Effects of *Paenibacillus xylanexedens* on growth performance, intestinal histomorphology, intestinal microflora, and immune response in broiler chickens challenged with *Escherichia coli* K88

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ABSTRACT This study investigated the effects of dietary *Paenibacillus xylanexedens* ysm1 supplementation on growth performance, intestinal morphology, immune response, and cecal microbiota of broiler chickens challenged with *Escherichia coli* K88. A total of 320 one-day-old male broiler chicks were randomly allocated to 4 treatments (8 floor pens, 10 birds/pen) including 1) negative control (**NC**) birds fed a basal diet and not challenged with *E. coli* K88; 2) positive control (**PC**) birds fed a basal diet and challenged with of *E. coli* K88; 3) *P. xylanexedens* ysm1 treatment (**PRO**) birds fed a basal diet supplemented with 1×10^9 *P. xylanexedens* ysm1 cfu/kg feed and challenged with *E. coli* K88; and 4) antibiotic treatment (**ANT**) birds fed a basal diet supplemented with 20 mg of colistin sulphate/kg of feed and challenged with *E. coli* K88. The *E. coli* challenge decreased ($P < 0.05$) BWG in PC birds compared with the ANT birds on days 21 and 28. The FCR was higher ($P < 0.01$) in PC birds compared with the NC, PRO, and ANT birds on days 14, 21, and 28. Compared with the NC, PRO, and ANT birds on day 28, PC birds had shorter villi and higher number of goblet cells in both jejunum and ileum ($P < 0.001$). Irrespective of the dietary treatments, the *E. coli* challenge reduced the number of PCNA-positive cells in both the jejunum and ileum on day 28. *Paenibacillus xylanexedens* ysm1 treatment resulted in higher concentration of mucosal sIgA in the jejunum as compared to the other treatment groups on days 14 and 28. The numbers of cecal *E. coli* were reduced ($P = 0.017$) in broilers treated with *P. xylanexedens* ysm1 or antibiotic in comparison with the PC group on day 28. In conclusion, the present study demonstrated that dietary supplementation of this new probiotic bacteria *P. xylanexedens* ysm1 improved broiler performance by modulating intestinal morphology, enhancing immune response, and reducing the number of *E. coli* in the cecum.

Key words: broiler, *Escherichia coli K88*, *Paenibacillus xylanexedens* ysm1, probiotic, intestinal histomorphology

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INTRODUCTION

Avian colibacillosis caused by enterotoxigenic *Escherichia coli* is an important infection that results in reduced performance, increased mortality, and significant economic losses in poultry production (Cao et al., 2013; Zhang et al., 2014). Antibiotics are typically used to treat or control bacterial diseases in the broiler industry. However, emergence of antibiotic-resistance bacteria and the possibility of antibiotic residues in meat and other animal products have put restrictions on the use of antibiotics (Zhang et al., 2016; Wang et al., 2017). Therefore, there is an increasing demand in the poultry industry for new alternative strategies to improve performance and disease resistance by establishing a favorable intestinal microbiota.

Probiotics influence the host health by maintaining the normal intestinal microbiota, preventing the growth of pathogenic microorganisms, promoting feed intake (**FI**) and digestion, and enhancing immune function (Kim et al., 2009; Lutful Kabir, 2009). Dietary use of probiotics significantly influenced broiler performance (Mountzouris et al., 2007, 2010; Mookiah et al., 2014), intestinal architecture (Awad et al., 2009; Sen et al., 2012), and the colonization of beneficial microorganisms in the intestines (Mookiah et al., 2014). Moreover, probiotics can decrease pathogen colonization and invasion of the intestinal tract to prevent several enteric infections in chickens (Cao et al., 2013; Wang et al., 2017).

Spore-forming probiotic bacteria, such as *Bacillus* spp., have been successfully used in animal nutrition and confirmed to promote animal performance and health (Kim et al., 2009; Cao et al., 2018). In general,

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endospore-forming probiotics have several advantages over *Lactobacillus* or *Bifidobacterium* as a probiotic feed additive (Grant et al., 2018). Thanks to their evolutionary advantage of spore-forming ability, these bacteria can withstand harsh environmental conditions, such as feed processing and pelleting, which makes these bacteria a suitable alternative growth promoter to use in the broiler industry (Shivaramaiah et al., 2011; Amerah et al., 2013). Lee et al. (2010) revealed that *Bacillus*-based direct feed microbials (strains 15AP4 and Bs27) alleviated the *Eimeria maxima* induced reduction of body weight gain and intestinal lesions. Teo and Tan (2006) reported that *Bacillus subtilis* PB6 positively influenced performance in broilers challenged with *E. coli*. *Bacillus*-based probiotics promote gut health and reduce signs of the enteric diseases via several different mechanisms such as competitive exclusion, antimicrobial peptide production, gut microflora modifications, and immune system stimulation (Grant et al., 2018).

Paenibacillus is a genus of facultative anaerobic, endospore-forming bacteria, which was previously distinguished from the other *Bacillus* groups by comparative 16S rRNA sequence analysis (Ash et al., 1993). Bacteria belonging to this genus have been isolated or detected in a variety of environments but the majority are found in soils often associated with plant roots (Grady et al., 2016). Plant-associated species of *Paenibacillus* serve as a plant growth promoter by producing several substances and fixing atmospheric nitrogen (Grady et al., 2016; Weselowski et al., 2016). In addition, they can competitively colonize plant roots and confer biocontrol against a diverse variety of phytopathogens by inducing host defense, producing biochemical substances (Grady et al., 2016), and also synthesizing polysaccharide-hydrolyzing enzymes (Nelson et al., 2009). In contrast to well-known probiotics, there have been limited reports on the effects of *Paenibacillus* sp. on animal performance and health. Our previous work showed that *Paenibacillus xylanexedens* ysm1 (*P. xylanexedens* ysm1) exhibited an adhesion pattern to enterocytes and ability to suppress *E. coli* proliferation under in vitro conditions (Calik et al., 2017). Moreover, dietary supplementation of *P. xylanexedens* ysm1 conferred beneficial effects in broilers by improving feed conversion ratio (**FCR**) and intestinal morphology (Calik et al., 2017). A very recent study showed that dietary use of *P. polymyxa* 10 improved the intestinal health of broilers by increasing intestinal barrier function, anti-oxidative capacity, and immune response, and by decreasing cell apoptosis (Wu et al., 2019).

Generally, probiotics are recognized as safe for animals and humans. However, due to the increasing number of newly isolated bacteria with probiotic features, assessment of safety of these new microorganisms becomes very important (Kumar et al., 2015). Regulation for novel or newly isolated probiotics may vary from one region to another and be subjected to an extensive safety assessment prior to approval in the markets (Kumar et al., 2015). For safety purposes, biological origin and genome, antibiotic resistance profile, studies on target species, potential toxic effects, and environmental risk are required for the assessment of probiotic feed additives (Anadon et al., 2006; Kumar et al., 2015). Based on previous findings that suggest the benefits of dietary probiotic administrations, and also our in vitro and in vivo study results, the current study hypothesized that dietary supplementation of new isolate *P. xylanexedens* ysm1 may be an effective method to maintain broiler performance and health by influencing intestinal morphology, gut microflora, and the immune system of broiler chickens challenged with *E. coli* K88.

MATERIALS AND METHODS

Animal Care and Use

All experimental procedures were approved by the Animal Ethics Committee of Gazi University (G.Ü.ET-15.049).

Probiotic Strain

The *P. xylanexedens* ysm1 strain was isolated from chyme samples of 8 different cattle between 2 and 4 yr of age and kept in the culture collection of Life Sciences Application and Research Center, Gazi University, Ankara, Turkey. After isolation, resistance to simulated gastric fluids, tolerance to bile salt, sporulation efficiency, spore formation, adhesion, and invasion properties were tested previously (Calik et al., 2017). Additionally, the antimicrobial activity of the isolates against pathogenic bacterial strains (*E. coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*) was evaluated, and the results were reported earlier (Calik et al., 2017).

Preparation of P. xylanexedens ysm1 as a Feed Additive

Cultures were grown in 50 mL Difco Sporulation Medium (**DSM**, USA) at 37°C, 250 rpm for 24 h and centrifuged at 1,280 × *g* for 5 min. Harvested spores were then inoculated in DSM and incubated at 37°C and 250 rpm for 48 h. After the incubation period, spores were centrifuged at 5,000 × *g* for 30 min; spore pellets were washed with 20 mL sterile distilled water and resuspended in 5 mL sterile water. Spores of the isolate were then mixed with 10% skim milk for further lyophilization. The lyophilized culture of *P. xylanexedens* ysm1 was added to the basal diet at 1×10^9 cfu/kg.

Birds, Diets, and Experimental Design

A total of 320 one-day-old male broiler chicks (Ross 308), with average weight of 40.51 ± 1.94 g (mean ± SD), were obtained from a commercial hatchery

Table 1. Ingredients and composition of basal diet.

	Basal diet	
	0 to 14 D	15 to 28 D
Ingredient, g/kg		
Corn	549.40	575.00
Soybean meal, CP 48%	375.00	342.40
Vegetable oil	33.00	44.00
Limestone	5.00	3.60
Dicalcium phosphate	24.50	23.40
DL-Methionine (98%)	3.60	3.15
L-Lysine HCl (78%)	3.00	2.35
L-Threonine	1.50	1.10
Salt	2.50	2.50
Vitamin premix[1]	1.00	1.00
Mineral premix[2]	1.00	1.00
Choline chloride	0.50	0.50
Total	1,000	1,000
Chemical composition (calculated)		
Dry matter, %	87.93	87.93
Crude protein, %	23.04	21.57
AME_n, kcal/kg	3,006	3,105
Lysine, %	1.44	1.30
Methionine + cysteine, %	1.08	0.99
Threonine, %	1.00	0.90
Calcium, %	0.97	0.88
Available phosphorus, %	0.48	0.44

[1]Provided per kilogram of complete diet: vitamin A, 15,000 IU; vitamin D3, 5,000 IU; vitamin E, 100 mg; vitamin K3, 3 mg; thiamin, 5 mg; riboflavin, 8 mg; pyridoxine, 5 mg; pantothenic acid, 16 mg; niacin, 60 mg; folic acid, 2 mg; biotin, 200 µg; vitamin B12, 20 µg.

[2]Provided per kilogram of complete diet: Cu, 16 mg; I, 1.5 mg, Co, 500 µg; Se, 350 µg; Fe, 60 mg; Zn, 100 mg; Mn, 120 mg; Mo, 1 mg.

(Beypiliç, Bolu, Turkey). The birds were randomly allocated to 4 experimental groups each comprising 8 replicate pens with 10 birds per pen. The treatments were as follows: negative control (**NC**) birds fed a basal diet and not challenged with *E. coli* K88; positive control (**PC**) birds fed a basal diet and orally challenged with *E. coli* K88; *P. xylanexedens* ysm1 treatment (**PRO**) birds fed a basal diet supplemented with 1×10^9 *P. xylanexedens* ysm1 cfu/kg feed and orally challenged with *E. coli* K88; and antibiotic treatment (**ANT**) birds fed a basal diet supplemented with 20 mg of colistin sulfate/kg of feed and orally challenged with *E. coli* K88. Birds were housed in a controlled environment for 28 day with the ambient temperature thermostatically controlled and gradually reduced from 34°C on the first day to 22°C at 3 wk, then maintained at 22°C thereafter. To prevent potential cross-contamination, birds within each treatment were placed in individual identical rooms, which had the same conditions throughout the study. The starter and grower diets were based on corn-soybean meal and fed from day 0 to 14 and day 15 to 28, respectively (Table 1). All diets were formulated to meet or exceed the NRC nutrient recommendations (NRC, 1994). Each pen was equipped with a manual plastic feeder and a nipple drinker. Water and the experimental diets (in mash form) were provided ad libitum throughout the study period. All chicks were individually weighed and FI was recorded at weekly intervals. Body weight gain (**BWG**), FI, and FCR were subsequently calculated based on performance values.

Escherichia coli K88 Preparation and Oral Challenge

Escherichia coli K88, which was previously isolated from an infected broiler flock, was obtained from the culture collection of Ankara University Faculty of Veterinary Medicine, Department of Microbiology. Birds in the PC, PRO, and ANT treatment groups were orally gavaged with 0.1 mL *E. coli* K88 (2×10^9 cfu/mL) on day 7 and 0.5 mL *E. coli* K88 (2×10^9 cfu/mL) on days 10, 14, and 21. The NC birds were administrated similarly with the same volume of 0.9% saline solution (Yang et al., 2008).

Sampling Procedures

At 14, 21, and 28 days of age, 1 bird from each replicate pen was selected based on average pen body weight. Birds were euthanized by exsanguination and the intestinal tract was immediately removed. Tissue samples were obtained from the jejunum and ileum for histomorphological analysis on days 14 and 28. A second sample was collected from the jejunum and snap-frozen in liquid nitrogen for sIgA determination on days 14 and 28. On days 14, 21, and 28, both ceca were ligated and aseptically removed from the gastrointestinal tract for cecal microbial analysis. Subsequently, the cecal content was collected in sterile 1.5 mL tubes and stored at –80°C until DNA isolation.

Morphological Measurements of the Jejunum and Ileum

Tissue samples in the formalin solution were dehydrated in graded ethanol solutions, cleared with xylol, and then embedded in paraffin. The intestinal segments were sectioned at a thickness of 5 μm with a microtome. Cross sections were prepared and stained with Mallory's triple stain, as modified by Crossman, in order to determine the jejunal and ileal morphometry (Culling et al., 1985). Villus height (**VH**) was measured from the top of the villus to the crypt mouth, and crypt depth (**CD**) was defined as the depth of the invagination between adjacent crypt mouths. Villus width was measured at the bottom of the villus. Goblet cells were analyzed by staining with combined Alcian Blue (**AB**) and periodic acid Schiff (**PAS**) reagent on day 28. Cells were identified as follows: acid mucin was stained by AB (blue), neutral mucin was stained by PAS (pink), and intermediate mucin, which includes both acid and neutral mucins, was stained by AB and PAS (purple) (Geier et al., 2011). All positive cells along the villus were counted, regardless of the mucin type.

A total of 10 well-oriented villi and crypts were randomly selected for histological measurements. Histological sections were examined under a light microscope (Leica DM 2500, Leica Microsystems GmbH, Wetzlar, Germany) and photographed with a digital microscope camera (Leica DFC450, Leica Microsystems GmbH,

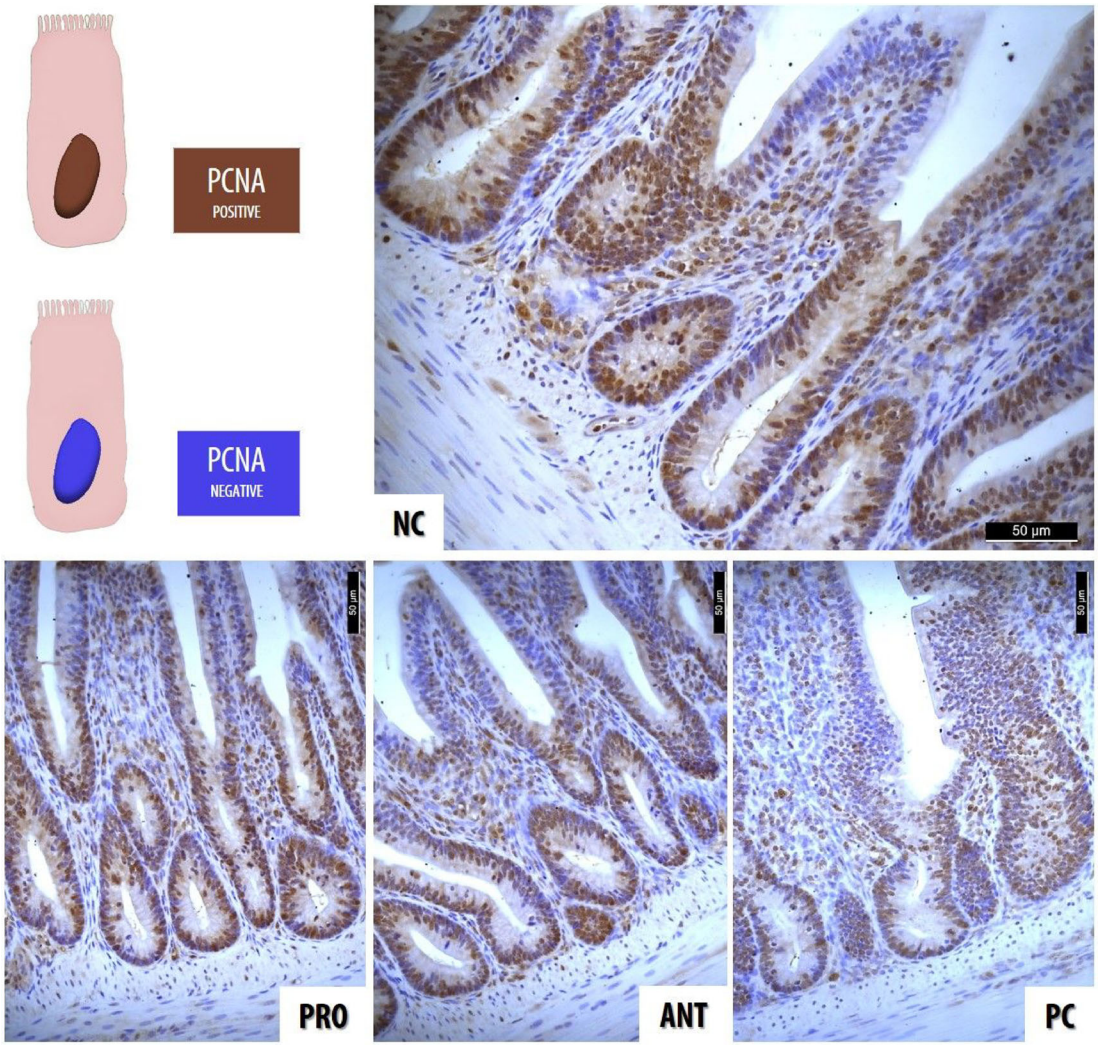

Figure 1. Immunohistochemical distribution of proliferating cell nuclear antigen positive cells on day 28.

Wetzlar, Germany). The images were evaluated using the ImageJ software (National Institute of Mental Health, Bethesda, Maryland, USA).

Proliferating Cell Nuclear Antigen Staining

Immunohistochemical staining was performed on the stored 5 μm thick formalin-fixed paraffin-embedded tissue sections. Tissue sections were placed on poly-L-lysine microscope slides (Thermo Scientific, Braunschweig, Germany), which were incubated at 37°C overnight and de-paraffinized with xylene and rehydrated through graded alcohols. Endogenous peroxidase activity was blocked by quenching with H_2O_2 (3% in methanol) for 30 min. The sections were pre-treated by heating for 20 min in 0.01 M citric acid buffer (pH 6) in a microwave oven at 800 W. After cooling for 20 min at room temperature, tissue sections were washed with PBS and incubated with 10% normal goat serum for 30 min for protein blocking to prevent the non-specific binding of antibodies, followed by incubation with the primary antibody to proliferating cell nuclear antigen (**PCNA**) (MAB424, mouse anti PCNA monoclonal antibody, PC10 clone; EMD Millipore, Darmstadt, Germany) at dilutions of 1:100 overnight at 4°C. After incubation with the primary antibodies, the tissue sections were washed with PBS and incubated with a biotinylated secondary antibody (Goat anti-mouse IgG, Invitrogen, CA, USA) for 30 min at room temperature. Background controls were included by replacing the primary antibodies with PBS. After a PBS wash, tissue sections were incubated using a streptavidin horseradish peroxidase kit (Histostain-Plus IHC Kit, HRP, broad spectrum, Invitrogen, CA, USA) for 30 min at room temperature. A final PBS wash was followed by incubation for color development with 3,3-diaminobenzidine tetrahydrochloride (DAB, Invitrogen, CA, USA) for 3 min at room temperature. Tissue sections were counterstained with Gill's hematoxylin, dehydrated in graded alcohols, applied to a coverslip using Entellan (Merck, Darmstadt, Germany), and examined with a Leica DM2500 light microscope. All images were captured with a digital camera (Leica DFC450, Leica Microsystems GmbH, Wetzlar, Germany) and processed with Image J. PCNA-positive nuclei (Figure 1) of total crypt epithelial cells on

10 different randomly selected intact crypts, regardless of the staining intensity, were counted as described by Bologna-Molina et al. (2011).

Secretory IgA Analysis

Jejunal mucosa was weighed as 0.5 g and diluted into 9 mL of 0.9% physiological saline. After centrifugation at 6,000 × *g* for 15 min at +4°C, supernatants were transferred into new 1.5 mL sterile microcentrifuge tubes. Secretory IgA (**sIgA**) ELISA Kit (Elabscience Biotechnology Co., Wuhan, PRC) was used in accordance with the manufacturer's instructions for sIgA concentration on days 14 and 28. Absorbance was read at 450 nm with an automated ELISA reader (BioTek ELx800 Absorbance Microplate Readers, Biotek, VT, USA).

Cecal Microbiota Analysis

DNA Extraction Genomic DNA from each sample was isolated from 200 mg of cecal content using GeneMatrix Stool DNA Purification Kit (EURx, Gdansk, Poland). All procedures were performed according to the manufacturer's instructions. The quantity of DNA was measured spectrophotometrically at 260 nm using Epoch Microplate Spectrophotometer (Biotek, VT, USA).

Preparation of External Standards and qPCR Analysis *Paenibacillus xylanexedens* ysm1 was incubated in nutrient broth, and *E. coli* (ATCC 2592 strain) was incubated in Triptic soy broth for 24 h at 37°C under aerobic conditions. DNA was extracted from bacterial colonies using the GeneMATRIX Bacterial & Yeast Genomic DNA Purification Kit (EURx, Gdansk, Poland). Standard curves were generated using PCR product of *E. coli* and *P. xylanexedens* ysm1. For each bacterial standard, dilutions of 1/10, 1/100, 1/1000, 1/10000, 1/100000 were prepared. The dilutions and each DNA sample from cecal contents were all subjected to the real-time PCR procedure in Qiagen Rotor-Gene Q (Qiagen, MA, USA). Amplicons from *E. coli* were used for quantification of total bacteria and cecal *E. coli*, and amplicons from *P. xylanexedens* ysm1were used for quantification of *P. xylanexedens* ysm1.

The amplifications were carried out using a final volume of 25 μL containing 12.5 μL GoTaq qPCR master mix (Promega, WI, USA), 100 ng DNA template, 0.5 μL each primer (0.1 μM) [BactF 5′-AGA GTT TGA TCC TGG CTC AG-3′ and BactR 5′-AAG GAG GTG ATC CAG CCG CA-3′ primers (Lane, 1991); *E. coli* F: 5′-CATGCCGCGTGTATGAAGAA-3′; R: 5′-CGGGTAACGTCAATGAGCAAA-3′ (Huijsdens et al., 2002) and *P. xylanexedens* ysm1 F: 5′-GTGAGCCATTACCCCACCAA-3′ and R: 5′-GCCCTCAAGTTTGGGACAAC-3′ (this study)], and nuclease free water. The qPCR conditions were 95°C for 2 min, followed by 40 cycles of 95°C for 15 s, 60°C for 60 s.

Table 2. Effects of *Paenibacillus xylanexedens* ysm1 on growth performance in broilers.[1]

	Dietary treatment[2]					
Item[3]	NC	PC	PRO	ANT	SEM	*P*-value
0 to 7 D						
BWG (g)	128.3	128.6	128.2	129.0	0.18	0.392
FI (g)	141.9	144.4	143.4	144.3	0.88	0.754
FCR	1.106	1.122	1.119	1.118	0.01	0.881
0 to 14 D						
BWG (g)	358.7	354.6	362.8	368.3	1.92	0.063
FI (g)	425.1[b]	446.8[a]	434.7[a,b]	439.7[a,b]	2.72	0.028
FCR	1.185[b]	1.261[a]	1.199[b]	1.194[b]	0.01	0.004
0 to 21 D						
BWG (g)	797.2[a,b]	751.7[b]	784.4[a,b]	803.4[a]	7.09	0.039
FI (g)	1,013	1,009	996.0	1,016	6.41	0.711
FCR	1.271[b]	1.343[a]	1.270[b]	1.267[b]	0.01	<0.001
0 to 28 D						
BWG (g)	1,382[a,b]	1,332[b]	1,407[a,b]	1,454[a]	13.48	0.007
FI (g)	1,823	1,870	1,845	1,890	14.69	0.417
FCR	1.320[b]	1.404[a]	1.311[b]	1.300[b]	0.01	<0.001

[a,b]Means with different superscripts in the same row are significantly different ($P < 0.05$).

[1]Data represent mean values of 8 replicates per treatment.

[2]NC: birds fed a basal diet and not challenged with *Escherichia coli* K88; PC: birds fed a basal diet and orally challenged with of *E. coli* K88; PRO: birds fed a diet supplemented with 1×10^9 *P. xylanexedens* ysm1 cfu/kg feed and orally challenged with *E. coli K88*; ANT: birds fed a diet supplemented with 20 mg of colistin sulfate/kg of feed and orally challenged with *E. coli* K88.

[3]BWG: body weight gain; FI: feed intake; FCR: feed conversion ratio.

Statistical Analysis

Data were analyzed using the ANOVA procedure of SPSS version 14.01 (SPSS Inc., Chicago, IL, USA). Significant differences among treatment groups were tested by Tukey multiple range tests. Statistical differences were considered significant at $P \leq 0.05$.

RESULTS

Growth Performance

The *E. coli* K88 challenge decreased BWG in PC birds compared to the ANT birds on day 21 ($P = 0.039$) and day 28 (P = 0.007) (Table 2). FI was higher ($P = 0.028$) in PC birds in comparison to NC birds on day 14. No significant differences in FI were observed on days 7, 21, and 28. The FCR was higher in PC birds compared with the NC, PRO, and ANT birds on days 14 ($P = 0.004$), 21 ($P < 0.001$), and 28 ($P < 0.001$). No significant mortality was observed during the entire experimental period.

Morphological Measurements of the Jejunum and Ileum

Day 14 Morphological measurements of jejunal and ileal tissues are shown in Table 3. No significant differences were observed among the dietary treatment groups in terms of VH, CD, and VH:CD ratio in the jejunum on day 14. However, the *E. coli* K88 challenge reduced ($P = 0.004$) ileum VH in the PC and PRO birds

Table 3. Effects of *Paenibacillus xylanexedens* ysm1 on intestinal morphology of the jejunum and ileum on days 14 and 28.[1]

Item	Dietary treatment[2]				SEM	*P*-value
	NC	PC	PRO	ANT		
Day 14						
Jejunum						
Villus height (μm)	496.8	455.6	485.1	487.9	6.78	0.154
Crypt depth (μm)	86.38	79.38	82.00	80.75	1.43	0.351
VH:CD[3]	5.77	5.75	6.01	6.05	0.10	0.653
Ileum						
Villus height (μm)	380.1[a]	357.9[b,c]	351.4[c]	374.6[a,b]	3.44	0.004
Crypt depth (μm)	68.64[a,b]	67.94[b]	73.20[a]	72.93[a]	0.73	0.006
VH:CD	5.54[a]	5.28[a,b]	4.80[c]	5.14[b,c]	0.06	<0.001
Day 28						
Jejunum						
Villus height (μm)	1,067[a]	910.4[c]	985.1[b]	1016[a,b]	13.58	<0.001
Crypt depth (μm)	108.0[a]	90.21[d]	96.10[c]	101.7[b]	1.33	<0.001
VH:CD	9.89	10.09	10.25	9.99	0.08	0.414
Ileum						
Villus height (μm)	532.8[a]	413.9[b]	511.8[a]	521.3[a]	9.07	<0.001
Crypt depth (μm)	95.43[a]	80.05[c]	91.06[b]	95.11[a]	1.16	<0.001
VH:CD	5.58[a]	5.17[b]	5.62[a]	5.48[a,b]	0.05	0.003

[a–d]Means with different superscripts in the same row are significantly different ($P < 0.05$).
[1]Data represent mean values of 8 replicates per treatment.
[2]NC: birds fed a basal diet and not challenged with *Escherichia coli* K88; PC: birds fed a basal diet and orally challenged with of *E. coli* K88; PRO: birds fed a diet supplemented with 1×10^9 *P. xylanexedens* ysm1 cfu/kg feed and orally challenged with *E. coli K88*; ANT: birds fed a diet supplemented with 20 mg of colistin sulfate/kg of feed and orally challenged with *E. coli* K88.
[3]Villus height to crypt depth ratio.

when compared with non-challenged birds. Dietary supplementation of *P. xylanexedens* ysm1 and colistin sulfate significantly increased CD ($P = 0.006$) and reduced VH:CD ratio ($P < 0.001$) in the ileum.

Day 28 Morphological measurements of intestinal tissues are shown in Table 3. In both jejunum and ileum, VH was decreased in the PC birds compared with the NC, PRO, and ANT birds ($P < 0.001$). Similarly, the lowest CD ($P < 0.001$) was observed in both jejunum and ileum of PC birds on day 28.

Goblet Cells and PCNA-Positive Cells

Goblet cells and PCNA-positive cell counts of the jejunum and ileum are shown in Figure 2 and Figure 3, respectively. The number of goblet cells was significantly higher ($P < 0.001$) in both jejunum and ileum of the PC group when compared with the other treatment groups on day 28. Irrespective of the dietary treatments, inoculation of *E. coli* K88 reduced ($P < 0.001$) the number of PCNA-positive cells in both jejunum and ileum on day 28.

Total sIgA Concentration in the Jejunum Mucosa

Dietary supplementation with *P. xylanexedens* ysm1 resulted in significantly higher concentrations of the jejunum mucosal sIgA (Figure 4A) as compared with the other treatment groups on both days 14 and 28 ($P < 0.001$).

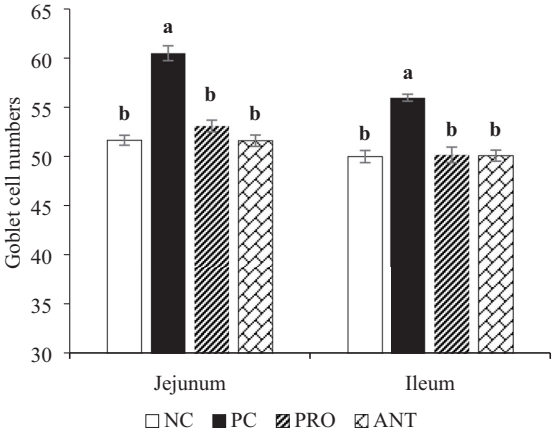

Figure 2. Effects of *Paenibacillus xylanexedens* ysm1 on jejunum and ileum goblet cell numbers on day 28. Each bar represents mean ± SE values of 8 replicates per treatment. Bars with different letters [(a,b)] differ significantly ($P < 0.001$).

Cecal Microbial Analysis

On days 21 and 28, the ratio of cecal *E. coli* was higher in the PC group compared to the NC group. As in the PRO group, there was no difference between *E. coli* cecal content on days 14 and 21 compared to the PC group. When comparing the cecal content of PC, PRO, and ANT groups on day 28, the amount of *E. coli* was lower ($P = 0.017$) in the PRO and ANT groups

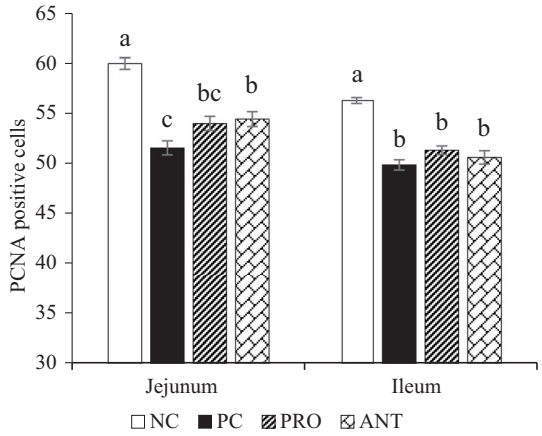

Figure 3. Effects of *Paenibacillus xylanexedens* ysm1 on proliferating cell nuclear antigen (PCNA) cell counts in jejunum and ileum on day 28. Each bar represents mean ± SE values of 8 replicates per treatment. Bars with different letters [(a-c)] differ significantly ($P < 0.001$).

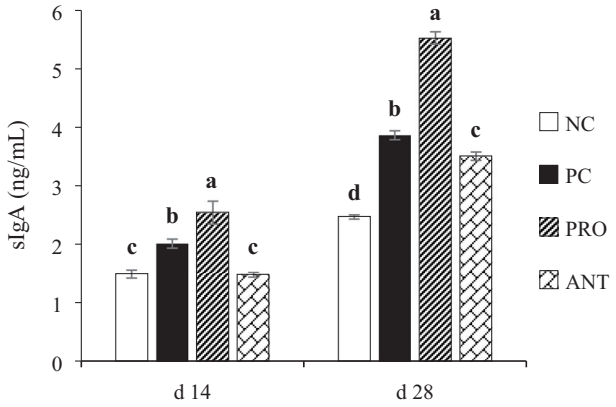

Figure 4. Effects of *Paenibacillus xylanexedens* ysm1 on concentration of sIgA in the jejunum of broilers. Each bar represents mean ± SE values of 8 replicates per treatment. Within the same day, bars with different letters [(a-d)] differ significantly ($P < 0.001$)

(Figure 5). The *P. xylanexedens* ysm1 was found only in cecal contents of the PRO group (data not shown).

DISCUSSION

Among the strategies to reduce the use of antibiotics in the broiler industry, the use of dietary probiotics is becoming an accepted alternative because of their beneficial effects on health and performance without having the risk of drug residues or antibacterial resistance. In this context, the present study investigated the effect of a new isolate *P. xylanexedens* ysm1 on broiler performance, morphology, gut microbiota, and immune response of broiler chickens challenged with *E. coli* K88.

As an expected outcome, the *E. coli* challenge retarded the growth of broilers in the PC group. However, dietary supplementation of *P. xylanexedens* ysm1 alleviated the growth suppression effect of the *E. coli* challenge but no significant differences were observed between the probiotic and antibiotic treated birds in terms of their BWG, FI, and FCR during the entire experimental period (day 0 to 28). Our results are in

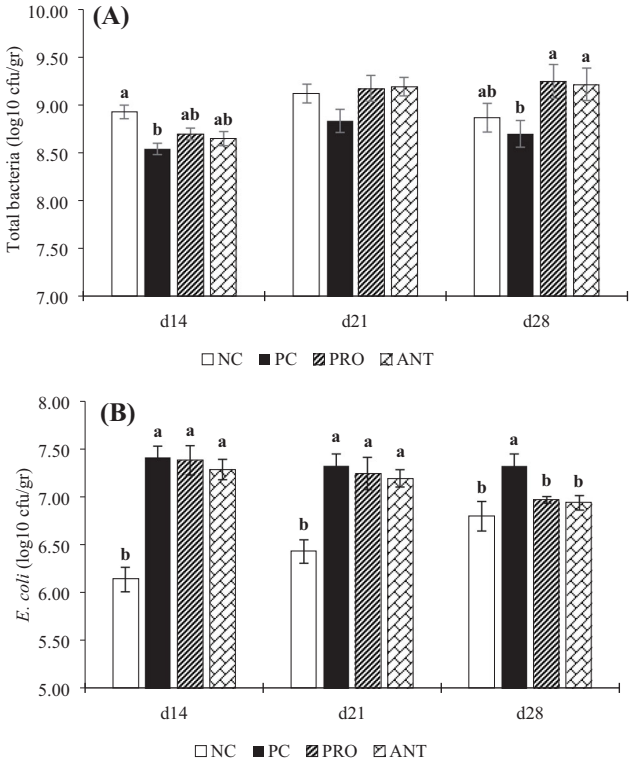

Figure 5. Effects of *Paenibacillus xylanexedens* ysm1 on cecal total bacteria (A) and *Escherichia coli* (B) populations in broilers. Each bar represents mean ± SE values of 8 replicates per treatment. Within the same day, bars with different letters [(a-b)] differ significantly ($P < 0.05$).

agreement with those of Calik et al. (2017) who reported that dietary supplementation of *P. xylanexedens* ysm1 (1×10^9 cfu/feed) resulted in improved performance from day 0 to 21. Other studies also revealed that dietary supplementation of several probiotics, such as *Enterococcus faecium* (Cao et al., 2013), *Clostridium butyricum* (Zhang et al., 2014), *Lactobacillus plantarum* B1 (Wang et al., 2017), and *B. subtilis* (Manafi et al., 2017), significantly improved broiler performance during an *E. coli* challenge by improving intestinal integrity and immune status of the broiler chickens. The exact mechanisms underlying the growth promoting effects of *P. xylanexedens* ysm1 remain unclear, as there has been no available information of its effect on broilers. However, *P. xylanexedens* ysm1 might affect the growth performance through several mechanism(s), such as competitive colonization, improved intestinal integrity, and selectively stimulating intestinal microbiota (Daudelin et al., 2011).

Morphological changes in the small intestine, such as increased villus height and villus height to crypt depth ratio, are important parameters that affect broiler performance by improving nutrient digestion and absorption (Calik and Ergun, 2015). Previous studies showed that a diverse variety of probiotics might be used to maintain intestinal integrity (Awad et al., 2009; Zhang et al., 2016). Based on the present findings, there was no improvement in villus height and VH:CD in jejunum and ileum on day 14, which was likely due to the acute phase of the infection or consecutive oral

gavaging with *E. coli* on days 7 and 10. However, as hypothesized in this study, dietary supplementation of *P. xylanexedens* ysm1 had a pronounced effect on villus height and VH:CD in both jejunum and ileum on day 28, which was accompanied by enhanced growth performance. Similar results were reported by Calik et al. (2017) where dietary application of *P. xylanexedens* ysm1 improved intestinal histomorphology in non-challenged broiler chickens. However, in contrast to well-established probiotics, there is limited reporting on the effects of *P. xylanexedens* ysm1 on broiler intestinal morphology during a pathogenic challenge. In agreement with our results, Cao et al. (2013) reported that dietary *E. faecium* supplementation increased jejunal villus height after an *E. coli* K88 challenge in broilers. More recently, Wang et al. (2017) concluded that *L. plantarum* B1 could alleviate the negative effects of colibacillosis on the intestinal epithelium. Such enhancements in intestinal integrity might be related to the antibacterial activity of the *P. xylanexedens* ysm1 against *E. coli* K88 and modification of the intestinal microbiota and increased abundance of bacterial metabolites such as butyrate, which induces enterocyte differentiation and proliferation.

The intestinal mucous layers, synthesized and secreted by goblet cells, cover the gut epithelium with compact viscous, permeable, and gel-forming mucin to provide a frontline defense against microbes, pathogenic microorganisms, environmental toxins, and other dietary components (Kim and Ho, 2010; Calik et al., 2017). Although mucin is continuously secreted at a basal level, the composition and thickness of this mucous layer can change rapidly in response to pathogenic microorganisms or microbial products and are modulated by the underlying innate and adaptive immune responses (Kim and Ho, 2010; Ashida et al., 2011; McGuckin et al., 2011). In addition to its adverse effect on intestinal morphology, our results revealed that the *E. coli* challenge also had a pronounced effect on goblet cells by increasing their numbers in the jejunum and ileum. In agreement with our results, previous studies reported an increase in the intestinal goblet cell numbers during *E. coli* (Almeida et al., 2013; Manafi et al., 2017) and *Salmonella enterica* serovar Typhimurium (Almeida et al., 2014) challenges. As an important finding of this study, dietary supplementation of *P. xylanexedens* ysm1 significantly reduced the number of goblet cells as efficiently as the antibiotic treatment, most likely due to the reduced population of *E. coli*, modification of the intestinal bacteria, or intestinal immune responses. Increase in villus height and decrease in the goblet cell numbers in the probiotic group might help to explain the improvements in growth performance, because thicker mucus may limit the diffusion of nutrients (Bontempo et al., 2006), and increased demand for mucosal secretion inevitably leads to increased endogenous loss, since it contains important nutrients such as serine, threonine, proline, and cysteine (Cowieson et al., 2004).

Intestinal epithelial cells have a short lifespan and need to be replenished rapidly and continuously via the replication of undifferentiated cells. Precursor cells or proliferative cells, which are being continuously generated in the crypt regions, terminally differentiate into secretory cells (goblet, enteroendocrine, and Paneth) or absorptive enterocytes (Fre et al., 2005; van der Flier and Clevers, 2009). These proliferating cells can be observed by immunohistochemical staining of an endogenous protein called PCNA, also known as cyclin or DNA-polymerase delta auxiliary protein (Foley et al., 1993; Uni et al., 1998; Gulbahar et al., 2005). In the current study, while challenged birds had similar numbers of PCNA-positive cells, birds in the probiotic group had higher intestinal villus height and lower goblet cell numbers compared to positive control birds. We could speculate that dietary supplementation of *P. xylanexedens* ysm1 might have influenced the cell fate decision, directly or indirectly, during the differentiation steps towards absorptive enterocytes rather than mucin-filled secretory goblet cells. The exact mechanisms of how the microbiota modulates the proliferation and differentiation of cell lineages to absorptive or secretary cells are still unclear. However, previous studies reported relationships between intestinal microbiota and cell fate decision pathways (Broderick et al., 2014; Troll et al., 2018). Further investigations on host–microbiome interaction, using RNA-seg methods, could help to reveal differentiation and proliferation dynamics of the intestinal epithelial cells.

The intestinal mucosal surface has a variety of defense mechanisms which protect the host against pathogens and toxins to maintain local homeostasis. Secretory IgA acts as a first line defense mechanism that promotes immune exclusion by entrapping harmful microorganisms and keeping the commensal bacteria in balance to ensure their controlled survival (Cerutti and Rescigno, 2008; Corthesy, 2013). The concentration of sIgA in the intestine is an important parameter to assess mucosal immunity (Wang et al., 2017). We observed a significantly higher level of sIgA concentration in the jejunum by dietary addition of the new probiotic *P. xylanexedens* ysm1. Previous studies revealed that dietary probiotic supplementation stimulated the intestinal sIgA production compared to control birds under challenge (Wang et al., 2017) and non-challenge (Amerah et al., 2013; Peng et al., 2016) conditions. Furthermore, increased levels of sIgA might help to increase nutrient absorption (Peng et al., 2016). Along with the improvements in intestinal morphology, this finding suggests that dietary addition of *P. xylanexedens* ysm1 appears to improve intestinal mucosa health, maintain barrier functions, and alleviate inflammatory response under *E. coli* K88 challenge.

Along with the modulatory effects on intestinal epithelial barrier and immune function, one of the well-known modes of action of probiotics is competitive exclusion or inhibition of pathogens in the gastrointestinal tract (Lebeer et al., 2010). On day 28 of the study,

despite the recurrence of *E. coli* challenge, probiotic and antibiotic administration had equivalent effects against *E. coli* K88 infection, considering the decrease in *E. coli* rates in PRO and ANT groups compared to the PC group. This can be correlated with our previous in vitro study results where *P. xylanexedens* ysm1 exhibited an antimicrobial activity against *E. coli.* Moreover, our findings are consistent with previous reports whereby administration of probiotics (such as *B. subtilis*, *E. faecium*, *Lactobacillus* sp., etc.) reduced colonization of *E. coli* (Cao et al., 2013), *Salmonella* Enteritidis (Mountzouris et al., 2009), and *Clostridium perfringens* (Jayaraman et al., 2013).

In conclusion, the present study demonstrated that dietary supplementation of a new *P. xylanexedens*-based probiotic improved broiler performance by modulating intestinal morphology, enhancing immune response, and reducing the number of *E. coli* in the cecum. Due to its spore-forming ability and potential beneficial effects on broiler performance and health, it might be used in the broiler industry as a promising alternative to antibiotic growth promoters.

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CONFLICT OF INTEREST STATEMENT

The authors declare that there are no conflicts of interest.

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