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Original Article

Effect of probiotic 379D as an alternative to antibiotics for feed supplementation in broiler chickens

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Antibiotics have been used to prevent disease, promote growth rate, and improve feed efficiency. However, the use of antibiotics in livestock has been restricted worldwide due to problems such as bacterial resistance. Therefore, probiotics among alternatives to antibiotics have gained attention in the livestock feed industry these days. This study was conducted to investigate the effects of dietary supplementation with probiotic 379D on safety, growth rate, and feed efficiency. In this study, bacterial strain 379D was isolated from soil and identified as a *Bacillus* sp. according to 16S rRNA sequence analysis. In an *in vitro* test, in-gel activity assay and antimicrobial susceptibility test were conducted to evaluate 379D. In an *in vivo* study, 379D was administered at concentrations of 0.1% and 1% to broiler chickens for 28 days. The results of in-gel activity assay and antimicrobial susceptibility test showed that strain 379D had broad spectrum antimicrobial activity. Furthermore, no adverse 379D-related effects were observed in 0.1% and 1% groups. Feed efficiency was higher in the 379D-treated groups than in the control group. In conclusion, 379D is expected to be used as a safe alternative to antibiotics in a feed supplement and will improve feed efficiency in broiler chickens.

Key words: probiotics, antibacterial effect, feed efficiency, feed supplementation, broiler chickens

Introduction

The food industry has developed dramatically following improved quality of life and economic growth. As a result, consumer interest in food safety has increased rapidly. In the livestock feed industry, animals become stressed from overcrowding, and the risk of disease has increased following the introduction of a factory farming system to achieve high levels of economic efficiency. Thus, antibiotics have been used worldwide as feed additives to prevent disease, promote growth rates, and improve feed efficiency [1-4]. However, adding antibiotics to animal feeds results in common problems such as bacterial resistance to antibiotics and antibiotic residue in animal products. Overuse of antibiotics has been restricted on farm animals worldwide [5-7]. Several studies have been carried out on alternatives to antibiotics such as probiotics, enzymes, hormones, organic acids and essential oils [5, 8, 9].

Among the alternatives to antibiotics, probiotics, which are viable micro-organisms used as supplements in animal feed, improve microbial balance, increase feed efficiency, and improve growth performance [10, 11]. Mainly *Lactobacillus, Streptococcus*, and *Bacillus* species have been used as probiotics in the livestock feed industry [5, 6, 8, 12].

Several studies have reported the effects of probiotics used as feed additives. The probiotic CS-A exhibits excellent antibacterial and anti-inflammatory effects *in vitro*. Moreover, supplementation with 0.1% CS-A results in improved feed efficiency and growth performance in broiler chickens [2]. Another study reported that supplementation with yeast cultures results in higher weight gain than those fed control diets [13].

Antibiotic use in food animals leads to resistance to antibiotics. Therefore, the demand for probiotics as a substitute for antibiotics in feed is increasing steadily in the livestock industry.

The production of antimicrobial peptides by *Bacillus* strains has been characterized, and many peptides produced by this group of bacteria are suitable for various applications [14]. *Bacillus* species are widely used to in-

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hibit infectious pathogens because their genetic and biochemical properties have been well-studied, and the antimicrobial substances produced by *Bacillus* species have low toxicity, high biodegradability, and are environmentally friendly. *Bacillus* species have also been granted the Generally Recognized as Safe status [15]. Bacterial strain 379D was isolated from the soil and was identified as a *Bacillus* sp according to 16S rRNA sequence analysis. Antimicrobial activity was found against various species of Gram-positive, Gram-negative bacteria, and antibiotic-resistant microorganisms.

Because probiotic 379D is expected to be an alternative to antibiotic used as feed supplements, we conducted this study to evaluate the effect of 379D on safety, feed efficiency, and growth performance in broiler chickens.

Materials and Methods

Test substance

Bacillus sp. 379D strain was used throughout this study. *Bacillus* sp. 379D was inoculated in 50 mL of medium in a 250 mL round flask. The medium composition was glucose 1%, beef extract 0.4%, peptone 0.4%, yeast extract 0.1%, and NaCl 0.25% (wt/vol). Fermentation was carried out in 400 mL of the medium in a 2 L flask at 37°C for 3 days on a shaking machine. Scale-up fermentation was carried out in 4 L of the same medium in a 7 L fermentor inoculated with 240 mL of seed culture at 37°C and 180 rpm on a shaker for 3 days. After harvest, the culture broth was freeze-dried for the animal based study.

Preparation of crude antimicrobial peptide

Bacillus sp. 379D was cultured for 48 hr in optimized medium (glucose 1%, beef extract 0.4%, peptone 0.4%, yeast extract 0.1%, and NaCl 0.25%). The culture supernatant was mixed with ammonium sulfate (10~90% saturation) and was kept at 4°C with overnight stirring. The following day, the precipitate was recovered by centrifugation at 6000 rpm for 30 min and was redissolved in 10 mM Tris-HCl buffer (pH 8.0). Following dialysis, it was purified with a Sepharose CL- 6B column (2.2 cm × 116 cm) using the same buffer. The active fractions were pooled and concentrated. The disk diffusion method was employed with the indicator strain *Mycobacterium smegmatis* ATCC9341 to assess antimicrobial activity.

In-gel activity assay for detecting bacteriocin activity

The in-gel activity assay was conducted according to a previous study [14]. The antimicrobial peptide was subjected to tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 16% polyacrylamide gel. The partially purified antimicrobial peptide was applied onto the gel in duplicate. After electrophoresis, a part of the gel along with molecular weight markers was stained with Coomassie Brilliant Blue R-250 to visualize the protein bands, whereas the unstained part of the gel was used for *in situ* detection of antimicrobial activity after fixation in a mixture of 2-propanol, acetic acid, and H_2O (25:10:65) for 15 min. The sample was then washed with sterile H_2O for 30 min repeatedly and placed in a sterile Petri dish, overlaid with 10 mL of soft agar (0.8%) containing the test strain *Mycobacterium smegmatis* ATCC9341.

Antimicrobial susceptibility test

Microorganisms were used as a reference (Table 1). Methicillin-resistant *Staphylococcus aureus* (MRSA) clinical strains were provided by Daewoong Pharmaceutical Co. and vancomycin resistant *Enterococcus* chicken intestinal strains were provided by Wonkwang University Hospital, Korea [16]. These strains were used as target microorganisms for the antimicrobial activity test of 379D. The antibacterial activity of the antimicrobial peptide was detected by the paper disc diffusion method. Each sample was run in triplicate.

Experimental birds and management

A total of 120, 1 day old Woorimatdag (Korean native chicken) chicks were obtained from Useuljae farm (Jeonbuk Buan, Korea). The chicks were randomly divided into three groups. The birds were raised in a well-ventilated room maintained at a temperature of 21~32°C and a relative humidity of 60~70% with artificial lighting from 08:00 to 20:00. The birds were bred in floor pens and allowed water and feed *ad libitum*. The Institutional Animal Care and Use Committee of Chonnam National University approved the protocols for the animal study, and the animals were cared for in accordance with the Guidelines for Animal Experiments of Chonnam National University [1].

Experimental groups

The chicks were randomly assigned to three experimental groups: two treatment groups of 379D receiving 0.1% (vol/wt) and 1% (vol/wt), respectively, and a control group. Each group consisted of 40 broiler chicks. The dietary treatments were: 1) basal feed (control), 2) basal feed plus 0.1% (vol/wt) or 1% (vol/wt) of 379D.

Clinical observations and mortality survey

All chicks were observed daily for clinical signs of toxicity and mortality.

Body weights and feed consumption measurements

The body weights of each chick were measured at the initiation of treatment (day 0), after 2 weeks (day 14), and after 4 weeks (day 28) during the treatment period. Feed consumption was measured during the treatment

period. The amount of food was calculated before it was supplied, and that remaining was measured the next day to calculate the difference, which was regarded as daily food consumption.

Statistical Analysis

The data were analyzed with the *Bonferroni assay* to evaluate the initial and final body weight using SPSS ver. 21.0 (SPSS, Inc., Chicago, IL, USA). A $P<0.05$ was considered statistically significant.

Results

Characterization of the antimicrobial peptide

The antimicrobial peptide produced by *Bacillus* sp. 379D was secreted into the culture supernatant and partially purified. Only one gel filtration column chromatography fraction showed antimicrobial activity (Fig. 1A). One step column chromatography yielded two major bands on SDS-PAGE, and one of the bands showed antimicrobial activity against *M. smegmatis* in the in-gel assay (Fig. 1B).

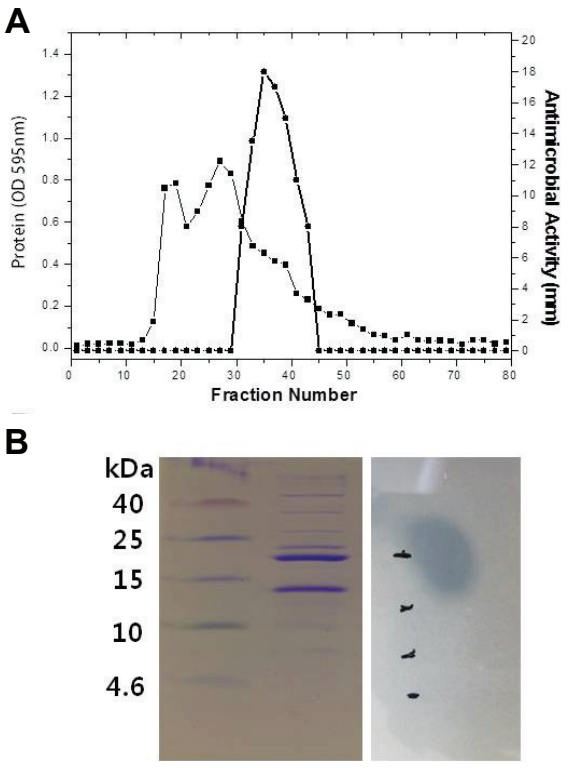

Fig. 1. (A) Gel permeation elution profile of 379D from a Sepharose CL-6B column (2.2 cm × 116 cm). -■-, protein concentration: -●-, antimicrobial activity (B) Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis and activity staining of 379D. Lane 1, protein size marker with corresponding value in kDa on the left; lane 2, partially purified 379D; lane 3, activity staining.

Inhibition spectrum and sensitivity of the antimicrobial peptide

The antimicrobial peptide displayed broad spectrum activity (Table 1). The antimicrobial peptide was active against *Mycobacterium smegmatis* ATCC9341, *Enterococcus faecalis* ATCC29212, *Candida albicans, Escherichia coli* KCTC1923, *Alcaligenes faecalis* ATCC1004, *Salmonella typhimurium* KCTC1925, MRSA U4, and IMP 129 (imipenem resistant isolate producing carbapenemase).

Clinical signs and mortality

No clinical signs of toxicity or mortality were observed in any of the groups.

Body weight changes, feed consumption, and feed efficiency

The body weight in the control group was lower than that in the treatment groups on day 0. Body weight increased in the 379D-treated groups compared with that in the control group. The 0.1% treated group had a greater body weight gain (16363.10 g) than that in the control group (15536.30 g), and average total body weight gain per chick in the 0.1% group was 20.67 g higher than that in the control group. The body weight in the 1% group increased to 17571.60 g, and average total body weight gain per chick was 50.88 g higher than that of the control group (Table 2).

The daily rate of weight gain during the experiment was evaluated in the control group (13.87 g/chicken/day), the 0.1% treated group (14.61 g/chicken/day), and the 1% treated group (15.69 g/chicken/day), and daily weight

Table 1. Inhibitory spectrum of *Bacillus* sp. 379D

Gram	Organisms	Susceptibility (cm)
+	*Enterococcus Faecalis* ATCC 29212	1.3
+	VRE 4	1.1
+	*Bacillus subtilis* ATCC6633	-
+	*Staphylococcus aureus* KCTC 1928	1.2
+	MRSA U4	1.2
+	*Micrococcus lutes* ATCC 9341	-
+	*Mycobacterium smegmatis* ATCC 9341	1.6
−	*Salmonella typhimrium* KCTC 1925	1.1
−	*Escherichia coli* KCTC 1923	1.1
−	*Pseudomonas aeruginosa* KCTC	-
−	*Alacligenes faecalis* ATCC 1004	1.1
	IMP 129	1.0

Activity was expressed as the diameter of inhibition zone against infectious strains.

gain was higher in the treatment groups than that in the control group (Table 3).

Feed efficiency was assessed through feed consumption and weight gain. Table 3 shows that feed efficiency in the 0.1% (0.53) and 1% (0.59) groups was higher than that in the control group (0.51). In particular, the 1% group had the highest feed efficiency (Table 3).

Discussion

As consumer interest in food safety has increased rapidly, probiotics rather than antibiotics are required as feed supplements to strengthen the competitiveness of animal products in the livestock industry [7-9]. Probiotics are viable micro-organisms that have beneficial effects for the host by improving the intestinal microbial balance [10, 11]. Antibiotics have been used as feed supplement to prevent disease, increase growth rate, and improve feed efficiency and growth performance. However, overuse and abuse of antibiotics have caused problems such as bacterial resistance to antibiotics and antibiotic residues in animal products. Probiotics are drawing attention as alternatives to antibiotics because they do not have these problems [5, 9, 12].

The new *Bacillus* strain 379D producing antimicrobial peptide was isolated from a soil sample. The optimal medium composition for production of the antimicrobial substance in *Bacillus* sp 379D was glucose 1%, beef extract 0.4%, peptone 0.4%, yeast extract 0.1%, and NaCl 0.25% (data not shown). Partial purification of the antimicrobial peptide (379D) was performed by ammonium sulfate precipitation followed by Sepharose CL-6B gel permeation chromatography. The antimicrobial activity of 379D against different pathogenic strains was assessed. Strain 379D exhibited broad spectrum antimicrobial activity against various pathogenic microorganisms and antibiotic-resistant strains. These results suggest that *Bacillus* strain 379D produces a high quality antimicrobial substance that might be very useful to control various pathogenic bacteria.

Therefore, this study was conducted to evaluate the effect of 379D as an alternative to antibiotics to improve safety, feed efficiency, and growth performance.

No clinical signs or death was observed in any of the broiler chickens. These results show that probiotic 379D has no harmful effects and is a safe substance to feed to chickens. In an acute/subacute toxicity study of CS682 in rats, the probiotic CS682 was identified as a very safe substance [17, 18]. The CS682-treated broiler chickens and weaning pigs showed no adverse effects [1]. Results of the present study were consistent with those of previous studies concerning other probiotics.

We measured the improvement of feed efficiency through feed consumption and weight gain to evaluate 379D as an alternative to antibiotics for growth promotion. Feed efficiency was lower in the control group than that in the 379D treated groups. In addition, the 1% treated group had higher feed efficiency than that in the 0.1% treated group. Therefore, it is concluded that 379D improved feed efficiency. This conclusion correspond with the theory that probiotics used as feed supplements improve the intestinal bacteria flora balance and help increase productivity [19]. In addition, adding probiotics to animal feeds increases growth rate and improves feed efficiency [1, 2].

In conclusion, the probiotic 379D is expected to be used safely as an alternative to antibiotics in a feed supplement and will help prevent disease, increase growth rates, and improve feed efficiency and growth performance.

Table 2. Body weights of chickens fed a diet containing 379D

		Group		
		Control	**0.1 %**	**1 %**
No. of chickens examined		40	40	40
Total body weight (g)	Day 0	1443.10	1521.10	1506.70
	Day 14	5889.60	5895.10	6227.50
	Day 28	16979.40	17884.20	19078.30
Weight gain		15536.30	16363.10	17571.60

Table 3. Feed efficiency of chickens fed a diet containing 379D (days 0~28)

	Group		
	Control	**0.1 %**	**1 %**
Initial body weight (g/chicken)	36.08 ± 2.06	38.03 ± 2.78	37.67 ± 2.31
Final body weight (g/chicken)	424.49 ± 69.87	447.11 ± 95.12	476.96 ± 64.67**
Weight gain (g/chicken/day)	13.87	14.61	15.69
Feed intake (g/chicken/day)	27.05	27.59	26.61
Feed efficiency (feed intake/weight gain)	1.95	1.89	1.70

Values are presented as means ± S.D.
**$P<0.01$ compared with the control group.

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