Effect of Gan Cao (*Glycyrrhiza uralensis Fisch*) polysaccharide on growth performance, immune function, and gut microflora of broiler chickens

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ABSTRACT *Glycyrrhiza uralensis Fisch*, also called Gan Cao, is a commonly prescribed herb in traditional Chinese medicine. Gan Cao is associated with immunemodulation and antitumor potential though its mechanism of action is not well-known. To explore the effects of different dietary levels of Gan Cao polysaccharide (**GCP**) on broilers, a total of 400 Avian broiler chickens were randomly divided into 4 groups with 10 replicates of 10 broilers each. The broilers in the control group were fed a basal diet, while those in the experimental groups were fed the basal diet supplemented with 0.5%, 1.0%, and 1.5% GCP, respectively, for 42 d. The results showed a significant increase in the growth performance in the GCP groups. The antibody titer of NDV and the phagocytosis index was higher in the birds with GCP treatment than in the control group, with the 1% GCP addition displaying the highest titer. The *Lactobacillus* and *Bifidobacteria* count in the cecum content of the birds in the 1% GCP group was higher compared to the other groups. In conclusion, dietary supplementation with GCP had a substantial impact on the growth performance, immune response, and microflora population in the cecum of the birds, especially at a level of 1% addition.

Key words: broiler, *Glycyrrhiza uralensis Fisch*, polysaccharide, growth performance, immunology

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INTRODUCTION

A variety of polysaccharides have been derived from different species of plants that elucidate the majority of functional activities. These plant polysaccharides display many biological functions, including improving growth performance and exhibiting antitumor, antibacterial, antioxidant, hypolipidemic, and hypoglycemic properties (Fiorito et al., 2018). In addition to the above functions, numerous investigations suggest that polysaccharides can enhance immune function. They can activate immune cells, regulate the production and secretion of cytokines, increase the proliferation of B lymphocytes and T lymphocytes, and increase the level of antibody production (Wang et al., 2014; Ayeka et al., 2016). Other study also confirmed that polysaccharides have immunomodulatory properties that enhance the function of macrophages (Wang et al., 2018). It was demonstrated that plant polysaccharides are relatively nontoxic and do not cause any significant side effects. Many additional pharmacological effects have been discovered as a result of ongoing polysaccharide research, which is attracting increasing interest in the fields of food and feed additives, as well as in the health care products industry (Li et al., 2020; Song et al., 2021).

China, being the birthplace of Gan Cao (*Glycyrrhiza uralensis Fisch*), has an abundance of it. In the past 10 year, Gan Cao has been widely used in commerce and medicine, and a lot of research has been done on active ingredients such as Gan Cao acid and Gan Cao flavonoids (Alagawany et al., 2019). Gan Cao polysaccharide (**GCP**) belongs to the α-D-pyran polysaccharide family, which is the principal active component of Gan Cao. A large number of studies have been performed over the years to assess GCP's functions, such as antitumor, antibacterial, antioxidation, and immune regulation (Hodgins et al., 2015; Ayeka et al., 2017; Lian et al., 2018; Wu, 2018).

Drug residue, poor disease resistance, limited immunological function, and susceptibility to external stressors are all issues that arise during the broiler industry's development phase. As a result, strengthening the body's immunity is significant for antipathogen infection and vaccination immunity. Many researchers have been exploring new feed additives which have immune enhancement functions (Vorwerk et al., 2004; Wu et al., 2017). However, there are few studies on the effect of

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GCP as an immunomodulator on the immune function of broilers.

According to its critical immune regulated function, we hypothesized that dietary supplementation with GCP may present beneficial consequences on the growth performance and immune function of broilers. In this study, we used a well-documented model to explore the effects on the immunity and growth performance in broiler chickens by different levels of dietary GCP supplementation. The data collected in this study will add to our existing understanding of plant extracts and identify a potential feed additive for the poultry industry.

MATERIALS AND METHODS

Ethical Statement

Experimentation with animals was approved by the Experimental Animal Management Methods of Xinxiang Medical University (Approval number 201206078) and followed Henan Authority's Experimental Animal Regulations.

GCP Preparation

Experimental Design, Animals, and Management A total of 400 1-day (d) old Avian commercial female broilers (Dayong Broiler Breeding Corp., Xinxiang, China) were used in the current study. The birds were randomly distributed into 4 treatments groups, with 10 replicate cages in a completely randomized design for a 42-d feeding trial. The birds were fed a basal diet supplemented with 0 (control group), 0.5%, 1%, and 1.5% GCP (Wouterlets, Lanzhou, China), respectively. The dosage of GCP was selected according to previous studies (Zhang et al., 2021).

All the birds were housed in electrically heated cages and exposed to 23 h of light and 1 h dark at first 2 wk and then 20 h light and 4 h dark in rest time with free access to food and water. The birds were kept at 33 to 34°C for the first week. Then the temperature was decreased by 2 to 3°C per week till 24°C was attained. This research lasted for 42 d. The ingredient compositions of the basal diet are shown in Table 1.

Vaccine and Serum Collection

The birds were administered the Newcastle disease (**ND**) virus vaccine at 1d (intranasal) and 14 d (intramuscular) of age. At 1 d, 21 d, and 42 d of age, 3 broilers from each cage were randomly selected and bled from the wing. These blood samples were kept at 37°C for 30 min for clotting and then rapidly centrifuged at 3,000 r/min for 10 min. The prepared serum was stored at −20°C for subsequent antibody titer detection.

Table 1. Composition of basal diet.

Composition	0−3 w	4−6 w
Corn (%)	52.50	58.59
Soybean meal (%)	38.70	32.90
Soybean oil (%)	4.60	4.54
Calcium hydrophosphate (%)	1.80	1.55
Mountain flour (%)	1.30	1.20
Salt (%)	0.35	0.35
Trace element premix (%)[a]	0.25	0.25
Multivitamin (%)[b]	0.03	0.03
Methionine (%)	0.10	0.08
Antioxidant (%)	0.03	0.03
Choline chloride (CC) (50%)	0.20	0.16
Metabolic energy (MJ/Kg)[c]	12.35	12.56
Crude protein (%)[d]	21.10	19.02
Calcium (%)	1.06	0.95
Available phosphorus (%)	0.45	0.40
Lysine (%)	1.10	0.96
methionine (%)	0.46	0.38

[a]Provided the following per kilogram of diet: vitamin A, 1500 IU; vitamin D, 3200 IU; vitamin E, 10 IU; vitamin K, 0.5 mg; vitamin B12, 0.01 mg; D-biotin, 0.15 mg; folic acid, 0.55 mg; niacin, 30.00 mg; D-pantothenic acid, 10.00 mg; pyridoxine, 3.50 mg; riboflavin, 1.80 mg; thiamine, 1.80 mg.

[b]Fe, 80.00 mg; Cu, 8.00 mg; I, 0.35 mg; Mn, 60.00 mg; Se, 0.15 mg; Zn, 40.00 mg.

[c,d]Measured value.

Growth Performance

The individual bodyweight of the chickens was determined on days 1 and 42. The feed intake by the groups was determined for each house. On the basis of the experimental data collected, the following basic parameters of production were calculated: average daily weight gain (**ADG**), feed intake (**FI**), and feed to gain ratio (**F/G**).

Antibody Titer

Antibody titers against NDV were measured by the hemagglutination inhibition (**HI**) method. The HI antibodies were then converted to log2.

Macrophage Phagocytosis Index

The phagocytosis index of the macrophage was performed by a slightly modified version of a previously reported method (Zhang et al., 2005). At14 d and 28 d of age, 2 broilers from each cage were randomly selected and injected colloidal carbon (drawing ink, Pelikan; 1 mL/Kg BW) intravenously. Two (T1) and 10 (T2) min after the injection, 0.2 mL blood was harvested from the wing and mixed with 2 mL sodium citrate, which was then centrifuged at 530 g for 5 min. The supernatant was used to measure the optical density (**OD**) at 600 nm. The chicken was then slaughtered, and the liver and spleen were weighed. The phagocytic index was calculated as follows: phagocytic index = body weight/(liver weight + spleen weight) × $K^{1/3}$. The carbon clearance (K) was calculated as follows: K = (lgOD1-OD2)/T2-T1. OD1 and OD2 were the optical density at 600 nm of the supernatant at T1 and T2, respectively.

Microflora in Cecum Analysis

The variation of microflora in the cecum was detected according to the previously reported method (Qorbanpour et al., 2018). On day 30, two broilers from each cage were randomly chosen and sacrificed. The contents of the cecum section were collected and weighted into a sterile centrifuge tube. After shaking for 30 min, 1 mL samples of the suspension were added into 9 mL normal saline for serial dilution at 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7}. Hundred microliter was then removed from the 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7} dilutions and added to the plating medium for microbial culture, which was incubated under anaerobic conditions at 37°C for 72 h. The Man Rogosa Sharpe (**MRS**) medium agar was used to culture *Lactobacilli*, Eosin Metilan-Blou (**EMB**) medium agar was used for *E. coli*, MacConky agar was used for *Salmonella*, and BBL was used for *Bifidobacteria*. The bacterial units were counted by a colony counter and adjusted to a 1 g sample.

Statistical Analysis

The experimental data were determined by one-way ANOVA analyses of variance using the general linear model (**GLM**) procedure of SPSS Statistics Version 20.0 (IBM Corporation. Somers, NY). The data were expressed as means ± standard error of mean (**SEM**). The differences between the groups were explored using the LSD multiple range test. The statistical significance was set at $P \leq 0.05$.

RESULTS

Growth Performance

The results of the impact of dietary supplementation of GCP on the weight gain of the broilers are presented in Table 2. It was demonstrated that feeding the broilers with diets containing different doses of GCP could increase their weight, and different doses of the polysaccharide have different effects on chicken weight gain. As displayed in Table 2, dietary GCP administration did not affect the feed intake (**FI**) ($P = 0.993$). However, the administration of 0.5% and 1% GCP tended to increase the average daily weight gain (**ADG**) and improve the feed to gain ratio (**F/G**) as compared to the control group throughout the entire experiment ($P < 0.001$). The highest ADG and F/G value was observed in the 1% GCP groups. These data suggest that GCP improves the broilers' growth performance.

Table 2. Effect of GCP supplementation on growth performance of broilers.

	GCP level (%)					
Items	0	0.5%	1.0%	1.5%	SEM	P-value
FI	82.15	82.00	82.36	82.45	0.495	0.993
BWG	43.11[a]	47.81[b]	50.48[c]	44.82[a]	0.801	<0.001
F/G	1.91[c]	1.76[a,b]	1.63[a]	1.84[b,c]	0.027	<0.001

BWG, body weight gain; F/G, Feed to gain ratio; FI, Food intake.
[a-c]Means that do not share similar letter in row are significantly different, $P \leq 0.05$.

Table 3. Antibody titers to Newcastle disease virus vaccine (log2).

	GCP level (%)					
Items	0	0.5	1.0	1.5	SEM	P-value
2d	2.74	2.72	2.75	2.74	0.029	0.997
21d	4.00[c]	6.40[a]	8.00[b]	7.75[b]	0.259	<0.001
42d	5.00[c]	8.2[a]	9.60[b]	8.4[a]	0.278	<0.001

[a-c]Means that do not share similar letter in row are significantly different, $P \leq 0.05$.

NDV Antibody Titer

The effect of the polysaccharide on the antibody titer of NDV was detected by the micro hemagglutination inhibition test. The hemagglutination inhibition titer (log2) of the broilers at 2 d, 21 d, and 42 d of age are presented in Table 3. A much higher antibody titer was observed in groups with GCP additive treatment than in the control group at 21 d and 42 d of age ($P < 0.001$). The optimal responses for antibody titer were found in the broilers fed with 1% GCP, which demonstrated the highest antibody titer both on 21 d and 42 d ($P < 0.001$). These data suggest that different amounts of dietary addition of GCP have different enhancement effects on the immunity of broilers.

The Phagocytosis of Macrophages

The carbon clearance test was used to observe the influences of GCP on the immune system in the chicken. The macrophage phagocytosis index at the age of 14 d and 28 d is shown in Table 4. It was demonstrated that broilers with different doses of polysaccharides showed an increase in the macrophage phagocytosis index compared to the control group. The highest phagocytosis index (9.48) at 14 d was observed in the group of 1% GCP dietary addition ($P < 0.001$). The same tendency was also observed at 28 d, and the highest phagocytosis index was (11.13) ($P < 0.001$). These data suggest that GCP can improve the phagocytosis ability of peritoneal macrophages in broilers.

Microflora in Cecum

The remarkable microbiota from the content in the cecum is displayed in Table 5. It was revealed that the

Table 4. Phagocytic index of macrophages in peripheral serum of broilers.

	GCP level (%)					
Items	0	0.5	1.0	1.5	SEM	P-value
14d	6. 67[a]	6.81[a]	9.48[b]	6.76[a]	0.207	<0.001
28d	7.32[a]	10.90[c]	11.13[b]	10.65[b]	0.344	<0.001

[a-c]Means that do not share similar letter in row are significantly different, $P \leq 0.05$.

Table 5. Microflora of caecum in broiler.

Items	GCP level (%) 0	0.5	1.0	1.5	SEM	*P*-value
Escherichia coli	8.52[c]	8.44[a,c]	8.15[a]	8.20[b]	0.086	<0.001
Salmonella	7.98[a]	7.75[b]	7.28[c]	7.42[c]	0.062	<0.001
Bifidobacterium	9.02[a]	9.24[b,c]	9.28[b,c]	9.13[a,c]	0.032	<0.011
Lactobacillus	8.56[a]	8.89[b]	8.96[b]	8.02[c]	0.071	<0.001

[a-c]Means that do not share similar letter in row are significantly different, $P \leq 0.05$.

enumeration of *E. coli, Salmonella, Bifidobacteria*, and *Lactobacilli* in the cecum was significantly different from that in the control group. *E. coli* and *Salmonella* decreased significantly in the GCP treatment groups, while *Bifidobacteria* and *Lactobacilli* increased in the GCP dietary addition groups ($P < 0.001$). Compared with the control group, the dietary supplementation of GCP at a 1% level displayed an optimal effect in regulating the cecum microflora. These data suggest that GCP can modulate the distribution of microbial population in the cecum.

DISCUSSIONS

Gan Cao is a common Chinese herbal medicine, widely used in the treatment of a cough, a weak spleen and stomach, gastric ulcers, and food poisoning. Multiple researches have shown that the main active substances of Gan Cao are polysaccharide, triterpenoid saponin, and flavonoids compounds. GCP can not only be used as a drug for disease prevention and control, but also as a feed additive in animal husbandry to enhance the growth performance of animals and improve immunity, and has broad application prospects. In this study, we proposed detailed evidence on the influence of Gan Cao polysaccharide (**GCP**) on the growth performance, antibody titer, macrophage phagocytic index, and caucus microbiota in broilers. The results of this study demonstrated that dietary supplementation of GCP improved growth performance, with the most pronounced results observed at a 1% GCP dosage. These findings are consistent with the previous findings of Zhang et al. (2021).

Macrophages play an important and indispensable role in the specific and nonspecific immunity of a body. Macrophages participate in the body's defense function, including destroying pathogens and eliminating foreign organisms and dead cells. The measurement of the phagocytic function of macrophages can reflect the level of the body's cellular immune function. A significant increase in the phagocytic index was observed in GCP-fed groups compared to the control group, especially in the 1% dietary addition group. It follows that GCP can increase phagocytic index of macrophages, which provides reference data for its application in immune regulation. Our results are consistent with previous research that found that macrophage-fed diets enriched with polysaccharides improved the phagocytic index (Wang et al. 2018).

In addition, it was found that the addition of GCP to the diet can increase the NDV antibody titer in broilers. The optimal responses for antibody titer were found in broiler fed 1% GCP. This high antibody titer after vaccination demonstrated that GCP make the broiler to create a humoral immune response in combating against infection. These data were in accordance with previous research that found that Bachu mushroom polysaccharides and Shenqi polysaccharides can increase the Newcastle disease antibodies titer. These data reconfirmed that polysaccharides can promote the production of immunoglobulins (Zhang et al., 2018). Thus, the addition of GCP to the diet can increase the NDV antibody titer and the phagocytosis index, which indicates that GCP is beneficial in enhancing the humoral and cellular immune function of broilers.

There are some individual differences in the type and the total number of bacteria in a chicken gut. Moreover, the bacterial community in the cecum is complex and age-dependent. In this study, 4 types of representative bacteria were detected in the cecum. Both *E. coli* and *Salmonella* belonged to Enterobacteriaceae, which is a pathogen in the animal gut and was reported to alter epithelial tight junction and gut barrier function (Ulluwishewa et al., 2011). It was reported that *Lactobacillus* strains could enrich the diversity of Lactobacillus flora in chicken jejunum and cecum by increasing the abundance and prevalence of Lactobacillus spp. inhabiting the intestine (Amit-Romach et al., 2004; Lan et al., 2004). Furthermore, *Bifidobacteria* and *Lactobacilli* can metabolize to produce organic acids such as lactic acid, acetic acid, propionic acid, and butyric acid, which can reduce the pH of the enteric cavity. The low pH could inhibit the growth of acid-intolerant pathogenic bacteria such as *E. coli* and *Salmonella* in the intestine (Clavijo and Florez, 2018; Lokapirnasari et al., 2019). In our research, the group with GCP exhibited a lower number of *E. coli* and *Salmonella* and a higher number of *Bifidobacteria* and *Lactobacilli* in the cecum contents. Our data were in consistent with previous research that AP (apple po) could promote the proliferation of B. longum. These data indicate that GCP benefits broilers by adjusting the enteric microflora balance and provide a further potential use in broiler health care.

In summary, our research found that dietary supplementation with GCP exert beneficial effects on the growth performance, antibody titer, phagocytosis index, and cecum microflora and the recommended addition dosage of GCP is 1% in broiler feed.

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DISCLOSURES

No part of this paper has been published or submitted elsewhere, and no conflict of interest exists in the submission of this manuscript. All the authors listed have approved the manuscript that is enclosed.

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