Orally delivered *Bacillus subtilis* expressing chicken NK-2 peptide stabilizes gut microbiota and enhances intestinal health and local immunity in coccidiosis-infected broiler chickens[1]

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ABSTRACT We recently reported a stable *Bacillus subtilis*-carrying chicken NK-lysin peptide (***B. subtilis*-cNK-2**) as an effective oral delivery system of an antimicrobial peptide to the gut with therapeutic effect against *Eimeria* parasites in broiler chickens. To further investigate the effects of a higher dose of an oral *B. subtilis*-cNK-2 treatment on coccidiosis, intestinal health, and gut microbiota composition, 100 (14-day-old) broiler chickens were allocated into 4 treatment groups in a randomized design: 1) uninfected control (**CON**), 2) infected control without *B. subtilis* (**NC**), 3) *B. subtilis* with empty vector (**EV**), and 4) *B. subtilis* with cNK-2 (**NK**). All chickens, except the CON group, were infected with 5,000 sporulated *Eimeria acervulina* (***E. acervulina***) oocysts on d 15. Chickens given *B. subtilis* (EV and NK) were orally gavaged (1×10^{12} cfu/mL) daily from d 14 to 18. Growth performances were measured on d 6, 9, and 13 postinfection (**dpi**). Spleen and duodenal samples were collected on 6 dpi to assess the gut microbiota, and gene expressions of gut integrity and local inflammation makers. Fecal samples were collected from 6 to 9 dpi to enumerate oocyst shedding. Blood samples were collected on 13 dpi to measure the serum 3−1E antibody levels. Chickens in the NK group showed significantly improved ($P < 0.05$) growth performance, gut integrity, reduced fecal oocyst shedding and mucosal immunity compared to NC. Interestingly, there was a distinct shift in the gut microbiota profile in the NK group compared to that of NC and EV chickens. Upon challenge with *E. acervulina*, the percentage of *Firmicutes* was reduced and that of *Cyanobacteria* increased. In NK chickens, however, the ratio between *Firmicutes* and *Cyanobacteria* was not affected and was similar to that of CON chickens. Taken together, NK treatment restored dysbiosis incurred by *E. acervulina* infection and showed the general protective effects of orally delivered *B. subtilis*-cNK-2 on coccidiosis infection. This includes reduction of fecal oocyst shedding, enhancement of local protective immunity, and maintenance of gut microbiota homeostasis in broiler chickens.

Key words: NK-lysin, chicken, *Bacillus subtilis*, coccidiosis, antimicrobial peptide

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INTRODUCTION

Emerging concerns of antimicrobial resistance have caused many countries to limit or ban the usage of in-feed sub-therapeutic antibiotics in the livestock industry, creating an urgent need to develop sustainable alternative antibiotic approaches to control enteric diseases such as coccidiosis (Lee and Lillehoj, 2011). Recently, we reported the clinical success of an oral-delivery system of *B. subtilis* spores expressing a novel chicken NK-2 antimicrobial peptide (**AMP**) as a novel alternative to antibiotics to combat avian coccidiosis (Wickramasuriya et al., 2021). To expand upon these findings, we conducted this current study to show the impact of increased dosage and lengthened dosage schedule. We further explored the direct impact of treatment on the chicken gut microbiome.

AMPs are biological molecules abundant in hydrophobic cationic residues and facilitate interaction with microbial outer membranes via their amphipathic structure (Wang et al., 2014; Gadde et al., 2017; Kim et al., 2017a). Also, AMPs have been shown to mediate the immunomodulation of inflammatory responses and gut homeostasis maintenance (Gubatan et al., 2021). Based

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on the AMP's effectiveness in microbial control, they are recognized as evolutionarily conserved defense proteins against a spectrum of microbes (Wang et al., 2016). Some AMPs, such as AMP-P5, AMP-A3, NK-Lysin, and cecropin A-D-Asn have been studied for use in broilers against several infections including coccidiosis (Wen and He, 2012; Lillehoj et al., 2014; Wang et al., 2016).

Among these AMP's, NK-lysin is an immunomodulatory AMP originally identified in the natural killer cells of porcine intestinal tissue (Andersson et al., 1995). It is an orthologue to human granulysin and has shown cytolytic activities towards microbes and tumor cells (Lee et al., 2014). Suggesting its role in parasite infection, chicken NK-lysin (**cNK-lysin**) was discovered in *Eimeria*-infected chicken intestinal lymphocytes (Hong et al., 2006a). In later studies, it was reported that the cNK-2 synthetic peptide with the core α-helical region is more effective than other cNK-lysin-derived peptides without this core region (Lee et al., 2013; Kim et al., 2017a). Moreover, the antiparasitic activity of cNK-2 against apicomplexan parasites such as *Eimeria* spp., via disrupting the parasitic membrane and modulating local inflammatory response when delivered to the gut, was reported using in-vivo and in-vitro experimental conditions (Hong et al., 2008; Lee et al., 2013; Kim et al., 2017b).

Avian coccidiosis is caused by several distinct species of intracellular *Eimeria* parasites and costs the global chicken industry more than 14 billion USD annually (Blake et al., 2020). Moreover, *Eimeria* spp. develop drug resistance quickly and few new drugs are being developed against these resistant strains. Hence, there is a timely need to develop antibiotic-independent strategies to mitigate losses due to coccidiosis. Previous studies have characterized the antiparasitic effects of cNK-2 (Hong et al., 2006a; Hong et al., 2008). However, knowledge pertaining to the successful oral delivery to preserve the functional activity of cNK-2 peptide in the chicken gut was not available until our recent report (Wickramasuriya et al., 2021). Our work demonstrated that oral administration of this AMP improved growth performance, enhanced gut integrity and reduced parasite fecundity in *Eimeria acervulina*-challenged broiler chickens. (Wickramasuriya et al., 2021).

It has been previously shown that changes in the intestinal microbiota occur in chickens treated with an AMP under different challenge conditions, including coccidiosis (Hume et al., 2011; Daneshmand et al., 2019; Wang et al., 2020; Madlala et al., 2021). Salavati et al. (2020) reported that a bioactive peptide derived from sesame meal elevated *Lactobacilli* population in the caecum of broiler chickens. Although *Eimeria* infection alone had little or no effect on α-diversity and caecal microbiota, *Firmicutes* (commensal bacteria) and *Streptococcus* (opportunistic pathogens) were altered, creating dysbiosis in the chicken gut (Madlala et al., 2021). To understand the underlying mechanism of protection mediated by oral *B. subtilis*-cNK-2 peptide delivery strategy, we investigated the chicken immune response and microbiota composition under *Eimeria* challenge conditions in infected broiler chickens.

MATERIALS AND METHODS

Chickens and Animal Care

A total of 100 one-day-old Ross broiler chickens (Ross 708) were obtained from a local hatchery (Longnecker Hatchery, Elizabethtown, PA). Up to 14 d of age, chickens were raised in Petersime brooder units housed in a temperature-controlled closed-house environment. After 14 d, chickens were moved to experimental grower cages. Chickens had ad libitum access to fresh clean water and nonmedicated commercial feed until the end of the experimental period. Experimental procedures were approved by the Beltsville Agricultural Research Center Small Animal Care Committee (Animal Protocol No. 20-002).

Experimental Design

On d 14, body weights were measured, and chickens were randomly allocated into the 4 treatment groups, maintaining the same body weight and weight distribution among treatments. Each treatment contained 5 cages with 5 chickens each. Experimental treatments included: 1) uninfected control (**CON**), 2) infected control without *B. subtilis* (**NC**), 3) infected treatment administered with *B. subtilis* empty vector (**EV**: 10^{12} cfu/d/chicken), and 4) infected treatment administered with *B. subtilis* expressing cNK-2 peptide (**NK**: 10^{12} cfu/d/chicken). The spores of recombinant *B. subtilis* expressing the cNK-2 peptide were produced by US Biologic, Inc. (Memphis, TN) as described previously (Wickramasuriya et al., 2021). From d 14 up to d 18, *B. subtilis* empty vector and *B. subtilis* expressing cNK-2 were orally gavaged (1 mL/chicken) to the chickens in respective treatments (Figure 1).

Freshly propagated *E. acervulina* (ARS Beltsville strain #12) was used to infect the chickens. On d 15 after hatching, all chickens except the CON chickens were infected with *E. acervulina* oocysts (5,000/chicken) through oral gavage.

Body Weight Measurement

All chickens were weighed individually on d 14, 21, 24, and 28 (-1, 6, 9, and 13 dpi) to measure body weight and to calculate weight gain.

Oocyst Shedding

From d 21 (6 dpi), fecal samples from every cage were collected until d 24 (9 dpi). The collected fecal samples were processed according to the method described by Lee et al. (2018). Briefly, feces collected from individual cages were ground and homogenized with 3 liters of water. Two subsamples from each cage were placed into

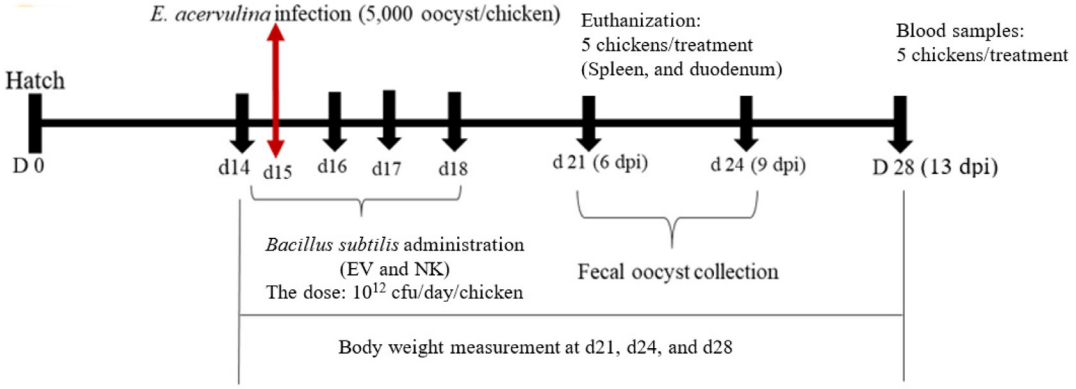

Figure 1. Schematic outline of the experimental design. All chickens except uninfected control (CON) were infected by oral gavage at d 15 with 5,000 oocysts/chicken of *E. acervulina*. *Bacillus subtilis* was administrated by oral gavage at - 1 to 4 d postinfection. Abbreviations: EV, *Bacillus subtilis* (empty vector) at 10^{12} cfu/d; NK, *Bacillus subtilis* expressing cNK-2 at 10^{12} cfu/d.

50 mL tubes for oocyst counting, and serial dilutions were made for the enumeration of oocysts for each sample. Three different scientists counted oocysts microscopically with a McMaster counting chamber using a sodium chloride flotation method (Lee et al., 2018; Wickramasuriya et al., 2021). The total number of oocysts shed per chicken was calculated using the following formula:

Total oocysts/chicken = (oocyst count × dilution factor × fecal sample volume/counting chamber volume)/number of chickens per cage.

Sample Collection

On d 21 (6 dpi), 5 chickens from each treatment group were randomly selected for sample collection. After humanely sacrificing chickens by cervical dislocation, the spleen, and the mid-duodenum were obtained. Collected intestinal samples were stored in plastic external thread cryogenic vials and immersed in liquid nitrogen immediately followed by storage at −80°C. Samples were sent to Clear labs (San Carlos, CA) for DNA isolation and whole microbiome sequencing. The remainder of the chicken duodenum tissues were scrapped aseptically to collect the mucosa using a tissue scraper (Wickramasuriya et al., 2021). Collected duodenum mucosa and spleen samples were stored in RNA stabilization solution (RNAlater solution, Invitrogen Corporation, Carlsbad, CA) at −20°C.

Blood samples were collected on d 28 for serum separation (5 chickens/treatment). Separated sera were stored at −20°C for antibody analysis.

RNA Extraction and qRT-PCR

Collected tissue samples were gently washed with ice-cold Hank's balanced salt solution (Sigma-Aldrich, St. Louis, MO) and homogenized using a handheld homogenizer (TissueRuptor; Qiagen, Hilden, Germany). Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) followed by DNase digestion as described (Park et al., 2020). Quantification and purity were assessed using a NanoDrop spectrophotometer (NanoDrop One; Thermo Scientific, Wilmington, DE) at 260/280 nm. Synthesis of cDNA was performed using a QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany) per the manufacturer's instructions. The cDNA samples were diluted to 1:5, and 5-μL aliquots were used for qRT-PCR amplification. The sample was analyzed using SYBR Green qPCR Master Mix (PowerTrack, Applied Biosystems, Vilnius, Lithuania) in triplicates using Applied Biosystems QuantStudio 3 Real-Time PCR Systems (Life Technologies, Carlsbad, CA). The following PCR conditions were followed: denaturation at 95°C for 2 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Glyceraldehyde 3-phosphate dehydrogenase was used as the reference gene for gene expression.

The gene expression levels of TJ proteins, such as junctional adhesion molecule 2 (**JAM-2**), occluding (**OCLN**), and zonula occludens 1 (**ZO-1**), and mucin (**MUC-2**) expression in the duodenum samples and antioxidant markers, including superoxide dismutase type 1 (**SOD-1**), heme oxygenase 1 (**HMOX-1**), and catalase (**CAT**) in both duodenum and spleen samples were investigated. The cytokine gene expression levels of duodenum mucosa including Interleukin (**IL**)-1β, IL-2, IL-17F, IL-22, IL-10, IL-6, interferon-gamma (**IFN-γ**), and tumor necrosis factor α (**TNF-α**) were also quantified. All oligonucleotide primer sequences used in this experiment are shown in Table 1. For the relative quantification of the gene expression levels, the logarithmic-scaled threshold cycle (**Ct**) values were used in the $2^{-\Delta\Delta Ct}$ method before calculating the mean and standard error of the mean (**SEM**) for the references and individual targets.

Antibody Enzyme-linked Immunosorbent Assay

For assessing serum antibody response to *Eimeria* infection, 3-1E antigen was used in enzyme-linked immunosorbent assay as described (Lillehoj et al., 2000; Ding et al., 2004). Briefly, flat-bottom microtiter plates

Table 1. Quantitative real-time PCR oligonucleotide primer sequences.

Target gene	Primer sequence	Accession no.
GAPDH	F 5′-GGTGGTGCTAAGCGTGTTAT-3′ R 5′-ACCTCTGTCATCTCTCCACA-3′	K01458
JAM-2	F: 5′-AGCCTCAAATGGGATTGGATT-3′ R: 5′-CATCAACTTGCATTCGCTTCA-3′	NM0,010,06257.1
OCLN	F: 5′-GAGCCCAGACTACCAAAGCAA-3′ R: 5′-GCTTGATGTGGAAGAGCTTGTTG-3′	NM205,128.1
ZO-1	F: 5′-CCGCAGTCGTTCACGATCT-3′ R: 5′-GGAGAATGTCTGGAATGGTCTGA-3′	XM01,527,8981.1
MUC-2	F: 5′-GCCTGCCCAGGAAATCAAG-3′ R: 5′-CGACAAGTTTGCTGGCACAT-3′	NM0,013,18434.1
HMOX-1	F 5′-CTGGAGAAGGGTTGGCTTTCT-3′ R 5′-GAAGCTCTGCCTTTGGCTGTA-3′	NM205344
SOD-1	F 5′-ATTACCGGCTTGTCTGATGG-3′ R 5′-CCTCCCTTTGCAGTCACATT-3′	NM205064.1
CAT	F 5′-ACTGCAAGGCGAAAGTGTTT-3′ R 5′-GGCTATGGATGAAGGATGGA-3′	NM001031215.1
IL-1β	F: 5'-TGGGCATCAAGGGCTACA-3' R: 5'-TCGGGTTGGTTGGTGATG-3'	NM_204524.1
IL-2	F: 5'-TACAGATAACTGGGACACTG-3' R: 5'-GTCTCAGTTGGTGTGTAGAG-3'	NM_204153.1
IL-6	F: 5'-CAAGGTGACGGAGGAGGAC-3' R: 5'-TGGCGAGGAGGGATTTCT-3'	NM_204628
IL-10	F 5′-CGGGAGCTGAGGGTGAA-3' R 5′-GTGAAGAAGCGGTGACAGC-3'	NM_001004414.2
IL-17F	F 5′-GAGAAGAGTGGTGGGAAAG-3′ R 5′-TCTACAAACTTGTTTATCAGCAT-3′	JQ776598
IL-22	F 5′-ACCCGTATGCTGAGGATGTGG-3′ R 5′-CTTGTTCCCTCCCTTCTTTGG-3′	NM_001199614.1
TNF-α	F 5′-CGCTCAGAACGACGTCAA-3′ R 5′-GTCGTCCACACCAACGAG-3′	MF000729.1
IFN-γ	F: 5'-AGCTGACGGTGGACCTATTATT-3' R: 5'-GGCTTTGCGCTGGATTC-3'	NM_205149.1

Abbreviations: CAT, catalase; F, forward primer; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HMOX-1, heme oxygenase 1; IFN-γ, interferon-gamma; JAM-2, junctional adhesion molecule 2; MUC-2, mucin 2; OCLN, occludin; R, reverse primer; SOD-1, superoxide dismutase 1; TNF-α, tumor necrosis factor α; ZO-1, zonula occludens-1.

(96-well; Costar, Boston, MA) were coated with 100 μL of purified 3-1E protein (10 μg/mL) in 0.1 M sodium carbonate buffer (pH 9.6) at 4°C overnight. Plates were washed 2 times with phosphate-buffered saline (**PBS**), containing 0.05% Tween 20 (**PBST**; pH 7.2,). The wells were blocked with 100 μL of blocking buffer (Pierce Protein-Free T20, Rockford, IL) for 1 h at room temperature, followed by 100 μL of serum for 2 h at room temperature. After washing the wells 6 times with PBST, the plates were incubated for 30 min at room temperature with 100 μL of horseradish peroxidase-conjugated antichicken immunoglobulin G (**IgG**) (Sigma) diluted 1:4,000 in 1% PBS. The wells were washed 6 times with PBST and developed with 100 μL of 0.01% (wt/vol) tetramethylbenzidine (Sigma-Aldrich, St. Louis, MO) in 0.05 M phosphate-citrate buffer, pH 5.0, for 10 min followed by 50 μL of 2 N H_2SO_4. Optical density values were (OD 450) determined with an absorbance microplate reader (ELx800, BioTek Instruments, Inc., Winooski, VT) using Gen 5 microplate reader and imaging software (Gen5 v3.11, Agilent Technologies, Inc., Carlsbad, CA).

Microbiome Sequencing

16S rRNA gene library preparation and high-throughput sequencing were performed at Clear Labs (San Carlos, CA) according to the method described by Bolinger et al. (2021). Briefly, DNA extraction was performed on 2 technical replicates of each sample in a 96-well format, following a modified version of the NucleoSpin Food Kit (Marcherey-Nagel, Düren, Germany) protocol, and optimized at Clear Labs. Following extraction, the amplification of multiple DNA bar-coding markers was conducted using proprietary primers strategically designed to bind the 16S ribosomal RNA region. Products obtained after amplification were purified and prepared for sequencing. Samples analyzed in this study were sequenced in replicates to ensure the quality of the results using an in-house Illumina MiSeq instrument (Illumina, Inc., San Diego, CA) with a 300-cycle MiSeq (version 3) reagent kit. Thereafter, sequence processing and microbial classification were performed using the Clear lab pipeline leveraging an in-house database.

Calculations and Statistical Analysis

Oocyst count and body weight data were analyzed using Mixed Model (PROC MIXED) in SAS. A pen used as the experimental unit for oocyst count. The individual chicken was considered as the experimental unit for all other parameters. The results are given as least-squares means and pooled SEM. P-values < 0.05 were considered to be significant. When the P-value between treatments was less than 0.05, homogeneous subsets were evaluated by the PDIFF option in SAS. For

analysis of the microbiome data, samples were first analyzed using all counts, including *Eimeria* read counts and subsequently analyzed after removing *Eimeria* read counts where all remaining bacteria were normalized to a total of 100%. Read counts for each chicken were grouped per treatment and means of percentage read counts were calculated within treatment groups for each phylum and order. Percentage read counts and principal components for principal component analysis were calculated, analyzed, and visualized using vegan, dplyr, ggplot2, Hmisc, devtools, and ggbiplot packages in R Studio (Vincent, 2011; Team, 2013; Wickham, 2016; Wickham et al., 2019; Harrell and Dupont, 2020; Oksanen et al., 2020; Wickham et al., 2020). Variation in microbiome composition of chickens in the 4 different treatment groups were analyzed using permutational multivariate analysis of variance, following the Morisita-Horn dissimilarity method using vegan package in R.

To calculate the species diversity in the microbiome, the following Shannon-wiener diversity function was used as described by Kim et al. (2017c). After calculation, Kruskal-Wallis pairwise test for alpha diversity was performed using GraphPad Software (GraphPad Prism version 8.0.0 for Windows, San Diego, CA).

Shannon-wiener diversity function:

$$H' = \sum_{\infty}^{s} (P_i)[\text{In } (P_i)]$$

H' = Shannon-Wiener index of diversity
s = number of species in the community
P_i = proportion of total abundance represented by i^{th} species

RESULTS

Growth Performance

No significant differences ($P > 0.05$) in chicken body weights were observed among the 4 treatment groups at the beginning of the experiment (d 14, 0 dpi) (Table 2). At 6 dpi and 9 dpi, chickens in both the NC and the EV groups showed lower ($P < 0.05$) body weights compared to the CON group. In contrast, the body weights of chickens in the NK group were improved and similar ($P > 0.05$) to those of the CON group at 6 dpi and 9 dpi. At 13 dpi, chickens in all groups showed similar ($P > 0.05$) body weights.

Chickens in *E. acervulina*-infected groups (NC, EV, and NK) showed lower ($P < 0.05$) average daily gain (ADG) compared to those of the CON group from −1 to 6 dpi. However, the ADG of chickens in the NK group were higher ($P < 0.05$) than those of NC chickens from −1 to 6 dpi. From 6 dpi to 9 dpi and 13 dpi, the ADG of the chickens were not different ($P > 0.05$) among treatment groups. Considering the period of −1 to 9 dpi, the ADG of chickens in the NK group were higher ($P < 0.05$) compared to those in the NC group and comparable to ($P > 0.05$) the ADG of chickens in the CON group.

Table 2. Growth performance of *Eimeria acervulina*-infected chickens following *Bacillus subtilis* expressing cNK-2 treatment.

Item	CON	NC	EV	NK	SEM	*P*-value
BW, g						
Initial (-1 dpi)	343	343	341	341	9.0	0.998
6 dpi	762[a]	676[b]	691[b]	720[ab]	18.2	0.021
9 dpi	1010[a]	902[c]	914[bc]	990[ab]	26.6	0.019
13 dpi	1341	1250	1260	1309	32.8	0.159
ADG, g						
-1 to 6 dpi	59.9[a]	47.1[c]	50.0[bc]	53.6[b]	2.0	0.003
6 to 9 dpi	79.9	75.9	72.8	83.1	3.7	0.227
9 to 13 dpi	83	86.9	86.9	80	3.8	0.503
-1 to 9 dpi	73.1[a]	62.9[b]	63.4[b]	70.0[a]	2.1	0.007
Overall	70.6	65.2	65.5	68	1.9	0.146

Abbreviations: ADG, average daily gain; BW, bodyweight; BWG, body weight gain; CON, uninfected control dpi, days postinfection; EV, *Bacillus subtilis* (empty vector) at 10^{12} cfu/d; NC, infected control; NK, *Bacillus subtilis* expressing cNK-2 at 10^{12} cfu/d.

All chickens except CON were infected by oral gavage at d 15 with 5,000 oocysts/chicken of *E. acervulina*. *Bacillus subtilis* was administrated by oral gavage at - 1 to 4 dpi.

[a-c]Means in the same row with different superscripts differ ($P < 0.05$) and the difference was evaluated by PDIFF option in SAS when *P*-value between treatments was less than 0.05.

Fecal Oocyst Shedding

Chickens in *E. acervulina*-infected groups (NC, EV, and NK) produced higher ($P < 0.05$) numbers of fecal oocysts compared to those of the CON group from 6 to 9 dpi (Figure 2). Similar oocyst shedding ($P > 0.05$) was observed among chickens in NC and EV groups, but oocyst shedding was significantly reduced ($P < 0.05$) in the NK group compared to the NC and EV groups.

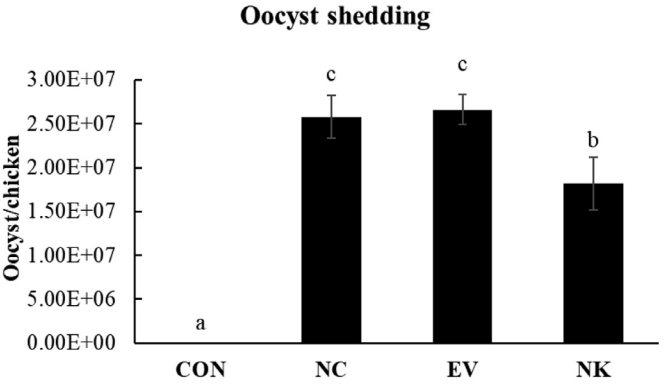

Figure 2. Fecal oocyst of *Eimeria acervulina*-infected chickens. All chickens except CON were infected by oral gavage at d 15 with 5,000 oocysts/chicken of *E. acervulina*. *Bacillus subtilis* was administrated by oral gavage at - 1 to 4 d postinfection. [a~c]Bars with no common letter differ significantly ($P < 0.05$). Each bar represents the mean ± SEM (n = 5). Fecal samples were collected from 6 to 9 d postinfection to calculate the oocyst shedding. Abbreviations: CON, uninfected control; EV, *Bacillus subtilis* (empty vector) at 10^{12} cfu/d; NC, infected control; NK, *Bacillus subtilis* expressing cNK-2 at 10^{12} cfu/d.

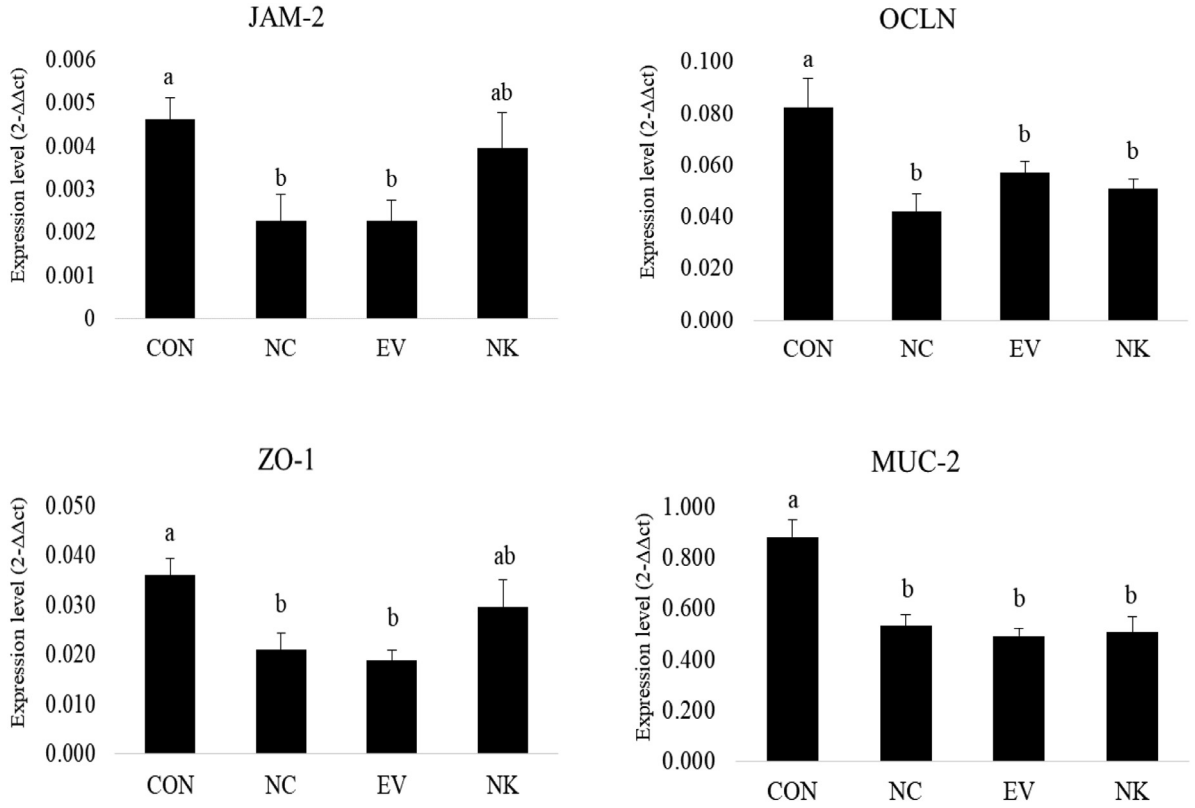

Figure 3. Tight junction gene expression in duodenal mucosa of *Eimeria acervulina*-infected broiler chickens (6 d postinfection). All chickens except CON were infected by oral gavage at d 15 with 5,000 oocysts/chicken of *E. acervulina*. *Bacillus subtilis* were administrated by oral gavage at - 1 to 4 d postinfection. Transcript levels of occludin (OCLN), zonula occludens 1 (ZO-1), junctional adhesion molecule 2 (JAM-2), and Mucin 2 (MUC-2) in duodenal mucosa were measured by quantitative RT-PCR and genes expression were analyzed using the $2^{-\Delta\Delta Ct}$ method. $^{a\sim d}$ Bars with no common letter differ significantly ($P < 0.05$). Each bar represents the mean ± SEM. Abbreviations: CON, uninfected control; EV, *Bacillus subtilis* (empty vector) at 10^{12} cfu/d; NC, infected control; NK, *Bacillus subtilis* expressing cNK-2 at 10^{12} cfu/d.

Gene Expression of Tight Junction Proteins and Mucin

Chickens in the NC group showed lower ($P < 0.05$) expression levels of JAM-2, OCLN, ZO-1, and MUC-2 genes compared to the CON group (Figure 3). Chickens in the EV group did not show any significant difference ($P > 0.05$) in the expression levels of tight junction protein and mucin gene compared to the NC group**.** Importantly, expression levels of JAM-2 and ZO-1 genes were elevated in NK chickens compared to the NC group and restored to levels similar ($P > 0.05$) to those in the CON group.

Antioxidant Gene Expression

No significant changes ($P > 0.05$) were observed for the SOD-1 expression levels among treatment groups on 6 dpi in the duodenal mucosa (Figure 4). However, the gene expression levels of CAT in the *E. acervulina*-infected-groups (NC, EV, and NK) were reduced ($P < 0.05$) compared to those of the CON group on 6 dpi, and HMOX-1 expression was increased in duodenal mucosa of chickens fed NK compared to NC ($P < 0.05$), restoring expression to levels observed in the CON group.

Similar to the findings in the duodenal mucosa, SOD-1 expression in the spleen of broiler chickens was the same ($P > 0.05$) among treatment groups at 6 dpi (Figure 5). No significant change ($P > 0.05$) was observed for CAT expression among treatments. However, broiler chickens fed NK showed higher expression ($P < 0.05$) levels of HMOX-1 compared to NC chickens, and the expression levels were similar ($P > 0.05$) in the EV, NK, and CON groups.

Mucosal Cytokine Gene Expression

Cytokine gene expression in the duodenal mucosa of broiler chickens in the present study was analyzed to assess differences in host immune responses between treatment groups (Figure 6). On 6 dpi, chickens in the NC group showed elevated ($P < 0.05$) IL-2, IL-10, IL-22, IL-1β, and IFN-γ expression levels compared to the chickens in the CON group. Compared to the NC group, broiler chickens fed EV or NK showed lower ($P < 0.05$) levels of IL-2, IL-10, IL-22, and IL-1β expression. IL-2, IL-10, and IL-22 expression in the mucosa of broiler chickens fed EV and NK were similar ($P > 0.05$) to chickens in the CON group. No significant changes ($P > 0.05$) were observed in IL-6, IL-17F and TNF-α gene expression in the duodenum mucosa on 6 dpi.

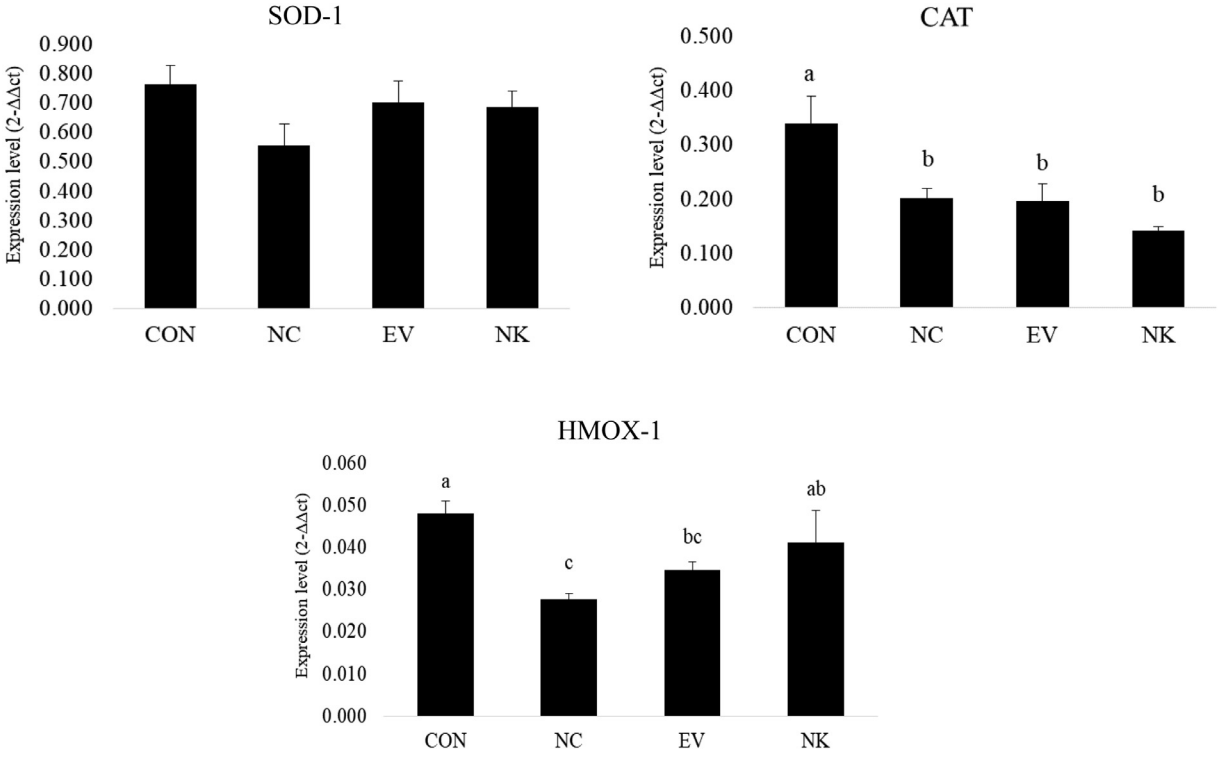

Figure 4. Antioxidant gene expression in duodenal mucosa of *Eimeria acervulina*-infected broiler chickens (6 d postinfection). All chickens except CON were infected by oral gavage at d 15 with 5,000 oocysts/chicken of *E. acervulina. Bacillus subtilis* were administrated by oral gavage at - 1 to 4 d postinfection. Transcript levels of superoxide dismutase 1 (SOD-1), catalase (CAT), heme oxygenase (HMOX-1) in duodenal mucosa were measured by quantitative RT-PCR and genes expression were analyzed using the $2^{-\Delta\Delta Ct}$ method. [a~d] Bars with no common letter differ significantly ($P < 0.05$). Each bar represents the mean ± SEM. Abbreviations: CON, uninfected control; EV, *Bacillus subtilis* (empty vector) at 10^{12} cfu/d; NC, infected control; NK, *Bacillus subtilis* expressing cNK-2 at 10^{12} cfu/d.

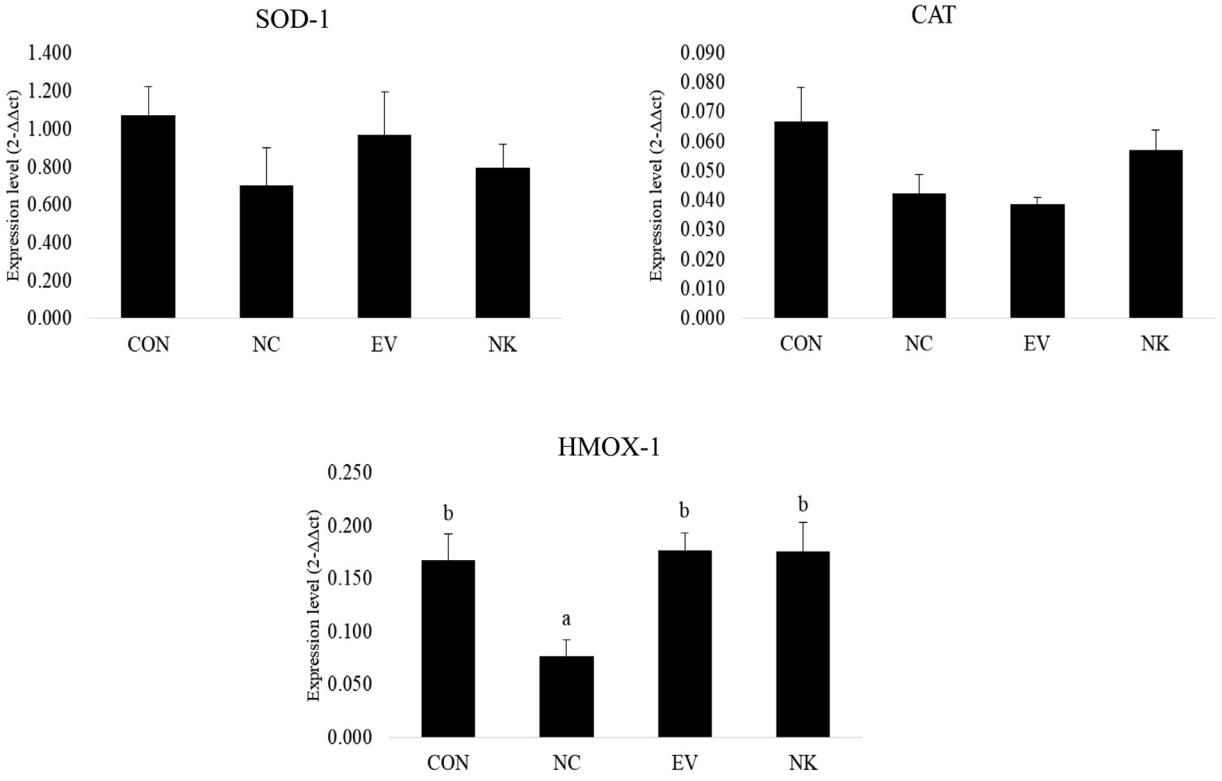

Figure 5. Antioxidant gene expression in spleen of *Eimeria acervulina*-infected broiler chickens (6 d postinfection). All chickens except CON were infected by oral gavage at d 15 with 5,000 oocysts/chicken of *E. acervulina. Bacillus subtilis* were administrated by oral gavage at - 1 to 4 d postinfection. Transcript levels of superoxide dismutase 1 (SOD-1), catalase (CAT), heme oxygenase (HMOX-1) in duodenal mucosa were measured by quantitative RT-PCR and genes expression were analyzed using the $2^{-\Delta\Delta Ct}$ method. [a~d] Bars with no common letter differ significantly ($P < 0.05$). Each bar represents the mean ± SEM. Abbreviations: CON, uninfected control; EV, *Bacillus subtilis* (empty vector) at 10^{12} cfu/d; NC, infected control; NK, *Bacillus subtilis* expressing cNK-2 at 10^{12} cfu/d.

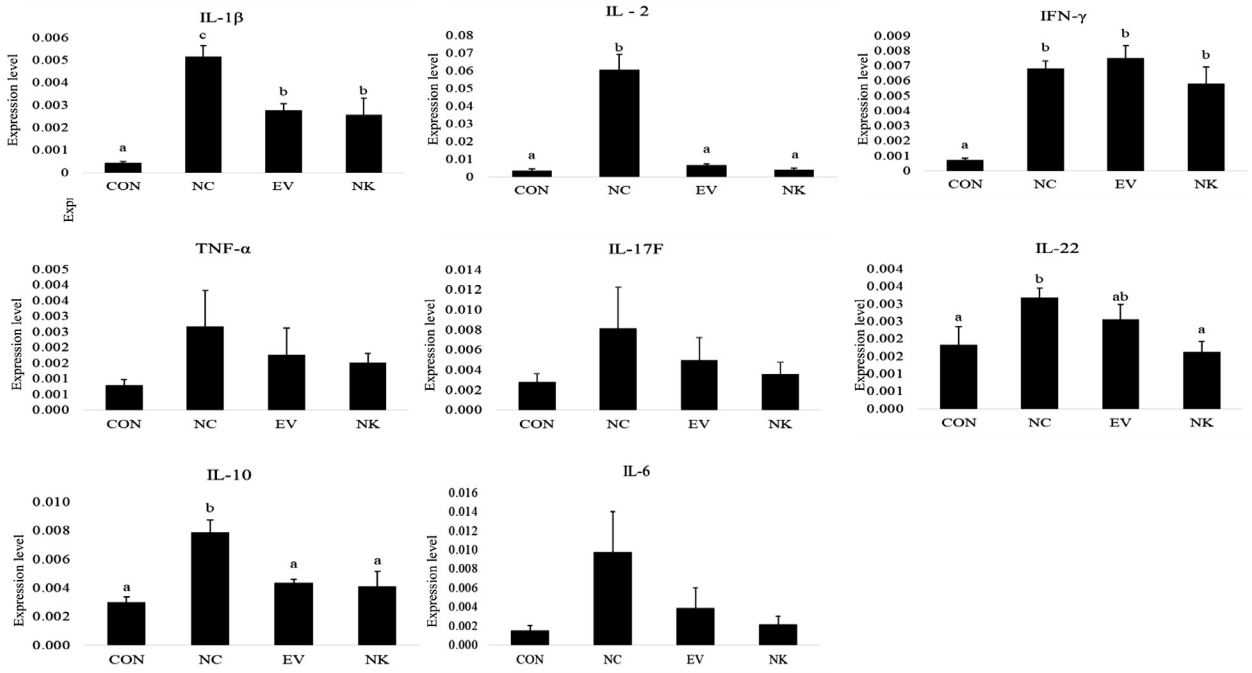

Figure 6. Cytokine gene expression in duodenal mucosa of *Eimeria acervulina*-infected broiler chickens (6 d postinfection). All chickens except CON were infected by oral gavage at d 15 with 5,000 oocysts/chicken of *E. acervulina*. *Bacillus subtilis* was administrated by oral gavage at - 1 to 4 d postinfection. Transcript levels of Interleukin-2 (IL-2), Interleukin 1 beta (IL-1β), and Interferon gamma (IFN-γ) in duodenal mucosa were measured by quantitative RT-PCR and genes expression were analyzed using the $2^{-\Delta\Delta Ct}$ method. $^{a\sim b}$Bars with no common letter differ significantly ($P < 0.05$). Each bar represents the mean ± SEM. Abbreviations: CON, uninfected control; EV, *Bacillus subtilis* (empty vector) at 10^{12} cfu/d; NC, infected control; NK, *Bacillus subtilis* expressing cNK-2 at 10^{12} cfu/d.

Serum Anti-Eimeria Antibody Response

At 13 dpi, serum 3−1E antibody levels of *E. acervulina*-infected broiler chickens were elevated ($P < 0.05$) compared to those in the CON group (Figure 7). Interestingly, chickens treated with NK were found to have higher ($P < 0.05$) 3−1E specific antibody levels compared to other *E. acervulina*-infected broiler chickens in NC and EV groups.

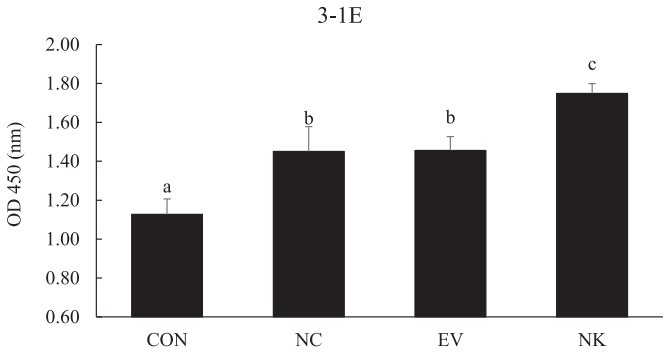

Figure 7. Serum 3−1E specific antibody levels of *Eimeria acervulina*-infected broiler chickens (13 d postinfection). All chickens except CON were infected by oral gavage at d 15 with 5,000 oocysts/chicken of *E. acervulina*. *Bacillus subtilis* was administrated by oral gavage at - 1 to 4 d postinfection. $^{a\sim c}$Bars with no common letter differ significantly ($P < 0.05$). Each bar represents the mean ± SEM. Abbreviations: CON, uninfected control; EV, *Bacillus subtilis* (empty vector) at 10^{12} cfu/d; NC, infected control; NK, *Bacillus subtilis* expressing cNK-2 at 10^{12} cfu/d.

Gut Microbial Composition and Diversity

Principal component analysis on read counts from the gut digesta samples, after removal of *Eimeria* counts, showed that microbiota populations in the chicken gut consisted predominantly of *Lactobacillus* (PC1; 58.9%). The presence of *L. salivarius* strongly captured variability among read counts between the groups (standard deviation = 2.92), showing a marked shift in untreated birds upon challenge with *Eimeria* (Figure 8A). The variability in *Enterococcus* read counts was captured in PC2 (18.0%, standard deviation = 1.62). The microbiomes of chickens treated with NK showed retention of the variation in microbiome composition similar to the microbiome of CON chickens (Figure 8B). In contrast, NC and EV chickens showed lower variation in the microbiome composition, when compared to CON and NK-treated chickens. The variation in microbiome composition was found to be significant between all 4 treatment groups (permutational multivariate analysis of variance, $P < 0.05$).

Looking more closely at the microbiota present in the duodenum at 6 dpi, *Lactobacillales* were dominant in the duodenal digesta of chickens in all treatment groups (Figure 9). In CON chickens, *Lactobacillales* and *Clostridiales* were dominated. In comparison to CON, chickens in the NC group showed higher *Lactobacillales, Spirulinales,* and lower *Clostridiales*. Chickens in the NK group showed *Lactobacillales* percentages similar to CON, and increased *Clostridiales* compared to NC, but reduced compared to CON.

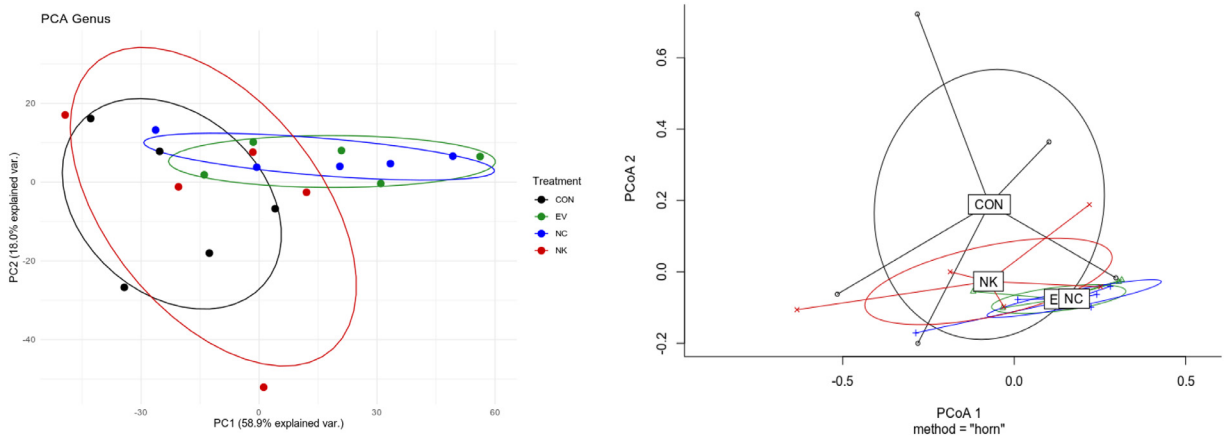

Figure 8. Variation in chicken microbiome composition. (A) Principal component analysis of percentage read counts for bacteria in the microbiome, grouped by genus, present in control and treatment groups. All bacteria present in the duodenum were analyzed, missing values were removed, and principal components were calculated. Each dot represents 1 microbiome sample, and are color coded according to treatment group. The ellipses show the grouping of the samples, with the tighter the ellipse, the closer the correlation between the individual samples. (B) To further analyze the variation observed in the composition of the microbiome between groups, a PERMANOVA was performed using the genus read counts, showing that there was a significant difference in the variance observed in chickens of the 4 different treatment groups ($P = 0.045$). The ellipses show the grouping of the samples, with the tighter the ellipse, the closer the correlation between the individual samples. All chickens except CON were infected by oral gavage at d 15 with 5,000 oocysts/chicken of E. acervulina. Bacillus subtilis were administrated by oral gavage at - 1 to 4 d postinfection. Abbreviations: CON, uninfected − control; EV, *Bacillus subtilis* (empty vector) at 1,012 cfu/d; NC, infected control; NK, *Bacillus subtilis* expressing cNK-2 at 10^{12} cfu/d; PERMANOVA, permutational multivariate analysis of variance.

At the phylum level in all different treatment groups (Figure 10), the duodenal microbiome was mostly composed of *Firmicutes* (order *Lactobacillales*) and *Cyanobacteria* (order *Cyanobacteria*). The most marked change was an increase in *Cyanobacteria* in NC chickens along with decreased *Firmicutes*. Those broiler chickens fed with oral *B. subtilis* (EV and NK), showed restoration in the balance between *Firmicutes* and *Cyanobacteria*. Additionally, broiler chickens fed with NK showed lower *Proteobacteria* abundancy compared to all other groups. The Shannon index of duodenum digesta confirmed that alpha diversity did not differ ($P < 0.05$) among the treatment groups (Figure 11).

DISCUSSION

Chicken NK-lysin peptide 2 (**cNK-2**) is a natural peptide with direct cytotoxicity against many parasites including coccidiosis-causing *Eimeria* protozoa. Characteristics and role of oral cNK-2 peptide treatment on avian coccidiosis have been reported previously (Lee et al., 2013; Lillehoj et al., 2014; Kim et al., 2021; Wickramasuriya et al., 2021). Recently, we developed a stable delivery system using *B. subtilis* spores carrying a modified sequence of the cNK-lysin peptide, which provided enhanced protection against *E. acervulina* infection in broiler chickens (Wickramasuriya et al., 2021). To our knowledge, that was the first documentation of

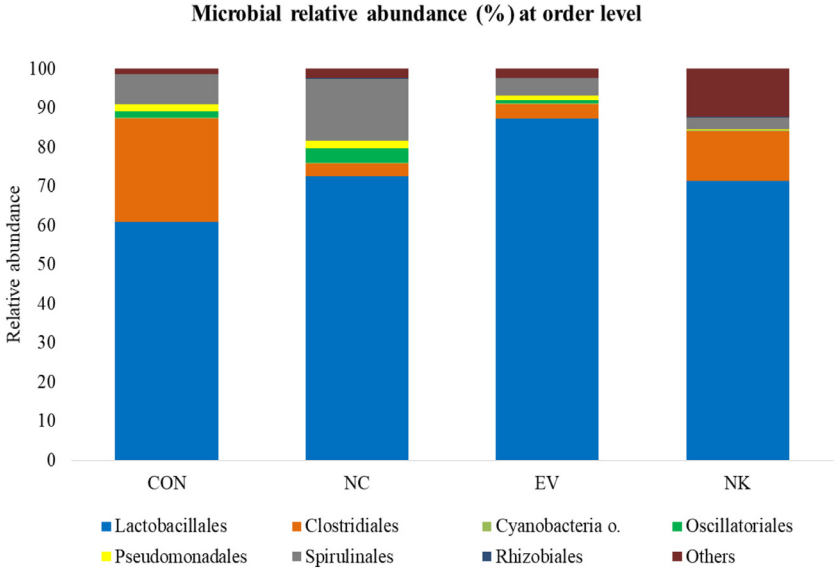

Figure 9. Comparison of microbial relative abundance (%) of duodenum digesta of *Eimeria acervulina*-infected broiler chickens (6 d postinfection) at order level. All chickens except CON were infected by oral gavage at d 15 with 5,000 oocysts/chicken of *E. acervulina*. *Bacillus subtilis* was administrated by oral gavage at - 1 to 4 d postinfection. Abbreviations: CON, uninfected − control; EV, *Bacillus subtilis* (empty vector) at 10^{12} cfu/d; NC, infected control; NK, *Bacillus subtilis* expressing cNK-2 at 10^{12} cfu/d.

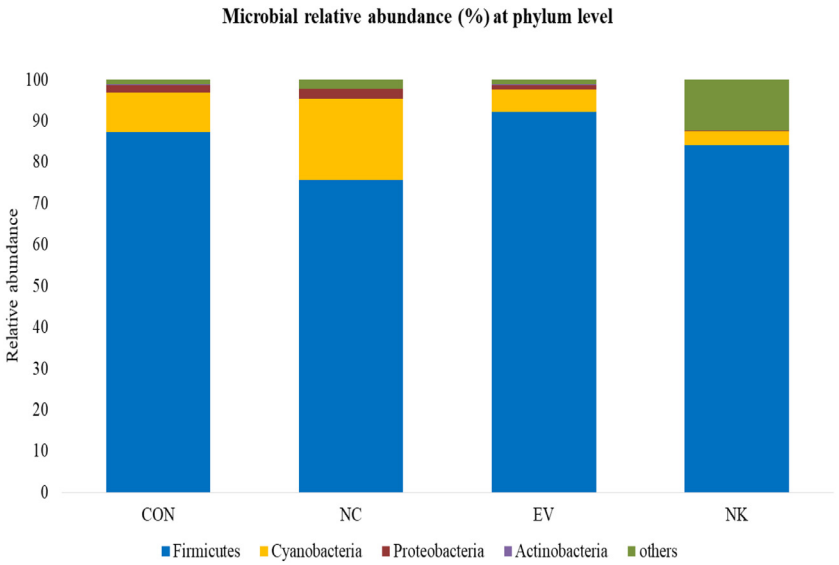

Figure 10. Comparison of microbial relative abundance (%) of duodenum digesta of *Eimeria acervulina*-infected broiler chickens (6 d postinfection) at phylum level. All chickens except CON were infected by oral gavage at d 15 with 5,000 oocysts/chicken of *E. acervulina*. *Bacillus subtilis* was administrated by oral gavage at - 1 to 4 d postinfection. Abbreviations: CON, uninfected − control; EV, *Bacillus subtilis* (empty vector) at 10^{12} cfu/d; NC, infected control; NK, *Bacillus subtilis* expressing cNK-2 at 10^{12} cfu/d.

successful oral delivery of cNK-2 AMP in a probiotic carrier, with activity in the gut against reducing parasite fecundity and mitigating coccidiosis-mediated gut damage. To better understand the beneficial effects of *B. subtilis*-cNK-2 on *E. acervulina*-induced stress on the gut microbiome, oxidative stress, and immunity, we further carried out the current in vivo trial. In the present study, we describe the effectiveness of *B. subtilis*-cNK-2 with respect to the growth performance and gut integrity with particular emphasis on the effects of cNK-2 treatment on intestinal immunity and gut microbiome in coccidiosis-challenged broiler chickens, based on increasing evidence of the close relationship between gut microbiota and local immune response (Durack and Lynch, 2019; Swaggerty et al., 2022).

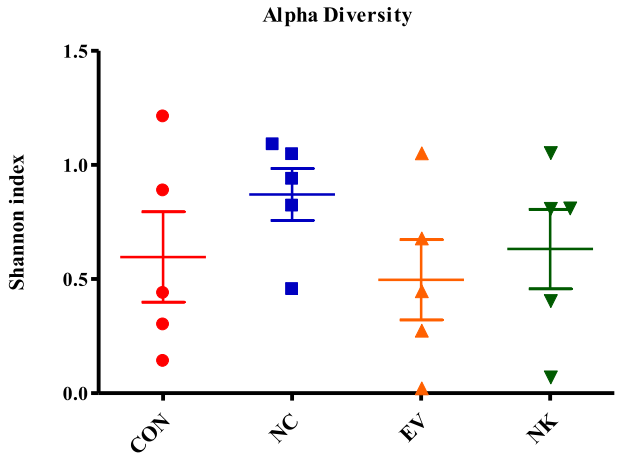

Figure 11. Alpha diversity analysis of duodenum digesta of *Eimeria acervulina*-infected broiler chickens (6 d postinfection). All chickens except CON were infected by oral gavage at d 15 with 5,000 oocysts/chicken of *E. acervulina*. *Bacillus subtilis* was administrated by oral gavage at - 1 to 4 d postinfection. Each bar represents the mean ± SEM. Each dot represents 1 microbiome sample, and are color coded per treatment group. Abbreviations: CON, uninfected control; EV, *Bacillus subtilis* (empty vector) at 10^{12} cfu/d; NC, infected control; NK, *Bacillus subtilis* expressing cNK-2 at 10^{12} cfu/d.

The onset of coccidiosis has several documented negative clinical impacts, including growth depression, bloody diarrhea, and mortality as the major adverse effects in chickens (Kim et al., 2013; Rochell et al., 2017; Wang et al., 2021). Indeed, in this study, *E. acervulina*-infected broiler chickens showed lower body weight and reduced ADG compared to uninfected chickens, confirming our experimental challenge was adequate to mimic field coccidiosis conditions. Gut epithelial cell invading *Eimeria* sporozoites disrupt normal gut physiology and nutrient metabolism, leading to the characteristic growth depression in coccidiosis-afflicted broiler chickens (Kim et al., 2013; Rochell et al., 2017; Chaudhari et al., 2020), along with decreased feed intake (Teng et al., 2021).

In this study, chickens treated with NK showed improved growth performance compared to NC chickens, with weights restored to that of CON chickens. Compared to our previously reported results (Wickramasuriya et al., 2021), the beneficial effects of orally delivered *B. subtilis*-cNK-2 on growth performance were enhanced with the higher dose of spores used (10^{10} vs. 10^{12} cfu/d/chicken) in this study. This dose effect, which was also observed in the previous study, further supports the beneficial effects of *B. subtilis*-cNK-2 in protecting against intestinal infection caused by *E. acervulina*.

Fecal oocyst count has been used as an indirect indicator of the severity of coccidiosis infection in broiler chickens (Leung et al., 2019; Chasser et al., 2020). Similarly, other researchers also reported the oocyst count changes in *Eimeria* challenge models (Leung et al., 2019). In this study, as well as in previous studies, our challenge model leads to high oocyst shedding by *E. acervulina*-infected broiler chickens (Lee et al., 2013; Lillehoj et al., 2014; Wickramasuriya et al., 2021), and chickens treated with

NK exhibit a significantly lowered oocyst shedding compared to chickens in other treatment groups. This further supports our preconceptions of oral *B. subtilis*-cNK-2 ability 1) to reach the small intestinal without losing its functional activity, 2) to reduce parasite fecundity, and 3) to suppress the severity of gut damage associated with coccidiosis. The strong cytotoxicity and disruption of sporozoite outer membrane integrity, followed by the release of intracellular contents as the effect of cNK-2 peptide, may explain the underlying mechanism of the lower oocyst shedding in this group as described previously (Lillehoj et al., 2014).

As we reported previously (Wickramasuriya et al., 2021), we observed that *E. acervulina* infection decreased the expression level of JAM-2, OCLN, ZO-1 and MUC-2, all of which are associated with intestinal epithelial barrier function and mucosal protection in chickens. Reduced expression of these tight junction genes depicts its association with adverse effects on chicken gut health (Leung et al., 2019), as the tight junction proteins are the key molecules that are responsible for maintaining proper intestinal barrier function and thereby maintaining the gut permeability (Ognik et al., 2020). By maintaining gut integrity, tight junction complexes enable ions to freely pass through the intestinal barrier, whereas pathogens and their toxins are prevented from entering cells. Observed lower tight junction gene expression in *E. acervulina*-infected broiler chickens is in line with other research studies demonstrating *Eimeria spp.* infection downregulates the gene expression of tight junction proteins in broiler chickens (Santos et al., 2020). Further, tight junction protein OCLN together with claudins and cadherins work together for proper intestinal barrier function, and therefore lower OCLN expression indicates increased gut permeability as an effect of *Eimeria* infection (Al-Sadi et al., 2011; Leung et al., 2019). Mucin 2 is a protein secreted by goblet cells present in the chicken intestine onto the mucosal surface forming a mucosal barrier between the lumen and the intestinal epithelium (Hansen et al., 2021). In the current study, EV and NK had no significant effect on the tight junction and mucin gene expressions compared to NC chickens on 6 dpi. However, it is of note that, although significance was not attained, the NK increased JAM-2 and ZO-1 gene expression to a level similar to uninfected chickens depicting its promising role in gut health improvement.

Under different field stress conditions, broiler chickens are continuously subjected to oxidative stress because of a disturbed redox balance in the chicken's body (Liu et al., 2020; Wickramasuriya et al., 2022). During parasitic infection, the enzymatic antioxidant system of broiler chickens was also reported to be affected causing oxidative stress (Lee et al., 2020). With the onset of oxidative stress conditions in chickens, growth performances are adversely affected as a matter of different metabolic disorders (Lv et al., 2018). To avoid adverse effects, antioxidant enzymes provide the first line of cellular defense against oxidative stress by scavenging oxygen free radicals (Mishra and Jha, 2019). The superoxide dismutase enzyme family includes SOD-1, SOD-2, and SOD-3, each of which converts superperoxide anions (O_2^-) into hydrogen peroxide (H_2O_2) and molecular oxygen (O_2) as a catalytic. Subsequently, CAT catalyzes the breakdown of H_2O_2 to H_2O and O_2 (Mondola et al., 2016; Oh et al., 2018). HMOX-1 works as a rate-limiting enzyme in the breakdown of heme to biliverdin, carbon monoxide, and free iron (Otterbein and Choi, 2000). Coccidiosis infection decreases intestinal SOD-1 and CAT activity in broiler chickens (Khatlab et al., 2019). In this study, we also observed *E. acervulina*-infected broiler chickens reduced CAT and HMOX-1 activity in duodenal mucosa after 6 dpi. Broilers fed with NK showed significantly increased HMOX-1 expression, which was like the levels of CON chickens, supporting beneficial cNK-2 activity towards gut health improvement. Similar to this study, we also observed higher HMOX-1 expression and no expression difference in SOD-1 in our previous study (Wickramasuriya et a., 2021). In our previous study, CAT expression levels were inconclusive, however, in the current study, a reduction in CAT expression in duodenal mucosa was observed in all challenge groups. This disparity may be explained by the different sampling times (13 dpi, vs. 6 dpi) in both studies.

As the spleen is a secondary lymphoid organ directly involved in immune reactions protecting from stress conditions, we evaluated the enzymatic antioxidant gene expression of the *E. acervulina*-infected broiler chickens fed with oral *B. subtilis*-cNK-2. The spleen tissues have been previously used to analyze the enzymatic antioxidant gene expression of broiler chickens under different stress conditions (El-Senousey et al., 2018; Yang et al., 2019; Wickramasuriya et al., 2021). As we reported previously, *E. acervulina*-infection did not impact enzymatic antioxidant SOD-1 gene expression in broiler chickens. In both studies, we observed that *E. acervulina*-infection reduced HMOX-1 gene expression in the spleen, whereas *E. acervulina*-infected broiler chickens fed EV or NK exhibited increased HMOX-1 gene expression, with levels restored to those of CON chickens. In our previous study, there was a more prominent effect between *B. subtilis*-cNK-2 and *B. subtilis*-EV fed chickens with regards to CAT and HMOX-1 activity. This discrepancy might be explained by the different time points (13 dpi vs. 6dpi) and the higher dose (10^{10} vs. 10^{12} cfu/d) used in the current experiment.

In broiler chickens, *Eimeria* parasites invasion through the intestinal epithelial cells leads to local inflammatory responses (Hong et al., 2006a; Kim et al., 2019). Because cell-mediated immunity has been shown to be critical for protection against coccidiosis, T cells produce various cytokines against avian coccidiosis (Kim et al., 2019; Lillehoj et al., 2019). Cytokines are immune regulatory peptides that mediate the communication between cells during immune responses and are recognized as a biomarker for intestinal health (Celi et al., 2019; Park et al., 2022). To assess mucosal immunity, we evaluated cytokine gene expressions in the duodenal mucosa of the chicken. Due to the site specificity of *E. acervulina* parasites (Blake and Tomley, 2019),

analyses were carried out on samples from the duodenum where the most intense gut damages were observed. Showing the impact of *E. acervulina*-infection on mucosal immunity in the duodenum, IL-1β, IL-2, IL-10, IL-22, and IFN-γ expressions were elevated in infected chickens compared to uninfected chickens. Previously, our laboratory (Park et al., 2020; Park et al., 2021) observed increased gene expression levels of IL-1β, IL-6, IL-10, and IFN-γ in *Eimeria maxima* infected chickens compared to uninfected chickens at 6 dpi. Rochell et al. (2017) also reported increased duodenal expression of IFN-γ, IL-1β, and IL-10 in *E. acervulina*-infected broilers at 6 dpi. Pro-inflammatory cytokines such as IFN-γ and IL-1β are associated with reduced *Eimeria* replication whereas anti-inflammatory cytokine IL-10 plays an immunoregulatory role (Hong et al., 2006b; Rochell et al., 2017). Broiler chickens fed with EV or NK reduced the expression levels of IL-1β, IL-2, IL-10, and IL-22, indicating protection from the detrimental immune effect of *E. acervulina* infection. Moreover, even without significant effect, TNF-α, IL-17F and IL-6 followed the same trend with other cytokine gene expression in duodenum mucosa of *E. acervulina* infected broilers at 6 dpi. cNK-2 peptide's ability to modulate the inflammatory responses was previously reported showing reduced expression of the pro-inflammatory cytokines in LPS-induced HD11 cells and monocytes (Kim et al., 2017a). Even though the NK effect was prominent on IL-22, expressions of IL-1β, IL-2, and IL-10, showed similar expressions to that of EV suggesting that *B. subtilis* could provide a level of protection on its own.

One hallmark of coccidiosis-induced immune response includes both antibody and cell-mediated immune responses in chickens (Lillehoj et al., 2019). Considering the markers of humoral immunity (*Eimeria*-specific antibodies), serum 3-1E antigen-specific antibody levels of *E. acervulina*-infected broiler chickens were investigated. The 3-1E antigen is located on the outer surface of the *Eimeria* sporozoites and merozoites (Lillehoj et al., 2000; Ding et al., 2004; Ma et al., 2011). *E. acervulina* infection increased the serum antibody levels compared to uninfected broiler chickens at 13 dpi, which is in concurrence with elevated serum antibody (IgA, IgG, and IgM) levels found in *E. maxima*-infected chickens (Yun et al., 2000). In this study, we observed increased serum 3-1E antibodies in NK broiler chickens supporting the role of cNK-2 in stimulating host humoral immunity against coccidiosis, and the utility of our oral delivery system as a biological defense against *Eimeria* infection in broiler chickens.

To fully understand the underlying mechanism and the mode of action of alternative antibiotics in chickens, and due to the known probiotic effects of *B. subtilis* in chickens (Grant et al., 2018; Chaudhari et al., 2020; Park et al., 2020), we investigated the effects of NK treatment on gut microbiome changes. Previous studies indicated a close interaction between gut microbiome and coccidiosis which cause intestinal dysbiosis (Lu et al., 2020; Madlala et al., 2021). In support of this notion, Gaboriaud et al. (2021) showed that the absence of gut microbiota (germ-free) hinders the development of *E. tenella* in broiler chickens due to a reduced number of sexual stages associated with delay in the gamogony stage appearance and the reduction of the excystation efficiency. We observed that the total microbiome composition was shifted in chickens infected with *E. acervulina* and that the microbiome composition of NK chickens is strongly resembling that of CON chickens, indicating a healthier gut colonization and suggesting a holistic protective effect of cNK-2. In contrast, lower variation in NC and EV chickens indicates that there was a larger effect of *Eimeria* colonization on the microbiome variability of these chickens.

A recent report described significant microbial shifts in the chicken gut upon *Eimeria* infection, which reduced the presence of commensal bacteria such as *Firmicutes* and increased the presence of *Streptococcus*-like opportunistic pathogens (Madlala et al., 2021). Altered cecal microbial composition and diversity together with reduced *Proteobacteria* and *Firmicutes* have been observed in *E. tenella*-infected broiler chickens (Zhou et al., 2020). Vieira et al. (2020) observed *Firmicutes* as the most abundant phylum in chickens challenged with 3 different *Eimeria* spp. Our report shows that indeed, *Firmicutes* showed the most relative abundance (>70%) in all chickens, and their relative abundance was decreased in *E. acervulina*-infected chickens at 6 dpi. Oral *B. subtilis* administration restored a relative abundance of *Firmicutes* in *E. acervulina*-infected broiler chickens, supporting the beneficial effect of *B. subtilis* on chicken gut microbiota.

The phylum *Firmicutes* has been reported to be linked to the energy-harvesting efficiency of animals (Vieira et al., 2020). Indeed, our study also shows a lowered relative abundance of *Firmicutes* in the *E. acervulina*-infected chickens predisposes the birds to pathogenic bacteria proliferation and consequently retard the growth performance. Phylum *Proteobacteria* includes potential gram-negative pathogenic bacteria which has been correlated with the pro-inflammatory cytokine profile of the chicken (Diaz Carrasco et al., 2019). The observed lowered relative abundance of the phylum *Proteobacteria* in NK chickens supports the attenuation of opportunistic pathogen proliferation in the coccidiosis-infected chicken gut by cNK-2.

At the order level, we observed *Lactobacillales* in more than 60% of the gut microbiome regardless of *E. acervulina* in this study. Broiler chicken in the EV group showed the highest relative abundance of *Lactobacillales* in the digesta. Nevertheless, the mean difference of *Lactobacillales* among treatments was not statistically significant. The order *Lactobacillales* belongs to the phylum *Firmicutes* and class *Bacilli*. Díaz-Sánchez et al. (2019) reported that *Lactobacillales* were associated with the production of bile salt hydrolase and poor weight gain in broilers, even though higher growth rates can be observed in the chicken when used as a probiotic. This study shows increased *Lactobacillales* in infected birds, which was only restored in NK-treated chickens.

As this group also performed most like unchallenged birds with regards to weight gain, and the retention of healthy microbiota abundances, cNK-2 could play a role in the observed protection from weight loss during *E. acervulina* infection in this group. The Shannon index considered uniformity in abundance in each treatment. In the present study, any significant difference was not observed in alpha diversity among treatment groups.

CONCLUSIONS

The results of this study demonstrate the effectiveness of an orally delivered stable cNK-2 peptide to control coccidiosis in commercial broiler chickens. In coccidiosis, *B. subtilis*-cNK-2 clearly provided beneficial effects because NK-lysin has a direct cytotoxic effect during the invasive stage of *Eimeria* parasites and reduces parasite fecundity and gut damage with resultant growth enhancement. These results also help to illustrate the ability of *B. subtilis*-cNK-2 to restore the *E. acervulina*-induced microbiota shift in the chicken gut. Further research determining the responses of different administration methods of *Bacillus* spores using different *Eimeria* challenges is warranted to develop effective antibiotic alternative strategies for commercial broiler chickens.

ACKNOWLEDGMENTS

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DISCLOSURES

JGvO, LR, and CP are employed by the US biologic, Inc. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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