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Anticoccidial Effect of CS 32 Compounds Against *Eimeria tenella* Infection in Chickens

Kwangsik Jang[1,2#], Se Eun Kim[1,2#], Kyung Mi Shim[1,2], Hye Ji Park[1,2], Suk Kim[3], Wongi Min[3], Jin Cheol Yoo[4] and Seong Soo Kang[1,2*]

[1]Department of Veterinary Surgery, College of Veterinary Medicine, BK21 Plus Project Team, Chonnam National University, Gwangju 61186, Korea
[2]Biomaterial R&BD Center, Chonnam National University, Gwangju 61186, Korea
[3]Institute of Animal Medicine, College of Veterinary Medicine, Gyeongsang National University, Jinju 52828, Korea
[4]Department of Pharmacy, College of Pharmacy, Chosun University, Gwangju 61452, Korea

#These authors contributed equals to this study.

Corresponding
Seong Soo Kang, DVM, PhD
College of Veterinary Medicine, Chonnam National University, 77, Yongbong-ro, Buk-gu, Gwangju 61186, Korea

Phone : +82-62-530-2877
Fax : +82-62-530-2881
E-mail : vetkang@chonnam.ac.kr .

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Coccidiosis-causing *Eimeria* species are transmitted in poultry via the oral-fecal route and can lead to hemorrhagic diarrhea and mortality. This results in enormous economic losses in the poultry industry. Furthermore, its resistance to some currently used antibiotics is increasing. This has prompted the development of new alternative drug therapies that address the issue of chemical-free meat production. Effective management of infectious diseases in veterinary practice includes the induction of protective and adaptive immunity by treatment with an alternative agent. In this study, we evaluated the anticoccidial effects of dietary supplementation of Chosun University (CS) 32 compounds (0.1% and 1.0%) against *Eimeria tenella*, which was isolated and purified from the supernatant of culture broth of *Bacillus* strain (KCTC18250P), as well as its effect on the growth rate and feed efficiency in chickens. Overall, we observed a decrease in lesion scores and oocyte output in CS 32 compounds-treated chickens. We concluded that 0.1% CS 32 compounds displayed anticoccidial effects against *E. tenella* infection.

Key Words: CS 32 compounds, anticoccidial effect, *E. tenella*, chickens

INTRODUCTION

Coccidiosis in chickens is a serious intestinal disease caused by 7 different species of *Eimera* protozoan parasites, which leads to significant losses in the poultry industry (1). Its mode of transmission is widely reported as an oro-fecal route. A total of 8 sporozoites are released from the 4 sporocysts contained within each oocyst upon ingestion. These rapidly adhere to the intestinal epithelium of the host. The invasion begins with a limited number of asexual cycles and the merozoites rapidly amplify. Eventually, after the merozoites are sexually differentiated, the male gametes fertilize the female macrogametes to create oocytes that are excreted in feces (2). *E. tenella* infection can lead to the development of hemorrhagic diarrhea, subsequent weight loss, and often death (2). As an obligate intracellular protozoan parasite, *E. tenella* possess a complex life cycle that is completed within 7 days. During this period, it undergoes intracellular development permitting distinctive

intracellular stages to proliferate within the cecal epithelium (3). Drug residues in poultry products have become an increasing concern with the emergence of multiple drugs for coccidiosis control. Concomitantly, the development of drug resistance has created a growing pressure within the government and consumer groups to evaluate, regulate, and even ban these drugs (2). Vaccination has been used in coccidiosis control for a long time; however, its application has been limited due to factors related to cost, safety, and the need for farmers or veterinarians to have technical expertise in administering vaccines (4). Thus, developing alternative drug-independent control strategies for avian coccidiosis is imperative.

Chosun University (CS) 32 compounds are food supplements, which are isolated from the culture medium of a new strain of *Bacillus*, *Bacillus* spp. strain CS 32, with a molecular weight of 5697.6 Da as determined by tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (tricine SDS-PAGE) (5). Previous studies demonstrated the probiotic effect of CS 32 compounds as antibiotic substitutes (6). Probiotic-containing diets can modulate immunity and are used as alternatives to antibiotics in Korean native chickens (7). However, the effects of CS 32 compounds as an alternative supplement against *Eimeria* infection have not been evaluated *in vivo*. In this study, we investigated the anticoccidial effect of CS 32 compounds in chickens infected with *E. tenella*.

MATERIALS AND METHODS

Isolation and production of biologically active antimicrobial peptides from *Bacillus* spp. CS 32

A. Optimization of the fermentation process

Bacillus strain CS 32 (KCTC18250P), which was isolated from the traditional Korean food (Kimchi), was used in this experiment to isolate biologically active antimicrobial peptide (5, 6). The antimicrobial peptide production of the strain was optimized by several culture parameters, such as the carbon level, nitrogen source, and mineral content. The effect of various carbon sources on antimicrobial peptide production was determined by combining 1% yeast extract with supplements such as glucose, mannitol, starch, lactose, fructose, sorbitol, sucrose, and maltose (1% each) in the media. Fermentation was performed in 250 mL Erlenmeyer flasks containing 50 mL of media with steady shaking at 180 rpm. Subsequently, the influence of nitrogen sources on peptide production was determined by using medium containing 1% glucose as the carbon source, combined with supplements such as beef extract, malt extract, tryptone, yeast extract, oatmeal, soytone, and peptone (1% each). In addition, the percentage of carbon and nitrogen sources to peptide production was valued as an optimized medium, including the best carbon sources (1% or 2% glucose), and the best nitrogen sources (0.5% or 1% beef extract and peptone) (6).

B. Preparation of *Bacillus* spp. CS 32-producing antimicrobial peptides

Bacillus spp. CS 32 was cultured in an optimized medium (1% glucose, 0.5% beef extract, and 0.5% peptone) at 37℃ and 180 rpm for 60 h. The cells were separated by centrifugation (6000×g rpm, 30 min, 4˚C), and peptides were precipitated from the supernatant at 4˚C overnight with ammonium sulfate of 60% saturation. The precipitate was collected by centrifugation (6000×g rpm, 60 min, 4˚C), resuspended in 10 mM Tris-HCl buffer (pH 8.0) and dialyzed using a 1-kDa cutoff membrane (Merk Millipore, Burlinton, MA, USA). The crude extracts were applied to a Sepharose CL-6B column (2.2×116 cm) (GE Healthcare, Chicago, IL, USA) and next to a Sephadex G-50 column (1.5×70 cm) (GE Healthcare, Chicago, IL, USA), and eluted with the same buffer. Active fractions were pooled and concentrated using a YM1 amicon filter (Merck KGaA, Darmstadt, Germany) and stored at −20˚C (6).

The CS 32 compounds used as anticoccidial food were supplied in the standard diet for 10 days before infection with *E. tenella*. The chickens were given a supplemental diet until the experiment was over. Starter diets using corn and soybeans,

which are commonly used in Korea and include corn as the major cereal and soybean meals made of protein concentrate, meet the National Research Council nutritional requirements for broiler chickens (NRC, 1994). The formulated starter diets consisted of 53.44% corn, 33.65% soybean meal, 4.68% soybean oils, 4.16% corn gluten meal, and 3.57% other ingredients. The calculated analysis value of the diets was 3100 kcal/kg metabolizable energy, 22% crude protein, 1.10% lysine, 1.00% calcium, 0.87% methionine plus cysteine, 0.50% available protein, and 0.05% methionine

Animal and animal experiments

Male Korean native chickens and female Rhode Island Red chickens were mated and fertilized eggs were obtained. The chickens were hatched from those eggs at the National Livestock Research Institute (Daejeon, Korea). Each group of chickens was bred with a commercial anticoccidial agent-free broiler feed and had access to coccidian-free water. Continuous 24-hour artificial lighting was maintained during the experiment. The initial temperature on the first day was set at 31~32°C and lowered by 2°C per week until the experiment was completed. All the animal experiments conducted in this study were reviewed and approved by the Animal Ethical Committee of Gyeongsang National University (Authorization Number GNU-120615-C0022).

The 112-day-old chicks were divided into four groups of 28 chickens each, and then randomly divided into four subgroups; the subgroups were further subdivided into four additional subgroups. In total, 16 cages containing 7 chickens each were used. Each group was treated as follows: control, uninfected and untreated (n = 28); untreated, infected and untreated (n = 28); 0.1% CS 32 compounds, infected and treated with 0.1% CS 32 compounds (n = 28); 1.0% CS 32 compounds, infected and treated with 1.0% CS 32 compounds (n = 28).

The chickens did not receive any vaccinations during the experiment. All treatments were initiated 10 days before the infection and sustained throughout the experiment.

The chickens were infected with *E. tenella* (Korean isolate 291-7) (8) and *E. tenella* oocysts were spread into specific pathogen-free chickens. Before infection, the virulence of the incipient stock of oocysts was assessed. The feces of specific pathogen-free chickens were collected to purify the new oocytes. The oocysts were then cultivated in a 2% potassium dichromate solution in a water bath (30℃) for 48 h to induce sporulation. After sporulation, the oocysts were maintained at 4℃ for 4 weeks. Just before infection, the oocysts were cleaned by dipping them into a 5.25% sodium hypochlorite solution. Then, the oocysts were washed in sterile phosphate-buffered saline three times. The counted oocysts were diluted to 1×10^5 oocysts/mL, the final concentration of the sporulation. A 5 cm esophageal cannula with 5×10^4 sporulated *E. tenella* oocysts was inserted into the crop of ten-day-old chicks for oral infection. The number of oocysts per gram of feces (OPG) was calculated by the following formula:

OPG = oocyst count from days 6 to 10 p.i. × dilution factor × (fecal sample volume / counting chamber volume) / number of birds per cage

Evaluation of drug treatment efficacy

We evaluated the efficacy of CS 32 compounds as an anticoccidial food supplement using the following indicators: body weight gain (BWG) and lesion scores (9).

The chickens were weighed every 5 days during the experiment to analyze BWG, *i.e.* at day 10 pre-infection and at days 0, 5, and 10 post infection (p.i.).

Seven chickens from each group were randomly selected and sacrificed on day 5 p.i. to determine the lesion scores. The cecum of each chicken was evaluated and the severity of the lesions was scored as 1 of 5 ranks (between 0 and 4) based on

the epithelial color, fluid accumulation, and overall general appearance of the intestine (serosal thickness, mucosal erosion, dilation, and similar factors) according to the method described by Johnson & Reid (Table 1) (10).

A total of 112 chickens in 4 subgroups (28 chickens per group) were measured to obtain the BWG data (day 5 p.i.). After sacrificing 7 chickens from each group on day 5 p.i., the remaining 84 chickens were analyzed to obtain the OPG data for the 4 subgroups, each by day 10 p.i..

Table 1. Chicken lesion score technique for *E. tenella* infection by Johnson and Reid

Score	Description
0	No gross lesions
+1	Very few scattered petechiae on the cecal wall; no thickening of the cecal walls; normal cecal contents present
+2	Lesions more numerous with noticeable blood in the cecal contents; cecal wall is somewhat thickened; normal cecal contents present
+3	Large amounts of blood or cecal cores present; cecal walls greatly thickened; little, if any, fecal contents in the ceca
+4	Cecal wall greatly distended with blood or large caseous cores; fecal debris lacking or included in cores (Dead birds scored as +4)

Statistical analysis

A general linear model procedure of SAS software (SAS Institute, Inc., Cary, NC, USA) was used to statistically analyze the mean and analysis of variance for BWG, lesion scores, and oocyst excretion. After natural log transformation, we analyzed the group OPGs to modify the heterogeneity of variance and to obtain, approximately, a normal distribution data. For estimating the significance of the differences between the 4 groups, a Mood-Brown median test was used to analyze lesion scores and oocyst excretion.

Mean values ± standard deviation for the different groups were used as results of measured indicators at different time periods throughout the experiment. Using GraphPad Prism™ software (GraphPad Software, San Diego, CA, USA), differences were considered significant at $P < 0.05$.

RESULTS

Evaluation of BWG

We examined the effect of CS 32 compounds on *E. tenella* inhibition by BWG. There were no significant differences in BWG between the 4 groups from day 5 to day 10 p.i. (Fig. 1).

Evaluation of oocyst output

Fig. 2 shows the oocyst output evaluation of chickens infected with *E. tenella* from days 6 to 10 p.i. Of note, the value of OPG in the 0.1% CS 32 compounds group decreased by approximately 33% compared to that of the untreated group (Fig. 2). However, the value of OPG in the 1% CS 32 compounds group was higher than that of either the 0.1% CS 32 compounds group or the untreated group (Fig. 2). The patterns of oocyst output in the 0.1% CS 32 compounds and 1.0% CS 32 compounds groups differed from those in the untreated groups.

Evaluation of lesion scores

After infection, lesion scores in the 0.1% CS 32 compounds and 1.0% CS 32 compounds groups were higher than those in the uninfected group; however, the 0.1% CS 32 compounds group was significantly lower than the untreated group when compared to the increasing pattern seen in the 1% CS 32 compounds group (Fig. 3). Similar to the results of oocyst output, significant differences were found in lesion scores between 0.1% CS 32 compounds and 1.0% CS 32 compounds groups before infection (Fig. 3).

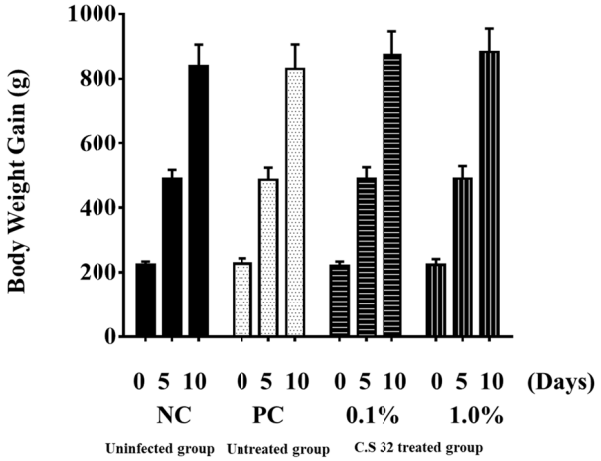

Fig. 1. Effects of dietary supplementation with CS 32 compounds on weight gains after challenged with *E. tenella*. NC: negative control (uninfected and untreated group); PC: positive control (infected and untreated group); 0.1%: 0.1% CS 32 compounds group (infected and treated with 0.1% CS 32 compounds); 1.0%: 1.0% CS 32 compounds group (infected and treated with 1.0% CS 32 compounds); n=28 for days 0 and 5, and n=21 for days 10.

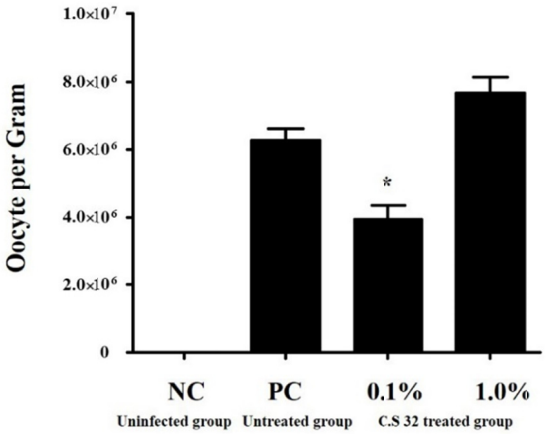

Fig. 2. Effects of dietary supplementation with CS 32 compounds on oocyte output after challenged with *E. tenella* from days 6 to 10 p.i.. NC: negative control (uninfected and untreated group); PC: positive control (infected and untreated group); 0.1%: 0.1% CS 32 compounds group (infected and treated with 0.1% CS 32 compounds); 1.0%: 1.0% CS 32 compounds group (infected and treated with 1.0% CS 32 compounds); n=21 for each group.

*Significant difference ($P<0.05$) between PC and 0.1% CS 32 compounds groups.

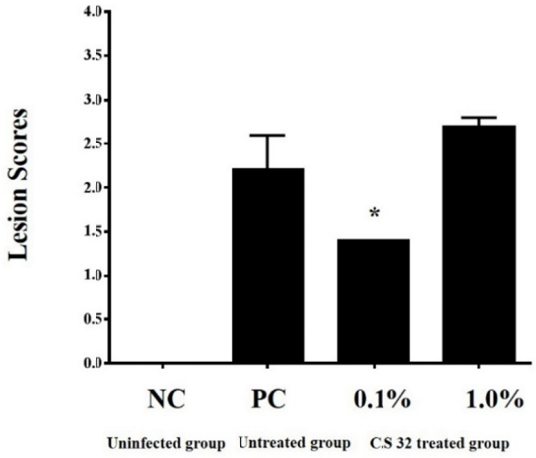

Fig. 3. Effects of dietary supplementation with CS 32 compounds on lesion score after challenged with *E. tenella* at 5 days p.i.. NC: negative control (uninfected and untreated group); PC: positive control (infected and untreated group); 0.1%: 0.1% CS 32 compounds group (infected and treated with 0.1% CS 32 compounds); 1.0%: 1.0% CS 32 compounds group (infected and treated with 1.0% CS 32 compounds); n=7 for each group.

*Significant difference ($P<0.05$) between PC and 0.1% CS 32 compounds groups.

DISCUSSION

Recently, probiotics, prebiotics, and synbiotics have been considered the most common alternatives to antibiotics (11). With growing concerns about antibiotic-resistant bacteria, there are a wider range of restrictions on the use of veterinary antibiotic growth promoters (AGPs). Consequently, interest in prebiotic oligosaccharides and probiotic microorganisms is increasing (12). Recently, several studies have shown that probiotics improve the growth rate of chickens, and many of these probiotics had growth-promoting effects similar to those of AGPs (13). Probiotics are a living microbial food additive that cause a positive change in the intestinal microbial balance (14-17). They can improve animal food efficiency and growth performance as supplemental probiotics (18). The CS 32 compounds used in this study were produced by *Bacillus* spp.; aerobic microflora such as *Lactobacillus* spp. have been widely used in the livestock industry (19).

Bacillus spp. have several characteristic features that make them suitable as possible biological control agents, such as availability in the soil and production of biologically-active metabolites (20) and peptide antibiotic compounds like circulin, colistin, and polymyxin (21). Bacitracin, the most important antibiotic product of *Bacillus* spp., mainly inhibits gram-positive bacteria (22). The aminopolyol antibiotic, zwittermicin A, may represent a new class of *Bacillus* antibiotics. *Bacillus* spp. also produce antibacterial and antifungal peptides (23, 24), lipopeptides (25, 26), and aminoglycosides (27, 28).

We studied the effect of *Bacillus* spp.-derived CS 32 compounds as a food supplement with probiotic activities for coccidiosis in chickens, because its antibacterial activity has been shown in previous studies (5). CS 32 compounds were prepared from the culture medium of *Bacillus* spp. CS 32, which showed effectiveness on gram-negative and gram-positive bacteria and MRSA (6). The anticoccidial effect of CS 32 compounds was evaluated by fecal oocyst shedding and cecal lesion score.

Further experiments are needed to define effects of dietary supplementation with CS 32 compounds on weight gains, as there were no significant differences between the negative control (uninfected and untreated group) and the positive control (infected and untreated group).

Supplementation with 0.1% CS 32 compounds significantly reduced fecal oocyst output and resulted in decreased cecal lesions in chickens infected with *E. tenella*.

Evaluating the pathogenic effects of *E. tenella* infection requires appropriate quantification of oocyst inoculation. The number of oocysts used for infection should induce statistically significant differences in the CS 32 compounds-untreated chickens and the CS 32 compounds-treated chickens. Excretion of oocysts in the feces is a parameter that is evaluated in coccidiosis. However, several researchers have disagreed on its significance and claim that counting oocysts is an unreliable and unsatisfactory way to assess the effect of anticoccidial therapy (29). Considering the reduction in fecal output of oocysts in the treated groups, CS 32 compounds treatment might correlate with an anticoccidial effect. Infection of *E. tenella* causes damage and swelling of the cecal wall and it leads to alterations in the normal function of the intestinal mucosa (30). When comparing the 0.1% CS 32 compounds-treated group with the 1.0% CS 32 compounds-treated group, OPG and lesion scores were increased in the 1.0% CS 32 compounds-treated group. This results are in accordance with those of Levine et al. (31). Treatment with 0.1% CS 32 compounds effectively lowered the lesion score caused by *E. tenella*. This finding shows that a diet supplemented with 0.1% CS 32 compounds reduces the gross pathological change induced by *E. tenella*.

Therefore, using 0.1% CS 32 compounds as a dietary supplement can improve host defense responses and have beneficial effects. Our results verified that 0.1% CS 32 compounds modulates host resistance to parasitic infections. This is the first study describing the anticoccidial effect of CS 32 compounds. However, the definite basic mechanism of CS 32 compounds-mediated immune response against coccidiosis is unclear. As concluded by lesion scores and fecal oocyst excretion, 0.1% CS 32 compounds as food supplements could effectively control *E. tenella* infection. Thus, in poultry, a CS 32 compounds-based diet may help prevent or treat coccidial infection. Further studies on other species of Coccidia are needed.

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