{c}	379.57^{a}	306.44^{b}	276.39^{c}	291.44^{c}	33.39	<0.01
VH:CD	8.45^{a}	4.79^{d}	7.02^{c}	7.93^{b}	7.70^{b}	0.71	<0.01
Jejunum							
VH (μm)	1303.44	1289.46	1291.92	1312.28	1316.33	86.12	0.119
CD (μm)	194.09^{a}	254.23^{b}	231.24^{ab}	201.06^{a}	196.17^{a}	31.09	0.092
VH:CD	6.72^{a}	5.07^{b}	5.51^{ab}	6.53^{a}	6.741^{a}	0.39	0.263
Ileum							
VH (μm)	942.02	916.18	936.16	920.77	926.56	68.04	0.213
CD (μm)	162.08^{b}	195.69^{a}	204.03^{a}	201.82^{a}	161.04^{b}	26.78	0.323
VH:CD	5.81^{a}	4.68^{b}	4.59^{b}	4.56^{b}	5.76^{a}	0.31	0.259

Note: In the same row, values with different lower-case letters (a-c) indicate statistically significant differences (p<0.05). PC (positive control): no FC supplementation, no *Eimeria acervulina* infection; NC (negative control): no supplementation, *E. acervulina* infection; FC1: 1% FC supplementation; FC3: 3% FC supplementation; AB: antibiotic treatment with Sulcox.

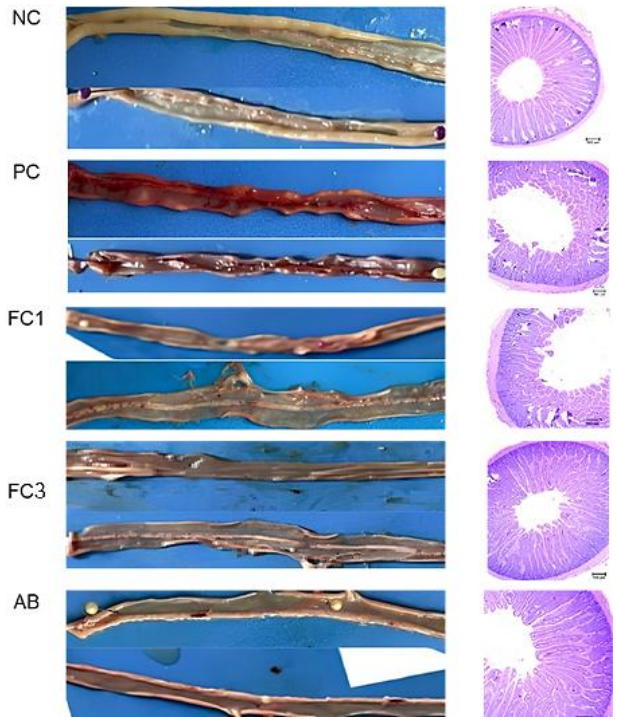

Figure 2. Illustrative images of small intestinal mucosal lesions and duodenal histopathology at 6 DPO showed severe lesions in chickens in the NC group and this was improved through lesion scores and more tightly and uniformly arranged villi in the FC1 group and especially the FC3 group (equivalent to the AB group) when supplemented with FC. NC (negative control): no supplementation, *Eimeria acervulina* infection; PC (positive control): no FC supplementation, no *E. acervulina* infection; FC1: 1% FC supplementation; FC3: 3% FC supplementation; AB: antibiotic treatment with Sulcox.

(p<0.05) compared to the other groups. In contrast, in the jejunum and ileum, only CD and VH:CD were significantly affected (p<0.05) compared to the other groups. Supplementation with 3% FC in the FC3 group improved VH (17.1%), VH/CD ratio (39.6%), and CD (27.2%) compared to the negative control (NC) group (p<0.05).

DISCUSSION

Productive Performance of of Broiler Chickens Supplemented with Fermented Chive

Eimeria infection causes severe intestinal damage, reducing nutrient absorption, which subsequently affects growth and production performance in chickens (Dalloul & Lillehoj, 2006). Previous studies have also indicated that coccidiosis impairs growth performance, energy metabolism, and intestinal structure in broiler chickens (Teng *et al.*, 2020).

The results of this study show that supplementation with FC, particularly in the FC3 group, significantly improved (p<0.05) body weight gain (BWG), feed intake (FI), and survival rate in chickens infected with *E. acervulina*. Overall, the FC3 group achieved performance similar to the antibiotic-treated group (AB), demonstrating the potential of FC as an antibiotic alternative in poultry production.

The improvement in weight gain may be related to enhanced palatability, better digestion, and nutrient absorption. Polyphenols, flavonoids, and organosulfur compounds in chives have antioxidant, antibacterial, and anti-inflammatory properties (Kothari *et al.*, 2020), helping to inhibit *E. acervulina* in the intestine. The fermentation process may enhance the biological activity of these compounds while reducing their

antinutritional effects, thus optimizing growth performance (Ahmed *et al.*, 2016). Supplementing fermented preparations also helps balance the gut microbiota, supporting weight gain and improving meat yield (Ignatova *et al.*, 2009). However, some studies have not observed clear benefits of probiotics for poultry infected with coccidia (Khan *et al.*, 2019). In this study, although both FC3 and FC1 improved production performance, there was no significant effect on feed conversion ratio (FCR), likely due to the low feed consumption in the NC group caused by the disease. Nevertheless, the production efficiency index (PEI) of FC3 and FC1 was significantly higher than that of the NC group, confirming the role of FC in improving poultry productivity during *E. acervulina* infection.

Immune Function of Broiler Chickens Supplemented with Fermented Chive

The immune organ index reflects the immune status of chickens, with increased organ weight indicating better immune function and lower disease risk (Zhu *et al.*, 2023). The FC3 group showed a significant increase in this index, possibly due to the effects of the *Lactobacillus* probiotics in FC, which help stimulate lymphoid tissue and support immune function through the antioxidant activity of flavonoids (Malematja *et al.*, 2022). Fermentation of chives also improves gut health, enhancing immune function (Ayana & Kamutambuko, 2024). Additionally, polysaccharides from FC, combined with probiotics, may function as prebiotics, balancing the gut microbiota and stimulating immune response, similar to the findings of Sugiharto and Ranjitkar (2019). Although thymus weight did not change significantly, the increased weight of the bursa of Fabricius and spleen in the FC3 group suggests improved immunity.

Supplementing FC also significantly increased IgA and IgG levels in the serum, reflecting enhanced mucosal and systemic immunity (Macpherson & Uhr, 2004). In contrast, IgM levels showed no significant change, consistent with its short-term role in immune responses (Trojan *et al.*, 2014). The NC group had the lowest IgA and IgG levels, indicating immune suppression without supplementation, which increases the risk of intestinal infections.

Immune Gene Expression of Broiler Chickens Supplemented with Fermented Chive

This study confirms the negative role of herbal supplementation in regulating immune responses in chickens infected with *E. acervulina*. The supplementation helps modulate the expression of pro-inflammatory and anti-inflammatory cytokines, while improving the intestinal epithelium's integrity, as shown by the increased levels of tight junction proteins ZO-1 and Claudin-2 (Turner, 2009).

The herbal supplementation group showed decreased levels of IFN-γ and TNF-α, reflecting an anti-inflammatory effect similar to that of Ryu *et al.* (2010). Elevated IL-4 levels in both the FC (FC3) and non-fermented (FC1) groups indicate the activation of Th2 immune responses (Mosmann & Coffman, 1989), which is also a reason for the reduced inflammation in the FC-supplemented groups. Meanwhile, the NC group had the highest levels of IFN-γ and TNF-α, reflecting severe inflammation (Wang *et al.*, 2000).

Supplementing with FC significantly increased IL-4 (83.67% and 69.51%) and decreased TNF-α and IFN-γ (~41% and 20%), showing immune modulation effects comparable to antibiotics. Bioactive compounds such as saponins, flavonoids, and polysaccharides, along with *Lactobacillus*, help stimulate IL-4, promote B cell development, and inhibit inflammation in broilers (Galli *et al.*, 2021). Fermented herbs also improve beneficial microbiota and modulate immune responses (Hai *et al.*, 2024). These results indicate that herbal preparations have the potential to enhance immune health and protect chickens from coccidia infections.

Oocyst Excretion in Excrete of Broiler Chickens Supplemented with Fermented Chive

No oocysts were detected in the excretion of the PC group, while the FC-supplemented groups (FC1, FC3) and the AB group showed a trend of decreasing oocyst numbers over time, although still higher in FC1, possibly due to insufficient strength or synchronization through the pathogen's developmental stages. Notably, on day 11, the AB group had the lowest oocyst count (9.3), indicating effective control of *E. acervulina* in the later stages.

Fructans from allium species can stimulate beneficial bacteria and inhibit pathogenic bacteria, aiding in receptor competition and pathogen elimination through the digestive tract (Zhao *et al.*, 2022). Additionally, phenolic compounds, allicin, and tannins in FC may affect the cell membrane of *Eimeria*, weakening its function and killing the parasite (Jiang *et al.*, 2024). Allicin from chives also has antioxidant activity, stimulates immunity, and helps destroy sporozoites (Wlaźlak *et al.*, 2023). Probiotics in FC can prevent pathogens from adhering to the intestinal mucosa, creating a "competitive exclusion" effect (Halder *et al.*, 2024). *L. plantarum* helps inhibit *Eimeria* by enhancing antioxidant enzymes, tight junction proteins, and improving serum chemical composition (Mohsin *et al.*, 2022). Furthermore, lipopeptides from probiotics, such as surfactin, have anti-coccidial activity against *Eimeria*, inhibiting oocyst formation and reducing oocyst count in the excretion of infected chickens, directly affecting oocyst structure (Ahmad *et al.*, 2024).

Gut Health of Broiler Chickens Supplemented with Fermented Chive

The intestinal damage score is an important indicator of coccidiosis infection. Both FC1 and FC3 groups significantly reduced ($p<0.05$) the damage score compared to the NC group, indicating the efficacy of lactic acid bacteria and antibiotics in limiting the development of *Eimeria* and protecting the intestinal mucosa. These results are consistent with the findings of Ghaniei *et al.*

(2023), showing that fermented preparations, including herbal components, reduce intestinal damage caused by *Eimeria* in broilers, with efficacy similar to antibiotics, supporting intestinal mucosa protection.

The gastrointestinal tract is a complex system where maintaining structural and functional integrity is crucial for nutrient absorption, infection resistance, and overall physiological performance (Celi *et al*., 2017). In this study, FC supplementation not only protected the mucosa but also promoted recovery, as indicated by increased VH and decreased CD in chickens infected with *E. acervulina*. The FC3 group significantly reduced villus damage, showing performance comparable to the antibiotic-treated group, suggesting optimized nutrient absorption (Shang *et al*., 2015). According to Liu *et al.* (2020), high VH and shallow CD reflect the integrity of the mucosal structure, which helps improve digestive function. FC may support recovery by producing short-chain fatty acids like butyrate from *Lactobacillus*, which helps maintain intestinal epithelial integrity (Zhu *et al*., 2023). Moreover, bioactive compounds in chives can inhibit pathogenic microorganisms, modulate immunity, and reduce inflammation (Navidshad *et al*., 2018). Additionally, herbal extracts also have the potential to regulate the apoptosis process of epithelial cells and promote cell maturation (Zhang *et al*., 2013).

The improvement in gut health observed in this study may be related to the direct inhibition of *E. acervulina*, reduced intestinal damage, and the promotion of rapid recovery in FC. Although the specific mechanisms by which herbal extracts improve the intestinal microstructure are not fully understood, a plausible hypothesis is that the herbal mixture may influence the process of epithelial cell renewal and support the maintenance of the intestinal barrier. The study results suggest that the herbal mixture improves intestinal morphology, thereby enhancing nutrient absorption efficiency. Higher VH:CD ratios may reduce the need to maintain intestinal structure, thus allowing more energy to be allocated to growth (Teng *et al*., 2020). These mechanisms may explain how FC mitigates the negative impact of *E. acervulina* on broiler growth. Furthermore, maintaining the integrity of the intestinal structure is closely related to the stability of the intestinal immune system (Ceylan *et al*., 2025).

Although the present study was conducted under controlled thermal conditions, the findings hold significant implications for poultry farming in tropical regions where heat stress represents a major challenge. Heat stress is known to impair immune function, increase susceptibility to diseases such as Coccidiosis, and decrease productivity (Hirakawa *et al*., 2020). Notably, heat stress can compromise intestinal barrier integrity, thereby increasing pathogen translocation (Zhang *et al*., 2017). Our results demonstrate that supplementation with 3% FC improved immunoglobulin levels, modulated immune gene expression, and enhanced gut health, which may help mitigate these adverse effects. These benefits could potentially support the maintenance of productivity and disease resistance in broiler chickens under heat-stress conditions. Future research should focus on evaluating the efficacy of fermented chives under high ambient temperatures to further validate this potential.

CONCLUSION

Supplementation with 3% *L. plantarum* 1582-fermented chives (FC3) significantly improved body weight gain, feed intake, and survival rate in *E. acervulina*-infected broiler chickens, matching the performance of antibiotic-treated birds. FC3 enhanced immune function by increasing IgA and IgG levels, upregulating tight junction proteins (ZO-1, Claudin-2), and modulating cytokine expression (increased IL-4, reduced TNF-α, IFN-γ). It also strengthened intestinal integrity by improving villus height and reducing lesion scores in the duodenum, jejunum, and ileum. Furthermore, FC3 significantly reduced *E. acervulina* oocyst output in excretion, demonstrating effective coccidiosis control. These results indicate that 3% FC is a viable antibiotic alternative for controlling coccidiosis, promoting sustainable broiler production.

CONFLICT OF INTEREST

We certify that there is no conflict of interest with any financial, personal, or other relationships with other people or organization related to the material discussed in the manuscript.

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