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Study on the preventive and curative effect of plant extract combined with probiotics on Eimeria chicken

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Abstract
The aim of this study was to investigate the effect of plant extracts combined with probiotics on the prevention and treatment of Eimeria tender infection in chicken. A total of 180 12-day-old coccidia-free chicks were randomly divided into 6 groups with 30 chickens in each group. The experimental groups were as follows: group A (blank control group), Group B (positive control group) (infected with coccidia without adding anti-coccidia drugs), group C (probiotic group), group D (plant extract test group), group E (probiotic + plant extract test group) and group F (dickezulil group). Except the blank control group, 5×10^4 oocysts of Eimeria teniculare infected orally at the age of 14 days, respectively. From the day of attack, groups A and B were fed a basic diet without anticoccidial drugs, groups C, D and E were fed diets containing corresponding additives, and group F was fed dickezulil in drinking water. The results showed that the addition of probiotics + plant extracts was superior to the addition of probiotics and plant extracts alone in the drinking water group to increase body weight, reduce cecal lesions and reduce the number of oocysts per gram of cecal contents of infected chicks, and the anticoccidial index was 160.91, which could achieve a good anti-coccidioides effect 160≤ACI < 180. It is concluded that plant extract combined with probiotics has a good effect on preventing and treating Eimeria teniculata.

Keywords: Coccidia, compound plant extract, probiotics, and coccidia-resistant index

1. Introduction
Coccidiosis of chicken is one of the important diseases that seriously harm the development of chicken industry. According to research, the global chicken industry suffers billions of dollars of economic losses every year due to chicken coccidiosis, mainly reflected in the increase of chick mortality, decreased growth performance and [1] reduced feed returns. Traditional chemicals such as diczulide are widely used in the control of chicken coccidiosis. However, many studies have shown that long-term use of chemicals leads to an increasing problem of coccidiosis resistance, and drug residues can also pose a threat to food safety.
With the increasing attention to food safety and environmental protection, the search for green and safe strategies for chicken coccidiosis control has become a research focus. Plant extracts and probiotics have attracted much attention due to their natural, low toxicity and no residue properties. It has been proved that the natural plant extracts involved in the field of anti-coccidia mainly include saponins, alkaloids, polyphenols, terpenoids, flavonoids and volatile oils [2, 3, 4, 5]. The mechanism of the anti-coccidian effect of these plant extracts is that they can inhibit the spore formation and proliferation of coccidian and the infection process of the body, and then reduce the number of coccidian in feces. At the same time, they also have multiple effects such as protecting the intestinal mucosa, enhancing the immune capacity of the body and improving the growth performance of animals, and finally realize the purpose [6] of anti-coccidian. Probiotics can regulate intestinal micro ecological balance and enhance body immunity. This study will explore the effect of plant extracts combined with probiotics on chicken eimeria, and provide new ideas and methods for the green prevention and control of chicken coccidiosis.

2. Materials and Methods
2.1 Test strain
Bacillus subtilis, 200 billion bacteria per gram, effective substance content 99.0%; Lactobacillus rhamnosus, 100 billion bacteria per gram, effective substance content 98.0%.

Yeast, 20 billion bacteria per gram, effective substance content up to 99.0%. Before the test, activate the probiotics, and dilute the mixture to 10× the use concentration, set aside.

2.2 Test the plant extracts
The plant extract consists of carvacrol, thymol, tannic acid, saponin, Astragalus polysaccharide, etc., purchased from a biotechnology company Limited. 0.5% Dicazuril solution was supplied by Huasao Biotechnology Co., LTD.

2.3 Coccidium oocysts
Eimeriatenella strains were provided by the laboratory of Hunan Agricultural University and stored in the Disease diagnosis Center of Hunan Bio-Mechanical and Electrical Vocational Technical College. The sporoidized oocyms were obtained from chickens artificially infected before the experiment, counted by Machella egg counting plate, and stored in 2.5% potassium bichromate solution in a refrigerator at 4 ℃ for later use. Before attack, Eimeriatenella strains were cultured according to 5×10^4 sporified oocysts/one inoculated chicken body.

2.4 Experimental animals
12-day-old egg-laying chicks provided by a farm in Hengyang, Hunan Province, have never received any immunization against coccidia. The chickens were given a basic diet free of anticoccidial drugs and antibiotics, and were guaranteed free access to food and water.

2.5 Experimental instruments and equipment
BM2000 microscope (Menji Biotechnology Co., LTD.); KDC-6000R Low speed refrigerated centrifuge (Guangzhou Jingying Chemical Technology Co., LTD.); GMB302 Electronic Balance (Zhuhai Tianchuang Instrument Co., LTD.); BKQ-B75 Autoclave (Beijing Rongxing Guangheng Technology Co., LTD.); Mackeria egg counting plate (Xiamen Keyi Instrument Co., LTD.); Potassium bichromate.

2.6 Experimental Methods
2.6.1 Grouping and treatment of experimental animals
A total of 180 12-day-old chicks were randomly divided into 6 groups (groups A, B, C, D, E and F) with 3 replicates per group and 10 chicks per replicate. Except group A (blank control group), all chicks in the other 5 groups (groups A, B, C, D and E) were orally inoculated with 1ml of spore Eimeriatenella oocysts (diluted concentration of 2×10^4 oocysts /ml) at 14 days of age.

The negative control group was given the same amount of normal saline. Since the day of insect attack, group A was the blank control group and 200μL PBS was given daily, group B was the single control group, group C was given 200μL probiotics per day, and group D was fed the basal diet supplemented with plant extract (500g/t). Group E was given compound probiotics 200μL+ plant extract in the basal diet (500g/t), and group F was administered with $0.2mL\bullet L^{-1}$ of dicranzulil solution by water as the positive drug control group. For 8 consecutive days, the death and clinical symptoms of the chicks were observed, and the fecal oocyst number (OPG), survival rate and relative weight gain rate were counted. The chicks were killed at 21 days of age, and the cecum was taken to observe the pathological changes.

Table 1: Treatment of experimental chickens

Groups	Treatment (treatment)
A	14 to 21 days of age, fasting administration of normal saline (200μL/ only)
B	14 to 21 days of age, fasting administration of normal saline (200μL/ only)
C	14 to 21 days of age basal diet supplemented with plant extracts (500g/t)
D	14-21 days of age fasting administration of complex probiotics (200μL/ only)
E	Fasting administration of complex probiotics (200μL/ animal) + basal diet supplemented with plant extract (500g/t) for 14 to 21 days of age
F	Add 0.5% dicazuril ($0.2mL\bullet L^{-1}$) to drinking water for 14 to 21 days of age

2.7 Detection of relevant indexes in the test
2.7.1 Blood stool score
The clinical manifestations and the degree of blood stool in the stool of the test chickens were observed every day during the experiment. The score of blood stool in each group was calculated by referring to the Soxun scoring system, and the score without blood in stool was 0; 1% ~ 25% feces with blood count 1 point; 26% to 50% fecal blood count 2 points; 51% to 75% fecal blood count 3 points; 76% to 100% fecal blood counts as 4 points.

2.7.2 Survival rate
The number of chickens killed by coccidia infection in each experimental group was counted during the experiment, and the survival rate was the ratio of the number of surviving chickens in each experimental group to the total number of chickens in each group.

2.7.3 Weight gain index: Chicks were weighed on an empty stomach at 14 days of age and 21 days of age, respectively, with the initial weight recorded at 14 days of age and the final weight recorded at 21 days of age, and the relative weight gain rate was calculated. Average weight gain = average final weight - average initial weight, relative weight gain rate = average weight gain of chicks in each group/average weight gain of negative control group × 100%.

2.8 Score for cecal lesions
Reid and Johnson scoring methods were used to calculate cecal lesions in the test. There were 5 grades of cecal lesions, the degree of which gradually deepened, corresponding with 0-4 scores respectively. The specific evaluation methods are shown in the table
2 as shown in Table 2. Cecal lesion value = average cecal score of chicks in each group ×10.

Table 2: The standard of Cecal lesion score

Scoring Criteria	Score value/points
Pathological changes of cecum were not obvious when observed by naked eye	0
The cecal wall was not thickened and the contents were normal	1
The number of intestinal wall bleeding is large, the contents are obviously bloody, and the cecal wall is slightly thickened, and the contents are normal	2
The cecum contained a large amount of blood, blood clots, and a banana-like grayish white cheese. The wall of the intestine was significantly thickened, and the fecal content in the cecum was less	3
The cecum is filled with a large amount of blood and the core is swollen, and the core contains fecal residue or no fecal residue, including dead chickens	4

2.9 Fecal oocysts (OPG count)

According to McMaster's improved method, feces from each group were collected from the 4th day after administration of sporized oocysts of Eimeria tenuis until the end [7] of the experiment. The feces of each group were thoroughly mixed, 2g of feces to be tested were put into beaker, 10mL of deionized water was added first, followed by 50mL of saturated salt water, and 1mL of feces were absorbed after mixing. It was injected into the McMaster counting plate and left for 5min. The number of eggs in the two calibration chambers was counted under the microscope. The average number of eggs in the two calibration chambers ×200 was the number of eggs in each gram of feces. Oocyst ratio = OPG in the medication group/OPG in the infection group. The calculation criteria of oocyst value are shown in Table 3[8].

Table 3: Oocysts valuecal culation method

Oocysts ratio /%	Oocyst value
0% to 1%	0
1% to 25%	5
26% ~ 50%	10
51% to 75%	20
76% to 100%	40

2.10 Coccidia Resistance Index (ACI)

ACI is an important index to evaluate the ability of various substances to resist Eimeria. In order to evaluate the effect of adding plant extracts to treat coccidia infection in chickens, ACI was introduced. ACI (Anti-coccidia index) = (relative weight gain rate + survival rate) - (egg sac value + lesion value). Relative weight gain rate = Weight gain of chicks in each experimental group/weight gain of chicks in the blank control group ×100%; Survival rate = number of chicks alive in each group/total number of chicks in each group ×100%; Lesion value = cecal lesion score of chicks ×10; Egg sac value calculation method: ratio of egg sac of chicks (%) = number of egg sac of chicks in blank control group/number of egg sac of chicks in each experimental group ×100%.

Table 4: ACI Evaluation Standards

ACI	Evaluation criteria
<120	Cannot achieve the effect of anticoccidia
120≤ACI < 160	Achieve moderate coccidium-resistant effect
160≤ACI < 180	Achieve good coccidioid resistance
ACI > 180	To achieve excellent coccidiosis resistance

2.11 Data statistics and analysis

The test data were expressed as "mean ± standard deviation", and SPSS 26 software was used for statistical analysis. p>0.05 meant no significant difference, p<0.05 meant significant difference, and p<0.01 meant extremely significant difference.

3. Results and Discussion

3.1 Clinical symptoms and survival rate

On the 5th day after infection with coccidia, the chicks of the healthy control group and the chemical control group showed no obvious clinical symptoms, and the chicks of the other experimental groups showed different degrees of clinical symptoms such as appetite loss, lethargy, bloody stool and so on. In the coccidia-infected group, chickens began to appear sporadic bloody stools on the 4th day after coccidia-infected day, the bloody stools were the most serious on the 5th day, and the symptoms of bloody stools were reduced or disappeared on the 6th to 8th day. The blood stool conditions of the groups supplemented with probiotics, plant extracts and both were lower than those of the coccidia-infected group. The blood stool scores of each experimental group were shown in Table 5. In the coccidia-infected group, 2 chickens died in the whole test period, and the survival rate was 93.3%.

Table 5: Blood fece scores of each experimental group

Groups	Score for blood stool after infection				Average Blood Stool scoring
	Day 4	Day 5	Day 6	Day 7	
A	0	0	0	0	0
B	1.00	3.00	2.00	2.00	2.00
C	0	1.00	2.00	1.00	1.00
D	0	1.00	2.00	1.00	1.00
E	0	1.00	1.00	0	0.50
F	0	0	0	0	0

3.2 Weight change results

The results showed that the body weight of chicks in group A (blank control group) was the largest, and the difference between group E (probiotic group + plant extract) and group E (probiotic group + plant extract) was significant (p< 0.05), and the difference between group B (single control group), group C (probiotic group) and group D (plant extract group) was extremely significant (p< 0.01). The body weight of chicks in group E (probiotic group + plant extract) was significantly higher than that in group C (probiotic group) and group D (plant extract group) (p<0.05), and was significantly different from that in group B (p<0.01). There was no significant difference in the body weight of chicks between groups C and D (p>0.05), but there was a significant difference between group C and group B (p<0.05). As shown in Table 6.

Table 6: Chickens weight in each group after coccidiosis vaccination

Groups	14 days of age first weight Average initial weight/g	21 days of age end weight Average final weight/g	Average weight gain (g) Relative weight gain/g	Relative weight gain rate Relative weight gain rate/%
A	100.24±2.39	178.67±9.39^{a}	78.43	100
B	101.30±1.89	146.62±10.95^{c}	45.32	57.78
C	100.98±0.37	154.38±7.85^{bc}	53.40	68.09
D	102.40±1.18	159.36±8.23^{bc}	56.96	72.63
E	103.54±0.89	168.88±8.78^{ab}	65.34	83.31
F	100.91±0.56	172.12±9.65^{a}	71.21	90.79

Note: There was no significant difference in the same column with the same lettering (p>0.05); There were significant differences among those with the same part of the shoulder marker (p<0.05), and extremely significant differences among those with different shoulder marker (p<0.01), and the whole text was the same.

3.3 Results of pathological changes of cecum

At the early age of 22 days, all chicks were slaughtered and the appearance and contents of cecal lesions were observed. The lesion values were calculated according to the observation and analysis of cecal lesions. It was found that the cecal lesions in group B (single control group) were very serious, and cecal puffiness, swelling, and blood clot and embolism were present. The degree of cecal lesions in group E (probiotic group + plant extract group) was lower than that in group C (probiotic group) and group D (plant extract group) (p< 0.05). There were a few hemorrhagic spots on the wall of cecum in group E (diczulil control group), and no other obvious lesions. The cecum lesions in groups C and D were less severe than those in group B (p<0.05), and the cecum lesions in the probiotic group, the plant extract group and the probiotic group plus plant extract addition group were reduced compared with the single insect attack group. As shown in Table 7.

Table 7: Cecal lesion scores

Groups	Cecal lesion score	Lesion value
A	0^{a}	0
B	3.65±1.08^{b}	36.5
C	2.87±0.98^{ab}	28.7
D	2.75±0.45^{ab}	27.5
E	1.24±1.12^{c}	12.4
F	0.5±0.68^{a}	5

3.4 Results of egg sac values in cecum contents after coccidia inoculation

At the 22nd day of age, all chicks in each group were slaughtered, cecal contents of chicks in each group were taken, and oocyst values were counted. Results The oocyst value in the contents of group E (probiotic group + plant extract addition group) was lower than that in group C (probiotic group) and group D (plant extract addition group), and the oocyst value in group F (dickezulil control group) was the least. The oocyst values in probiotic group, plant extract group and probiotic + plant extract group were all lower than those in single attack group (p< 0.05). The values were shown in Table 8.

Table 8: The value of oocysts in cecum of each group chickens

Groups	OPG value (x 10^5)	Oocyst ratio	Oocyst value
A	0.00±0.00^{c}	0.00	0
B	12.02±0.28^{a}	100.00	40
C	6.88±0.84^{b}	57.24	20
D	6.15±0.53^{b}	51.16	20
E	5.24±0.84^{b}	43.59	10
F	0.57±0.18^{c}	4.74	5

3.5 Test the ACI results of each group of chicks

According to the statistics of the above indexes, the anti-coccidius index of each group was calculated. The results showed that the ACI of the probiotic group plus plant extract addition group (group E) was higher than that of the C group (probiotic group) and the D group (plant extract addition group), and the ACI of the D group (plant extract addition group) could reach a medium level (120≤ACI < 160). The ACI of E (probiotic group + plant extract addition group) could reach a good level (160≤ACI < 180), but was lower than that of decazuril group (ACI > 180). See Table 9.

Table 9: Anticoccidial index

Categories	Survival rate	Relative weight gain rate	Egg sac value	Lesion value	ACI
A	100	100	0	0	200
B	93.3	57.78	40	36.5	74.58
C	100	68.09	20	28.7	119.39
D	100	72.63	20	27.5	125.13
E	100	83.31	10	12.4	160.91
F	100	90.79	5	5	180.79

4. Discussion

Coccidiosis is one of the more serious diseases in the chicken industry, and it often spreads in a fulminant fashion [9, 10, 11]. The United States Department of Agriculture has listed it as one of the five most serious diseases affecting poultry. Eimeria tenuis is a proto [12] zoa disease caused by E. Tenella parasitizing the chicken cecum. The disease is widespread throughout the world and mainly affects chicks between 2 and 9 weeks of age. The incidence of the disease ranges from 50% to 70%, and the case fatality rate is generally 20% to 30%, and in severe cases, the case fatality rate can exceed 80% [13].

In this experiment, the plant extracts used were made of carvacrol, tannic acid, saponin, Astragalus polysaccharide and other plant extracts. Many studies have shown that plant additives can be used as an effective substitute for anti-coccidian drugs in broilers. Lee *et al.* [14] also conducted a related study. They added 60 g/t and 120 g/t carvacro-based and thymol groups to broiler diets, respectively, and the results showed that the plant extract could successfully

counteract the growth inhibition induced by coccidiosis vaccine in broilers, and at the same time improve the intestinal status of broilers. Galli *et al*. [15] added 100 g/t carvacrol, thymol and other components to the diet of broilers, and found that the experiment proved that the addition could effectively reduce the excretion of coccididium in the feces of broilers. After Tonda *et al*. [16] added 100 g/t tannic acid to the diet of broilers, the feed to gain ratio of broilers was improved, and the immunity and disease resistance of broilers were also improved. Bafundo *et al*. [17] added 250 g/t and 500 g/t saponins to the diets of broilers vaccinated with coccidiosis vaccine, and the results showed that the addition can improve the production performance of broilers, improve carcass quality, and significantly reduce the mortality and coccidiosis disease score. Felici *et al*. [18] found through *in vitro* experiments that carvacrol, thymol and saponin could not only effectively inhibit the infection of M. eimeria spores to MDBK cells, but also had a synergistic effect among these components.

Yin *et al*. [19] 's study confirmed that Astragalus polysaccharide can improve intestinal health, enhance immunity against coccidia, improve antioxidant properties and other functions, as well as induce feeding and improve nutrient digestibility, thus improving the production performance of chickens. Cao Jianwen *et al*. [20] reported that although the addition of probiotics only showed an ineffective anti-coccidia effect, it improved the pathological changes of cecum induced by E.terella, and significantly increased the survival rate and average weight gain of infected chickens.

In this study, plant extracts combined with probiotics showed certain effects on the prevention and treatment of E. eimeria in chickens. The synergistic effect of probiotics and plant extract was better than that of single probiotics or plant extract group in alleviating blood stool symptoms, reducing cecum lesion degree and reducing oocyst value. This may be because probiotics regulate intestinal microecological balance, inhibit the growth of harmful bacteria, and create a more favorable intestinal environment for plant extracts to play the anti-coccidioid role. At the same time, many components in plant extracts, such as carvacrol, thymol and tannic acid, play their roles in different ways, such as inhibiting the growth and reproduction of coccidia and regulating the body's immunity, respectively. The combination of the two has a synergistic effect.

However, compared with diczulil control group, there was still a gap in the improvement of chick weight and coccidiosis resistance index in the plant extract combined with probiotics group. This may be due to the fact that diczulide, as a chemically synthesized anticoccidial, can rapidly and directly inhibit the physiological activities of coccidids, while plant extracts and probiotics play a relatively slow role and the mechanism of action is more complex. Carvacrol and thymol can interact with cell membranes to change the permeability of cations to cell membranes.

As a result, cellular components leak out, causing an imbalance of water in the cell, membrane potential collapse, and inhibition of ATP synthesis, leading to cell death. During coccidia development, carvacrol and thymol interfere with the sporogenesis of oocysts, alter the morphology and size of oocysts, and reduce the number of sporangium. Tannic acid can penetrate the wall of coccidium oocysts, destroy the cytoplasm of coccidium, and inactivate the endogenous enzymes of coccidium oocysts. Saponins will combine with 4-sterol molecules on the cell membrane of coccidia, disturb the lipid of the cell membrane of coccidia, affect the enzyme activity and metabolism of coccidia, and eventually lead to cell death, effectively prevent the sporozoite from invading intestinal epithelial cells and the sphaera from entering the schizozoon stage [6]. All of these plant extracts can reduce the number of coccidids in the stool and alleviate intestinal damage.

At present, the problem of drug residue and drug resistance is increasingly serious, plant extracts combined with probiotics as a green prevention and control strategy of chicken coccidiosis has broad development prospects. Follow-up studies should focus on optimizing the formulation and application of plant extracts and probiotics, as well as expanding the situation of multi-coccidiosis co-infection, in order to more comprehensively evaluate the anti-coccidiosis effect of plant extracts combined with probiotics. To provide more effective technical support for the healthy and sustainable development of chicken industry.

5. Conclusion

The results of this study showed that the addition of probiotics combined with plant extracts was superior to the addition of probiotics and plant extracts alone in increasing body weight, reducing cecal lesions, and reducing oocysts per gram of cecal contents, and the obtained anti-coccidioid index was 160.91, which could achieve good anti-coccidioid effect.Plant extracts have broad prospects in the application of anti-coccidia in animal husbandry.

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