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Effects of dietary lysozyme supplementation on growth performance, nutrient digestibility, intestinal microbiota, and blood profiles of weanling pigs challenged with *Escherichia coli*

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Abstract

The aim of this was evaluate the efficacy of lysozyme on growth performance, nutrient digestibility, excreta microflora population, and blood profiles of weanling pigs under *Escherichia coli* (*E. coli*) challenge. A total of 30 piglets weaned at 25 days, 7.46 kg body weight, were assigned to three dietary treatments, composed of five replications, two piglets per replication, for 7 days. The dietary treatment groups were negative control (NC; without antibiotics and lysozyme), positive control (PC; NC + antibiotics), lysozyme (NC + 0.1% lysozyme). All piglets were challenged orally with 6 ml suspension, containing *E. coli* K88 (2×10^9 CFU/mL). Dietary supplementation with lysozyme and PC resulted in no significant differences in average daily gain and gain to feed efficiency. Weanling pigs fed with *E. coli* challenge with lysozyme and PC treatments had significantly enhanced nutrient retentions of dry matter and energy ($p < 0.05$); however, there was a tendency to increase nitrogen digestibility. Furthermore, dietary inclusion of lysozyme and antibiotics treatment groups had a beneficial effect on excreta, ileal, and cecal of the fecal microbial population as decreased *E. coli* ($p < 0.05$) counts, without effects on *lactobacillus* counts. A significant effect were observed on a white blood cells, epinephrine and cortisol concentrations were reduced in piglets fed diets containing *E. coli* challenge with lysozyme and antibiotics supplementation comparison with the NC group. Therefore, the present data indicate that lysozyme in diet could ameliorate the experimental stress response induced by *E. coli* in piglets by decreasing intestinal *E. coli*, white blood cells and stress hormones and improving nutrient digestibility.

Keywords: Lysozyme, *Escherichia coli* challenge, Intestinal microflora, Weaning pig

INTRODUCTION

Young piglets have the most common diarrhea because their digestive system is not completely mature, and this is probably the most severe threat due to their high mortality rate. The intestinal tract is

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Availability of data and material
Upon reasonable request, the datasets of this study can be available from the corresponding author.

Authors' contributions
Conceptualization: Park JH, Kim IH.
Data curation: Park JH, Sureshkumar S.
Formal analysis: Park JH.
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This research has been approved by the Institutional Animal Care and Use Committee (IACUC) of Dankook University (DKU-IRB-2019-1839).

microbiologically sterile at weanling pigs' birth and has no immunity to species developing diseases. Bacterial species, including possibly pathogenic strains of *Escherichia coli* (*E. coli*) and *Clostridium perfringens* (*C. perfringens*), tend to colonize the intestines soon after birth and becoming healthy representatives of the gut microbiota in the intestinal tract. Pathogenic *E. coli* commonly causes intestinal disorders such as edema disease syndrome and diarrhea in weaner pigs.

Lysozyme is an enzyme, 1,4- β -N-acetylmuramidase that cleaves the glycosidic bond between the N-acetylmuramic acid and N-acetylglucosamine in bacterial peptidoglycan of the cell wall, resulting in the loss of cellular membrane integrity and cell death [1]. lysozyme is a generic enzyme that is commercially derived from an avian ingredient (egg white) abundant in many tissues, tears, and secretions such as animal milk [2]. Previously, some studies have reported lysozyme significant function as a protector against bacteria in different species [3,4]. In the body's defense mechanisms, lysozyme functions are associated with the monocyte macrophage system and immunoglobulins [5]. Furthermore, lysozyme is an important antibacterial agent and through its direct bacteriolytic activity, it is used as a mediator or activates macrophage phagocytic activity [3,6]. lysozyme has been studied as a potential alternative to antibiotics for animals in recent years. An *in vitro* experiment conducted by Zhang et al. [7] showed that lysozyme (200 μg/mL) has not only completely inhibited the growth of *C. perfringens* but also inhibited the development of alpha-toxin that induces necrotic enteritis (NE)-associated lesions in chickens. It has also been reported that lysozyme has shown changes in metabolite profiles, intestinal microbiota, and intestinal morphology in pigs, broiler chickens, and mice fed lysozyme [8–11]. Currently, lysozyme is not extensively used as a feed additive in the animal industry; however, few studies are available regarding lysozyme is use as an alternative to antibiotics for pigs. Therefore, this study was designed to evaluate lysozyme effects on growth performance, nutrient digestibility, intestinal microbiota populations, and blood profiles in *E. coli* experimentally infected weaning pigs.

MATERIAL AND METHODS

The experimental protocol (DK-2-1839) used in the current research was approved by the Animal Care and Use Committee of Dankook University, Korea.

Experimental design, animals and diets

A total of 30 piglets ([Yorkshire × Landrace] × Duroc; 7.46 ± 0.67 kg) weaned from sow at 25 days of age were used in a 7-d trial. Weaner pigs were allocated five replication pens per treatment with two piglets (2 m × 2 m) to one out of three dietary treatments following to their initial body weight and sex. All piglets were orally dosed with 6 mL suspension which contains 2×10^9 CFU/mL of *E. coli* K88 to cause mild diarrhea. The dosage of *E. coli* K88 was based on a previous study [12]. The dietary treatments were 1) negative control (NC; without antibiotics, and lysozyme), 2) positive control (PC; NC + antibiotics 55 mg/kg feed [Aureo S-P 250]), 3) Lysozyme (NC + 0.1% lysozyme [Cell Tech, Eumseong, Korea]). All diets used in the present study were formulated in order to meet or little exceed the estimated nutrient requirements for weanling pigs recommended by NRC [13] (Table 1). Weaner pigs were housed in an environmentally controlled room with a mechanical ventilation system and slatted plastic flooring, although the lighting was automatically regulated to provide 12 h of artificial light daily. The starting temperature within the room was kept up at 30℃ ± 1 and humidity at around 60%. Each pen was prepared with a one-sided stainless-steel self-feeder, and one nipple drinker to allow weaner pigs to feed and ad libitum water during the experiment.

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Table 1. Formulation and chemical composition of experimental diet

Items	
Ingredients (%)	100
Extruded corn	47.80
Soybean meal (dehulled)	18.00
Fermented soybean meal	8.00
Fish meal	2.70
Soy oil	3.20
DCP	1.34
Limestone	0.74
Sugar	2.00
Whey protein	8.00
Lactose	6.70
L-Lysine HCl	0.46
DL-Met	0.17
Threonine	0.29
Choline chloride 50%	0.10
Salt	0.10
Mineral premix[1)]	0.20
Vitamin premix[2)]	0.20
Nutrients (%)	
Protein	19.0
Fat	4.80
Calcium	0.75
Phosphorus	0.65
DE (kcal/kg)	3,900
Lys	1.50
Met	0.45
Lactose	12.0

[1)]Provided per kg diet: Fe, 100 mg as ferrous sulfate; Cu, 17 mg as copper sulfate; Mn, 17 mg as manganese oxide; I, 0.5 mg as potassium iodide; and Se, 0.3 mg as sodium selenite.

[2)]Provided per kilograms of diet: vitamin A, 10,800 IU; vitamin D_3, 4,000 IU; vitamin E, 40 IU; vitamin K_3, 4 mg; vitamin B_1, 6 mg; vitamin B_2, 12 mg; vitamin B_6, 6 mg; vitamin B_{12}, 0.05 mg; biotin, 0.2 mg; folic acid, 2 mg; niacin, 50 mg; D-calcium pantothenate, 25 mg.

DCP, dicumyl peroxide; Met, methionine; DE, digestible energy; Lys, lysine.

Sample collection and laboratory procedures

The body weight of piglets was record at the beginning and at the conclusion of the experiment. Feed intake and residual was also recorded on a pen basis until the experiment in order to calculate average daily gain (ADG), average daily feed intake (ADFI), and gain:feed ratio (G:F).

Piglets were fed diets mixed with 0.5% Cr_2O_3 (chromic oxide) as an indigestible marker to determine apparent total tract digestibility for dry matter (DM) and nitrogen during the experimental period. On day 7, fecal samples were collected from all piglets in each pen via rectal massage. Before analysis, fecal samples were dried at 60℃ for 3 days in drying oven; subsequently, they were pulverized to pass through a 1-mm screen. Then all feed and fecal samples were analyzed to determine DM, energy, and nitrogen by the Method of 930.15; AOAC [14]. The DM was calculated according to the indicator method, with the concentration of chromium being analyzed by UV absorption spectrophotometry (UV-1201, Shimadzu, Kyoto, Japan) following the

methods of Williams et al. [15]. The feed and feces' gross energy was determined using a 6100 Parr calorimeter (Model 1241, Parr Instrument, Moline, IL, USA). Nitrogen was determined by Kjectec 2300 Nitrogen Analyzer (Foss Tecator AB, Hoeganaes, Sweden).

At the end of the trial, fresh fecal samples were collected from 2 piglets in each treatment, placed on ice for transportation to the research laboratory, and microbial counts were analyzed. After piglets were killed, ileal and cecal contents were also taken for microbial analysis. We first took a one-gram fecal sample for microbial analysis and diluted it with 9 mL of 1% peptone broth (Becton, Dickinson, Franklin Lakes, NJ, USA) and then homogenized with a vortex mixer. After10-fold serial dilution, 0.02% peptone solution were poured into MacConkey agar plates (Difco Laboratories, Detroit, MI, USA) and Lactobacilli medium III agar plates (Medium 638, DSMZ, Braunschweig, Germany) and kept in incubation (at 37℃) for one day, and *E. coli* colonies were counted and recorded. On the next day *Lactobacillus* agar plates were taken out from incubation (37℃), and the colonies were counted and recorded for statistical analysis. For blood profile assay, all pigs selected from each pen for blood samples were taken by anterior vena cava puncture 24 h after challenge. Blood samples were collected into either 5-mL vacuum tubes without and with K_3EDTA coating (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ, USA).

Serum samples were analyzed, approximately 3 mL blood samples were centrifuged at 3,000×g for 15 min at 4℃, and serum hormones (epinephrine, norepinephrine, cortisol) were assessed using enzyme-linked immunosorbent assay kits (LDN GmbH & Co., Nordhorn, Germany) following to the manufacturer's protocol. White blood cells (WBC), lymphocytes, and red blood cells were quantified using a Hemavet hematology analyzer (Drew Scientific, Dallas, TX, USA).

Statistical analysis

All data were analyzed using the PROC MIXED procedure of SAS (SAS Inst., Cary, NC, USA) in a randomized complete block design. The experimental unit was the pen and block was the sex. The statistical model for growth performance, nutrient digestibility, and blood profiles included effects of dietary treatment as a fixed effect and sex as a random effect. For microbial counts, data were log-transformed prior to statistical analysis. Results are given as means ± SEM. Statistical significance and tendency were considered at $p < 0.05$ and $0.05 \leq p < 0.10$, respectively.

RESULTS

Dietary supplementation of lysozyme with *E. coli* challenge did not significantly differ on body weight gain (BWG), ADG, and G:F during the overall experiment, respectively. Levels of ADFI did not differ among lysozyme, antibiotics, and NC treatments (Table 2). At the end of the experiment, apparent total tract digestibility of DM ($p = 0.009$), and energy ($p = 0.046$) showed a significant increase in dietary supplementation of lysozyme and antibiotics of weanling pigs challenged with *E. coli*; however, there was a tendency to increase in N digestibility (Table 3). Furthermore, significant effects on beneficial effects on the fecal ($p = 0.018$), ileal ($p = 0.027$), and cecal ($p = 0.020$) microbial population as decreased *E. coli* counts with dietary inclusion of lysozyme and antibiotics of weaning pigs challenged with *E. coli*, without effects on *lactobacillus* counts in fecal, ileal, and cecal microbiota (Table 4). A significant effect was observed on WBC ($p = 0.018$), and epinephrine ($p = 0.002$) and cortisol ($p = 0.001$) concentrations were reduced in piglets challenged with *E. coli* fed diets containing lysozyme and antibiotics supplementation. As well, there were no significant differences in RBC, lymphocytes, norepinephrine concentrations in piglets fed lysozyme or PC diets (Table 5).

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Table 2. Effect of lysozyme supplementation on growth performance in weaning pig

Items	NC[1)]	PC[1)]	Lysozyme[1)]	SEM	*p*-value
Initial weight (kg)	7.48	7.45	7.46	0.06	0.524
Final weight (kg)	9.35	9.48	9.46	0.09	0.486
ADG (g)	267	290	286	7.25	0.272
ADFI (g)	428	430	428	9.43	0.847
G:F	0.624	0.674	0.669	0.123	0.182

[1)]NC, basal diet; PC, NC + antibiotics; Lysozyme, NC + 0.1% lysozyme.

NC, negative control; PC, positive control; ADG, average daily gain; ADFI, average daily feed intake; G:F, gain:feed.

Table 3. Effect of lysozyme supplementation on nutrient digestibility in weaning pig

Items (%)	NC[1)]	PC[1)]	Lysozyme[1)]	SEM	*p*-value
Dry matter	78.15^{b}	82.08^{a}	81.61^{a}	0.50	0.009
Nitrogen	77.26	79.65	78.57	0.59	0.096
Energy	78.07^{b}	80.43^{a}	80.31^{a}	0.52	0.046

[1)]NC, basal diet; PC, NC + antibiotic 55 mg/kg feed (Aureo S-P 250); Lysozyme, NC + 0.1% lysozyme.

[a,b]Means in the same row with different superscript differ significantly ($p < 0.05$).

NC, negative control; PC, positive control.

Table 4. Effect of lysozyme supplementation on microbial in weaning pig

Items (Log_{10}CFU/g)	NC[1)]	PC[1)]	Lysozyme[1)]	SEM	*p*-value
Feces					
Lactobacillus	7.09	7.64	7.70	0.03	0.510
E. coli	5.58^{a}	4.07^{b}	4.09^{b}	0.12	0.018
Ileum					
Lactobacillus	7.38	7.96	7.00	0.04	0.420
E. coli	5.50^{a}	4.37^{b}	4.39^{b}	0.10	0.027
Cecum					
Lactobacillus	8.64	8.26	8.79	0.04	0.603
E. coli	5.76^{a}	4.60^{c}	4.77^{bc}	0.13	0.020

[1)]NC, basal diet; PC, NC + antibiotic 55 mg/kg feed (Aureo S-P 250); Lysozyme, NC + 0.1% lysozyme.

[a–c]Means in the same row with different superscript differ significantly ($p < 0.05$).

NC, negative control; PC, positive control; *E. coli, Escherichia coli*.

Table 5. Effect of lysozyme supplementation on blood profile in weaning pig

Items	NC[1)]	PC[1)]	Lysozyme[1)]	SEM	*p*-value
WBC (10^3/μL)	18.7^{a}	14.7^{b}	14.3^{b}	0.47	0.018
RBC (10^6/μL)	6.4	5.7	5.9	0.12	0.244
Lymphocyte (%)	69.8	60.4	62.1	1.36	0.083
Epinephrine (pg/mL)	658^{a}	382^{b}	357^{b}	38	0.002
Norepinephrine (pg/mL)	1466	1151	1292	162	0.172
Cortisol (μg/dL)	5.7^{a}	2.1^{b}	2.0^{b}	0.35	0.001

[1)]NC, basal diet; PC, NC + antibiotic 55 mg/kg feed (Aureo S-P 250); Lysozyme, NC + 0.1% lysozyme.

[a,b]Means in the same row with different superscript differ significantly ($p < 0.05$).

NC, negative control; PC, positive control; WBC, white blood cells; RBC, red blood cells.

DISCUSSION

Escherichia coli is an important causative agent of porcine diarrhea, causing mortality, morbidity, and low growth rates of infected pigs, causing many economic losses to treatment and prevention costs. Lysozyme has been tested in some studies with animals with varying responses, depending upon the different sources or dietary concentration of added lysozyme, or induced disease challenge [9,16,17]. In inconsistent with previous reports, in this study, the *E. coli* challenge was not successful that it increased diarrhea score moderately (data not shown) and did not reduce ADG after inoculation [18]. Moreover, contrary to expectations, lysozyme and antibiotics did not improve the growth performance of piglet to an oral challenge of *Escherichia coli* K88. Previous studies have reported that dietary lysozyme supplementation indicated improved growth performance and feed efficacy in pigs [9] and poultry [19]. Liu et al. [20] reported that exogenous lysozyme addition decreased the *C. perfringens* concentration in the intestinal lesion score and ileum, increased feed conversion ratio and body weight gain of chickens challenged with *C. perfringens* type A during days 14 to 28. Xiong et al. [21] stated that pigs fed with 1.0 g/kg^{-1} lysozyme supplementation had higher average weaning weight during a 14 days experimental trial. Furthermore, it has been reported that nursery pigs consuming lysozyme or antibiotics gained weight approximately 8% faster and pigs consuming either lysozyme or antibiotics had improved feed efficiency of approximately 7% for 28 days [9, 10]. Contrastingly, Nyachoti et al. [17] and Garas et al. [22] observed that lysozyme treatment did not influence the ADG and G:F or ADG of weaned pigs receiving supplements after oral challenge with enterotoxigenic *E. coli*. The exact mechanisms involved in the relationship between lysozyme and improvement in performance are still not fully understood. The different observations due to feeding lysozyme on *E. coli* may be due to the different sources of lysozyme, different species of *E. coli*, or the presence of a direct *E. coli* K88 challenge [9].

In the current study, DM and energy retention were higher in lysozyme and antibiotic treatments, than in no lysozyme treatments. Studies assessing the effects of lysozyme in piglets are limited. The morphology of the small intestinal is frequently used to measure digestion and nutrient absorption as a marker. Brundige et al. [23] and Cooper et al. [24] reported piglets fed lysozyme supplementation had a beneficial effect on villi height in the ileum and villi wider in the duodenum than those reared on control milk. Similarly, pigs consuming lysozyme (100 mg/kg diet) showed villus height was increased and crypt depth was decreased in the jejunum, resulting in an increased villus height to crypt depth ratio [9]. Xiong et al. [21] reported that the inclusion of 1.0 g kg^{-1} lysozyme had higher villus height of jejunal than those in the control groups after the 14-day treatment. Furthermore, Nyachoti et al. [25] observed pigs fed lysozyme (egg white source) had improved the villi height of ileum at 17 days of an experimental trial. Altogether, these results show that small intestinal morphology is enhanced by lysozyme supplementation. Although the small intestine morphology has not been investigated in this study, the development of villi by lysozyme corresponds to an increased intestinal surface area and, therefore, may result in nutrient digestion and gastrointestinal absorption.

Intestinal microflora affects host health and disease through symbiotic interactions with the host body. It is known to participate in the defense against pathogen invasion and immune system development and maturation, and regulate host metabolism by producing short-chain fatty acids through vitamin synthesis and fermentation of polysaccharides and supplying them as nutrients [26]. Previously, Maga et al. [27] stated that lysozyme was efficient modulating the bacterial species of both goats and piglets in the duodenum and ileum. Liu et al. [20] reported that exogenous lysozymes inclusion significantly reduced the *E. coli* counts and increased the *Lactobacillus counts* in the ileum and intestinal bacteria translocation to the spleen after challenge *C. perfringens* in pigs.

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Xiong et al. [21] reported that *Fibrobacteres, Bacteroidetes*, and *Proteobacteria* were dominant relative abundance phyla in pigs fed with the highest dosage of lysozyme supplementation. In addition, 0.1%. lysozyme had been shown to reduce enterotoxigenic *E. coli* in challenged-piglets [25]. The current study also demonstrated that challenged-piglets fed lysozyme supplemented diet led to lower *E. coli* concentration in feces, ileum, and cecum. Therefore, lysozyme could suppress the growth of *E. coli* and lead to healthy intestinal development in challenged-piglets.

It has been reported that hematological parameters could be used as indicators of the stress condition during the lipopolysaccharide challenge. Stress reduction has been reported as one of the causes that affect the levels of lymphocytes, heterophils, and overall white blood count [28]. Faas et al. [29] demonstrated that WBC migrates directionally inflammatory sites while an animal is infected with bacteria and secrete many chemokines, adhesion factors, and pro-inflammatory cytokines to eliminate corresponding pathogens in a coordinated way. Wolmarans [30] stated that results in an inflammatory reaction that culminated in an increase in WBC level in the blood serum. An increasing number of WBC levels are very beneficial for the host to prevent invasion by bacteria. Piglets fed lysozyme showed the lowest value for WBC counts, and this may be due to the relief of the immune system by the immunogenic property of the lysozyme used in this treatment. Furthermore, cortisol is the primary hormone of the hypothalamic-pituitary-adrenocortical axis, responding to stress [31]. Increased of serum cortisol was observed in pigs under stress conditions, including lipopolysaccharide challenges [32]. The endocrine stress response involves the secretion of catecholamines, epinephrine, norepinephrine, and adrenal steroid cortisol [33]. Chronic or repeated cortisol elevations in the blood are eventually immunosuppressive, and may have major deleterious growth effects. In the current study, supplementation of lysozyme to challenged-piglets led to faster normalization of stress hormones such as epinephrine and cortisol. Therefore, this suggests that consuming lysozyme may alleviate the severity of the infection.

CONCLUSION

It is concluded that lysozyme dietary supplementation resulted in increased DM and energy retention, and reduced fecal and intestinal *E. coli* counts, WBC, and stress hormone concentrations of weanling pigs challenged with *E. coli*, although there was no change in growth performance. It can be suggested that lysozyme could help partially relieve the response to stress conditions by challenging *E. coli*, similar to antibiotic treatment. However, the future investigation should focus on the mechanism of action and understanding the effect of different concentrations of lysozyme in the weaning pigs diet by with challenging or without challenging *E. coli* for different phase feeding.

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