Evaluation of *in ovo Bacillus* spp. based probiotic administration on horizontal transmission of virulent *Escherichia coli* in neonatal broiler chickens

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ABSTRACT This study evaluated the effect of *in ovo Bacillus* spp. base probiotic (**BBP**) administration on hatchability, Gram-negative bacteria (**GNB**) recovery, performance, and microbiota composition in 2 independent trials using a virulent *E. coli* seeder challenge model. In each trial, one hundred and eighty 18-day-old embryos were allocated into 1 of 2 groups: Control and treated group (inoculated with 10^7 BBP). On day 19 of embryogenesis, seeder embryos (n = 18) were inoculated with 4.5×10^4 *E. coli*/mL+272 μg/mL tetracycline and segregated into mesh hatching bags. Twelve chicks per group were euthanized at hatch and at day 7 to evaluate the gastrointestinal composition of total GNB or total aerobic pasteurized bacteria. Also, in trial 2, ceca content from five chickens at day 7 were collected to evaluate microbiota composition. Embryos inoculated with BBP showed a significant ($P < 0.05$) reduction in the total number of GNB at day-of-hatch (**DOH**) and day 7. Probiotic treatment increased BW at DOH and day 7, and BW gain (days 0 to 7) when compared with Control chickens. Proteobacteria phylum was significantly reduced, while the Firmicutes was significantly increased by the BBP as compared to the Control ($P < 0.05$). At family level, Enterobacteriaceae was significantly decreased, while the Lachnospiraceae was significantly elevated in the BBP as compared to the Control group ($P < 0.05$). The genus *Oscillospira* was significantly enriched in the BBP group, whereas the unidentified genus of family *Enterobacteriaceae* in the Control group ($P < 0.05$). The BBP group increased the bacterial species richness, although there was no significant difference between treatments ($P > 0.05$). Interestingly, beta diversity showed a significant difference in bacterial community structure between Control and BBP groups ($P < 0.05$). The results of the present study suggest that *in ovo* administration of a BBP can reduce the severity of virulent *E. coli* horizontal transmission and infection of broiler chickens during hatch. The reduction in the severity of the transmission and infection by the BPP might be achieved through alterations of microbiota composition and its community structure.

Key words: broiler, *Escherichia coli*, hatchers, *in ovo*, probiotic

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INTRODUCTION

Beneficial bacterial species surpass the number of pathogenic species, complementing the biology and physiology of metazoans (Kikuchi et al., 2009). One example of such beneficial mutualism is found in the gastrointestinal tract (**GIT**; Xie et al., 2010). The gathering of the gut microbiota is regulated by the elaborate and combinatorial host–microbial and microbial–microbial interactions (Xu and Gordon, 2003). Several studies have described how the microbiota modulates the gut-associated lymphoid tissue (Martin et al., 2010), instructs the immune system (McFall-Ngai, 2007), improves the intestinal integrity (Duerkop et al., 2009), regulates the proliferation and differentiation of the enterocytes (Moran, 2007), regulates blood flow (Sekirov et al., 2010), and activates the enteric nervous system (Tlaskalová-Hogenová et al., 2011). In mammals, colonization of the microbiota initiates at birth and continues throughout life (Di Mauro et al., 2013). Under

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commercial conditions, chickens hatch in an environment that contains potential pathogens where these pathogens can colonize and shift the microbiota prior to beneficial bacteria colonization. While the natural route of transmission of enteropathogens is fecal-oral (White et al., 1997; Galanis et al., 2006), published studies have also suggested that airborne transmission of enteropathogens in poultry is possible (Kallapura et al., 2014a,b,c). Recently, we demonstrated that the *in ovo* administration of a probiotic mixed with a Marek's disease (**MD**) vaccine had no effect on the effectiveness of the MD vaccine, but reduced intestinal *Salmonella* Enteritidis counts and improved BW and intestinal integrity (Teague et al., 2017). Furthermore, our laboratory has developed a novel *in ovo* challenge model for virulent *Escherichia coli* strains (Graham et al., 2019). In that study, we have shown that co-administration of a virulent *E. coli* strain with tetracycline allows for the hatch of directly challenged chicks and effective horizontal transmission to contact chicks during the hatching process, as evidenced by reduced performance and altered selected enteric bacterial recovery at day 7. Therefore, considering that the 21 d of embryogenesis plus the first 7 d represent 50 to 74% of the commercial life of chickens (Cherian, 2011), in the present study, we evaluated the effect of *in ovo* administration of a *Bacillus* base probiotic (**BBP**) on hatchability, Gram-negative bacteria recovery, performance, and microbiota composition during the first 7 d after hatch, using our published *E. coli* horizontal infection model in the hatching cabinet in broiler chickens.

MATERIALS AND METHODS

Bacillus Based Probiotic

Norum^{TM} (Eco-Bio/Euxxis Bioscience LLC, Fayetteville, AR) is a *Bacillus* spore DFM culture, consisting of three isolates: two *Bacillus amyloliquefaciens* and one *Bacillus subtilis* (Latorre et al., 2015b, 2016). The product contains a concentration of stable *Bacillus* spores ($\sim 3 \times 10^{11}$ spores/g). Aliquots of vegetative bacterial strains from Norum^{TM} were maintained in 50% glycerol frozen stocks at −80°C. In the present study, 100 µL of a frozen aliquot of each strain were added to 10 mL of tryptic soy broth (**TSB**, Sigma-Aldrich, St Louis, MO, USA) and incubated at 37°C for 24 h. Then, bacteria were washed 3 times, combined, resuspended in sterile phosphate-buffered saline (**PBS**) and adjusted to an optical density (OD_{600}) of 0.8 to 0.9. This combination of BBP was diluted in sterile saline to an expected concentration of 5×10^7 cfu/mL for *in ovo* injection of 0.2 mL into the amnion. Actual colony-forming units from each trial were reported, which were determined retrospectively from spread plating on tryptic soy agar (**TSA**).

E. coli Culture and Challenge

A lactose negative *E. coli* strain known to cause respiratory disease and mortality in both chickens and turkeys (Huff et al., 1998, 2002, 2003) was used in the present study. For that, 100 μL of *E. coli* were removed from a frozen aliquot and added to 10 mL of TSB. The culture was incubated at 37°C for 18 h. Post incubation, bacterial cells were washed with sterile 0.9% saline by centrifugation at 1800 × g for 15 min and reconstituted in saline. The wash procedure was completed three times. *E. coli* cfu enumeration was determined by serial dilution and plating on MacConkey agar (MacConkey Agar, cat. no. 89,429–342, V.W.R., Suwanee, GA 30,024) to determine the stock concentration and then cells were held overnight at 4°C. Approximately 16 h later, the culture was serially diluted to desired cfu concentration for *in ovo* challenge (day 19 of embryogenesis) with the selected dose of tetracycline hydrochloride (Tetracycline hydrochloride, cat. no. 64,755, Sigma, St. Louis, MO 63,103). Actual *E. coli* challenge dose (cfu/mL) was confirmed as described above and reported. Relative minimal inhibitory concentrations of tetracycline were determined *in vitro* (data not shown) and then adjusted in subsequent trials based on *in vivo* results.

Experimental Design

Two independent trials with 18-day-old of embryogenesis Ross 308 embryos were conducted. Embryos were candled and randomly allocated into one of two groups (n = 180 embryos per group) and placed into separate hatchers (G.Q.F. 1550 Digital Cabinet Egg Incubator) based on treatment group, and inoculated via *in ovo* injection into the amnion with 0.2 mL of sterile PBS or with 5×10^7 cfu/mL (1×10^7 cfu/0.2 mL) of the BBP. Hatchers were housed separately to prevent possible cross-contamination between treatments during the hatch. On day 19 of embryogenesis, seeder embryos (n = 18 seeders/hatcher or 10%/hatcher) were inoculated with *E. coli*/tetracycline treatment via *in ovo* injection into the amnion and segregated into mesh hatching bags (reusable mesh nylon netting, I.D.S., Amazon). Seeder embryos were challenged with a dose of 4.5×10^4 cfu/mL *E. coli* + 272 μg/mL tetracycline. On day-of-hatch (**DOH**), dry chicks were removed from hatchers, and hatchability was determined. Then, the contact-challenged chicks were weighed, and 90 chickens were selected to be placed into pens (3pens/group with 30 chicks each). No seeders were placed. The BW on the day of hatching was normalized so that the differences in weight were due to the treatment and not to the higher initial weight of any of the groups. BW allocation was achieved by normalizing the means between all pens and treatments by standard deviation. In each trial, body weight gain (**BWG**) from d0 to day 7 was determined for the duration of each trial (7-d trial period). Furthermore, 12 chicks per group were

euthanized on **DOH** and day 7 to evaluate the gastrointestinal composition on selective media and enumerate total Gram negative. At day 7, ceca content of five chickens were collected to evaluate microbiota composition. Chickens were provided *ad libitum* access to water and a balanced, unmedicated corn and soybean diet meeting the nutritional requirements for broilers recommended by Aviagen. This study was carried out in accordance with the recommendations of the Institutional Animal Care and Use Committee (**IACUC**) at the University of Arkansas, Fayetteville. The IACUC approved protocol #17,073 at the University of Arkansas, Fayetteville for this study.

Enumeration of Bacteria

In both independent trials, the whole gut (ventriculus to the cecum) of 12 chickens per group was aseptically removed and collected into sterile bags as previously described (Tellez et al. 2015). Samples were weighed, homogenized, and 1:4 wt/vol dilutions were made using sterile 0.9% saline. Ten-fold dilutions of each sample, from each group, were made in a sterile 96 well Bacti flat bottom plates and the diluted samples were plated on culture media to evaluate the total number of Gram-negative bacteria on MacConkey agar (MacConkey Agar, cat. no. 89,429–342, V.W.R., Suwanee, GA 30,024). Following heat treatment, 10-fold dilutions of the feed samples were plated on TSA. All plates were incubated at 37°C for 18 h, and bacterial counts were expressed as Log_{10} cfu/g of sample (Tellez et al. 2015).

Microbiota Analysis

Sample Processing, DNA Extraction, PCR, Library Preparation, and Sequencing One gram of cecum content from 5 chickens at day 7 was transferred into collection tubes containing a lysis and stabilization buffer. DNA extraction, amplification, and library preparation were performed as described earlier (Almonacid et al., 2017). Briefly, samples were lysed through bead-beating and DNA was extracted by guanidine thiocyanate silica column-based purification method using a liquid-handling robot in a class 1,000 clean room (Cady et al., 2003). The 515F (5'-GTGCCAGCMGCCGCGGTAA) and 806R (5'-GGACTACHVGGGTWTCTAAT) primers that contained Illumina tags and barcodes were used for amplification of the V4 variable region of the 16S rRNA gene (Caporaso et al., 2011). PCR products were then pooled, column-purified, and size-selected through microfluidic DNA fractionation (Minalla et al., 2001). Consolidated libraries were quantified by quantitative real-time PCR using the Kapa Bio-Rad iCycler qPCR kit on a BioRad MyiQ before loading for sequencing. Sequencing was performed in a pair-end modality on the Illumina NextSeq 500 platform rendering 2 × 150 bp pair-end sequences.

16S rRNA Gene Sequences Analysis After sequencing, demultiplexing of samples was performed using Illumina's BCL2FASTQ algorithm. Forward and reverse reads obtained in each of the 4 lanes per sample were filtered using the following criteria: both forward and reverse reads in a pair must have an average Q-score > 30. Primers and any leading random nucleotides (used to increase the diversity of the library being sequenced) were trimmed, and forward reads were capped at 125 bp and reverse reads are capped at 124 bp. After quality filtering as described above, the Deblur (Amir et al., 2017) workflow was applied for the forward reads to generate a feature table and representative sequences using "qiime deblur denoise-16S" method implemented in QIIME2 version 2019.1 (Bolyen et al., 2018). The features that were present only in a single sample were removed from the feature table. Naive Bayes classifier (Pedregosa et al., 2011) was trained using Green genes 13_8 99% O.T.U.s (DeSantis et al., 2006), where the sequences were trimmed to include only 125 bases from the region of the 16S rRNA gene bound by the 515F/806R primer pair. This pretrained classifier was used to assign taxonomy to the representative sequences using q2-feature-classifier plugin. Microbial diversity analyses were performed using q2-diversity-plugin of QIIME2 using the even sampling depth of 14,610. The alpha diversity as computed by observed OTUs metric and Shannon's index (Shannon, 1948) and beta diversity as calculated by unweighted UniFrac (Lozupone et al., 2011) distance metrics were reported. All figures were created using ggplot2 packages (Wickham, 2016) on R version 3.5.3.

Statistical Analysis

All data were subjected to one-way analysis of variance as a completely randomized design using the GLM procedure of SAS. (SAS Institute, 2002). Data were expressed as mean ± SE. Significant differences ($P < 0.05$) among the means were further separated using Duncan's multiple range test for bacterial recovery, BW, and BWG. Hatchability was compared using the chi-squared test of independence to determine the significance for these studies (Zar, 1984). Wilcoxon test was performed for statistical analysis of alpha diversity and bacterial taxonomic groups (phylum, family, and genus) between the 2 treatments. However, PERMANOVA (Anderson, 2001) test was used to calculate significant difference in beta diversity between two treatments.

RESULTS

The results of the effect of *in ovo* administration of BBP on microbial composition in the GIT of hatching broiler chickens, hatchability and BW in a virulent *E. coli* horizontal transmission challenge in the hatching cabinet in broiler chickens are summarized in Table 1. In both trials, BBP significantly reduced ($P < 0.05$) the

Table 1. Effect of *in ovo* administration of *Bacillus* spp. base probiotic (BBP) on microbial composition in the gastrointestinal tract of hatching broiler chickens, hatchability, body weight (g), and horizontal transmission of virulent *E. coli* during hatch.

Treatment	Gram-negative bacterial recovery DOH (Log_{10} cfu/g)	Gram-negative bacterial recovery day 7 (Log_{10} cfu/g)	Hatchability (%)	Average BW DOH (CV)	Average BW day 7 (CV)	Average BWG days 0 to 7 (CV)
			Trial 1			
In ovo PBS Control	5.70 ± 0.28[a]	7.43 ± 0.12[a]	174/180 (96.66%)	40.03 ± 0.07[b] (0.089)	164.56 ± 2.52[b] (0.120)	116.93 ± 2.63[b] (0.144)
In ovo 10^7 cfu/mL BBP	4.32 ± 0.91[b]	4.11 ± 0.47[b]	175/180 (97.22%)	47.77 ± 0.86[a] (0.086)	175.15 ± 2.71[a] (0.097)	127.38 ± 2.69[a] (0.112)
			Trial 2			
In ovo PBS Control	4.92 ± 0.32[a]	6.34 ± 0.33[a]	176/180 (97.77%))	41.30 ± 0.03[b] (0.099)	161.31 ± 2.68[b] (0.140)	111.81 ± 1.91[b] (0.137)
In ovo 10^7 cfu/mL BBP	3.41 ± 0.81[b]	3.89 ± 0.35[b]	178/180 (98.88%)	42.77 ± 0.11[a] (0.120)	181.15 ± 2.71[a] (0.150)	138.38 ± 3.69[a] (0.160)

Hatchability total: hatched chickens/total embryos placed (%). Body weight (BW), n = 30.
[a,b]Indicates significant difference between columns ($P < 0.05$).

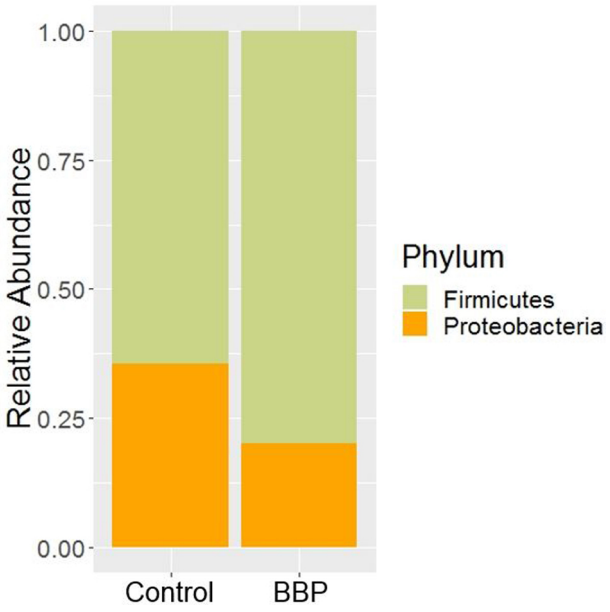

Figure 1. Relative abundance of bacterial phyla recovered from Control and *Bacillus* base probiotic (BBP) in trial 2.

recovery of Gram-negative bacteria on DOH and day 7 as compared to the *in ovo* Control group in both trials (Table 1). No significant differences in hatchability were observed in both trials. Nevertheless, *in ovo* administration of the BBP significantly increased the average BW at DOH and day 7, as well as BWG (days 0 to 7) when compared with Control PBS group (Table 1).

Bacterial Composition at the Phylum Level

At the phylum level, Firmicutes and Proteobacteria were the only 2 phyla detected from the 2 treatment groups, whose relative abundances are shown in Figure 1. Firmicutes (Control 64.38 ± 5.51%, BBP 79.88 ± 3.74%) was the predominant phylum in both groups followed by the Proteobacteria (Control 35.62 ± 5.51%, BBP 20.12 ± 3.74%). The Proteobacteria was significantly reduced, while Firmicutes was significantly increased in BBP group as compared to the Control (Table 2; Wilcoxon test, $P < 0.05$).

Table 2. Differentially abundant bacterial taxa at different levels of taxonomic classification in 2 treatment groups: Control and BBP (Wilcoxon test, $P < 0.05$).

Control		BBP
	Phylum Level	
Proteobacteria		Firmicutes
	Family Level	
Enterobacteriaceae		Lachnospiraceae
	Genus Level	
Unidentified genus		*Oscillospira*

Bacterial Composition at the Family Level

The relative abundance of different bacterial families recovered from Control and BBP groups is shown in Figure 2. In both groups, Lachnospiraceae (Control 40.72 ± 1.84%, BBP 53.30 ± 4.26%) as found the highest percentage followed by Enterobacteriaceae in the Control group (Control; 35.62 ± 5.51%; BBP; 20.11 ± 3.74%), while Ruminococcaceae in the BPP group (Control; 15.01 ± 5.01%, BBP; 20.83 ± 4.44%). Other notable bacterial families were Lactobacillaceae (Control 4.26 ± 2.02%, BBP 1.21 ± 0.60%) and Erysipelotrichaceae (Control 3.73 ± 1.25%, BBP 2.53 ± 0.63%). The Clostridiaceae, Enterococcaceae, and Peptostreptococcaceae were observed as minor members whose average relative abundance was less than 1% in both groups. The statistical analysis revealed that the Enterobacteriaceae was significantly decreased, while the Lachnospiraceae was significantly elevated in BBP as compared to the Control group (Table 2; Wilcoxon test, $P < 0.05$).

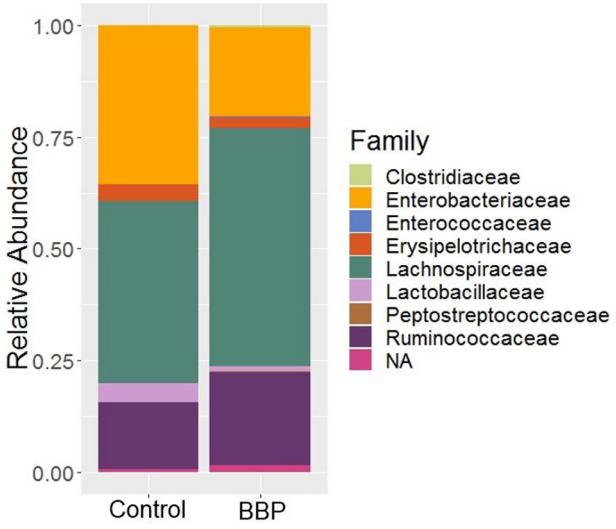

Figure 2. Relative abundance of bacterial families recovered on two treatment groups: Control and *Bacillus* base probiotic (BBP) in trial 2. NA represent those sequence reads which were not assigned at the family level, however, were assigned at the higher level of taxonomic classification.

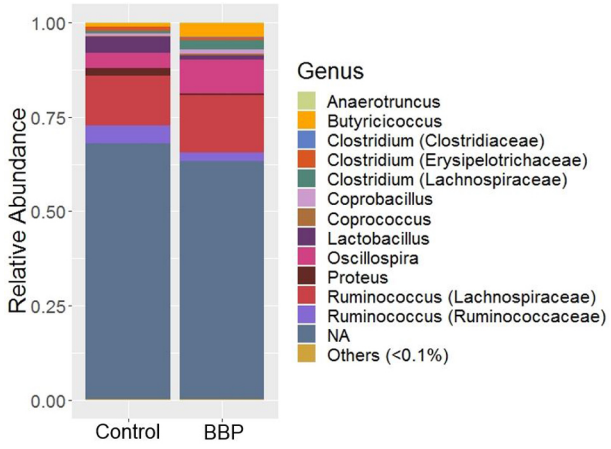

Figure 3. Relative abundance of bacterial genera recovered on2treatment groups: Control and *Bacillus* base probiotic (BBP) in trial 2. NA represent those sequence reads which were not assigned at the genus level, however, were assigned at the higher level of taxonomic classification. Others represent minor bacterial genera whose average relative abundance across all samples was <0.1%.

Bacterial Composition at the Genus Level

The relative abundance of dominant bacterial genera in Control and BBP groups is shown in Figure 3**.** Majority of the reads (>50%) were not assigned at the genus level, however, they were assigned at the higher taxonomic level, and are grouped under NA (Figure 3). Among those identified genera, the genus *Ruminococcus* that belong to the family Lachnospiraceae was found the highest in both Control (13.25 ± 6.24%) and BBP (15.15 ± 4.82%) groups. This was followed by the *Ruminococcus* (4.67 ± 1.72%) of family Ruminococcaceae in the Control, while the *Oscillospira* (8.86 ± 2.12%) in the BBP group. The *Ruminococcus* in the BBP group and the *Oscillospira* in the Control group were 2.39 ± 0.65% and 3.92 ± 0.74%, respectively. The genus *Oscillospira* was significantly enriched in the BBP group, whereas the unidentified genus of family Enterobacteriaceae in the Control group (Table 2; Wilcoxon test, $P < 0.05$). Other important numerical observations were the increase of *Lactobacillus* and *Proteus* in the Control group, and *Butyricicoccus* in the BBP group. Also, the genus *Clostridium* that belongs to the families Clostridiaceae and Lachnospiraceae was numerically increased in the BBP group while the *Clostridium* of family Erysipelotrichaceae was elevated in the Control group (Figure 3).

Alpha Diversity

The alpha diversity of Control and BBP group as calculated by observed OTUs metric and Shannon's index are shown in Figure 4A and 4B, respectively. Although the species richness was increased by the BBP group (Figure 4A), there was no significant difference between the 2 groups, as shown in Figure 4 (Wilcoxon test, $P > 0.05$).

Beta Diversity

The beta diversity between Control and BBP groups as calculated by unweighted UniFrac distance metric is illustrated in the PCoA plot (Figure 5). As shown in Figure 5, there was a significant difference in bacterial community structure between Control and BBP groups (PERMANOVA, $P < 0.05$).

DISCUSSION

The spread of antibiotic resistance genes has created public and scientific concerns leading to new regulations that limit the use of antibiotics as growth promoters, creating a need to evaluate different alternative products. Hence, the use of probiotics as alternative tools to antibiotic growth promoters has been increasing, and many investigators around the world have demonstrated their efficacy. Probiotics regulate the immune system (Lyte, 2011; Molinaro et al., 2012), exert anti-oxidant properties (Tao et al., 2006), and enhance barrier integrity (Yu et al., 2012). Recent studies published by our laboratory demonstrate that 90% of *Bacillus* spp. probiotic spores germinate within 60 min in the crop having full cycle from spores to vegetative cells to spores in different sections of the GIT (Latorre et al., 2014). After spore germination, bacteria become metabolically active to produce enzymes and other compounds that are beneficial to the host.

Moreover, the inclusion of this selected *Bacillus*-DFM (Norum^{TM}) that produce a different set of extracellular enzymes using different poultry diets, significantly reduce both viscosity and *C. perfringens* proliferation (Latorre et al., 2015b). Further studies confirmed that this multiple enzymes producing

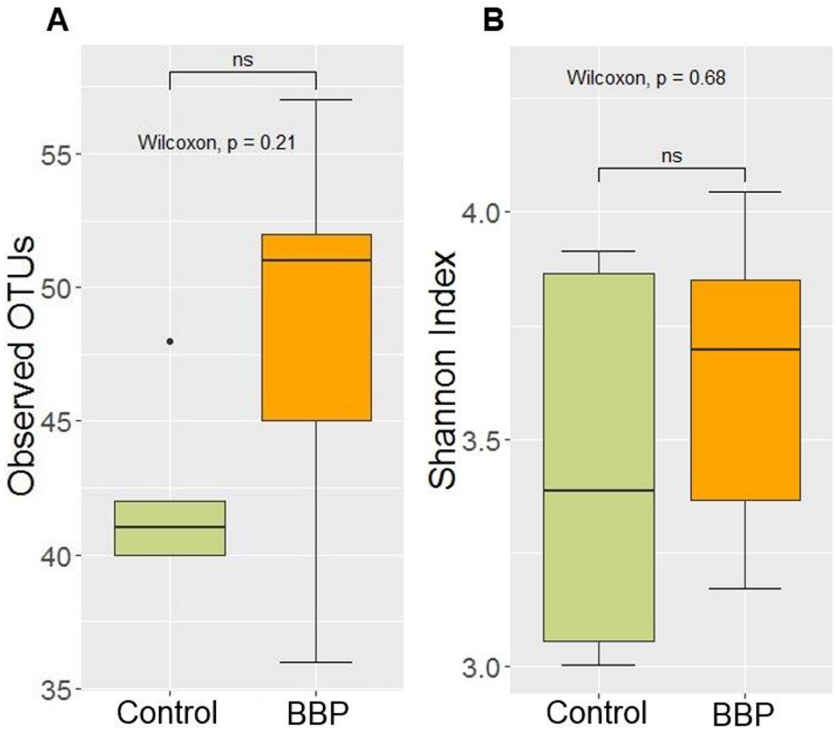

Figure 4. The alpha diversity of two treatment groups: Control and *Bacillus* base probiotic (BBP) in trial 2. The alpha diversity was calculated by observed OTUs metric (A) and Shannon's index (B), where the statistical significant difference between treatment groups was calculated by Wilcoxon test. NS represent non-significant difference between treatment groups ($P > 0.05$).

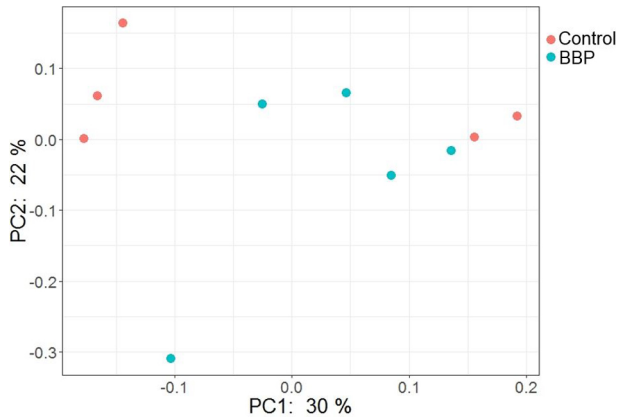

Figure 5. The PCoA plot showing the significant difference in beta diversity between 2 treatment groups: Control and *Bacillus* base probiotic (BBP) in trial 2. PERMANOVA, $P < 0.05$.

Bacillus-based DFM improved growth performance, digesta viscosity, bacterial translocation, microbiota composition, and bone mineralization in broiler chickens fed with a rye-based diet (Latorre et al., 2015a) as well as mitigate the negative impacts of necrotic enteritis in broiler chickens (Hernandez-Patlan et al., 2019). In the present study, we evaluated for the first time, the *in ovo* application of the vegetative *Bacillus* spp. strains contained in Norum^{TM} against experimental horizontal infection of *E. coli* in the hatching cabinet. Virulent *E. coli* strains can invade the host via the respiratory tract, leading to septicemia and airsacculitis (Dziva and Stevens, 2008). Under commercial conditions, chicks may be exposed to virulent *E. coli* strains during the hatch, indicating a need for a laboratory model allowing for evaluation of the effects of exposure during the hatching process.

In chickens, metagenome sequencing has shown that there are 4 major microbial phyla (Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria), which represent 99% of the intestinal microbiota. These phyla fall into 3 main groups of strict extremophile anaerobes: *Bacteroides, Clostridium* cluster XIVa, and *Clostridium* cluster IV (Oakley and Kogut, 2016). *Clostridium* and *Bacillus* are both in the phylum Firmicutes, but they are in different classes, orders, and families. Also, *Clostridium* is distinguished from *Bacillus* by being strict anaerobes. *Clostridium* genus contains over 100 commensal species, but only a few of them such as *Clostridium perfringens* and *Clostridium tetani* are pathogenic and produce some of the most potent toxins known in nature. Interestingly, most of Clostridia have a remarkable commensal and central relationship with their metazoan host, playing decisive roles in the physiology, immunology, and even cognitive activities as some of the essential butyric acid-producing bacteria of the GIT (Lopetuso et al., 2013; Liu et al., 2015; Zhong et al., 2015).

In both trials of the present study, hatchability was not affected by the treatment. However, embryos inoculated with BBP showed a significant reduction in the total number of Gram-negative bacteria in the GIT on DOH and day 7. Probiotic treatment increased BW DOH, BW day 7, and days 0 to 7 BWG when compared with control chickens. Moreover, the bacterial

composition at the phylum level revealed that Proteobacteria was significantly higher in the PBS Control, while Firmicutes was significantly higher in the BBP. The gut microbiota complements the biology of metazoans playing important roles in animal overall health and productiveness (Wei et al., 2013; Tellez, 2014). Several studies have documented some mechanisms by which BBP can balance the gut microbiota and improve performance (Latorre et al., 2015a; Qin et al., 2018).

At the family level, *in ovo* administration of the BBP increased the presence of Lachnospiraceae but showed a significant reduction of Enterobacteriaceae when compared with the PBS Control group. Lachnospiraceae (phylum Firmicutes, class Clostridia) is abundant in the digestive tracts of many mammals, are also important within the group because of the production of butyric acid (Meehan and Beiko, 2014; Schnabl and Brenner, 2014). In this context, at the genus level, BBP group also showed a significant increase in *Oscillospira* and a significant reduction in unidentified genus of family Enterobacteriaceae when compared with the PBS Control group. Even though alpha diversity richness was increased by the BBP group, it was not significant when compared with the control group. Interestingly, beta diversity showed a significant difference in bacterial community structure between Control and BBP groups. *Oscillospira* is an anaerobic bacterial genus from Clostridial cluster IV, and this genus was found to reduce significantly in the gut of human having enteric inflammation, whereas positively correlate with the leanness (Konikoff and Gophna, 2016; Gophna et al., 2017). In chickens, *Clostridium* and *Ruminococcus* are some of the most predominant genera found in the cecum (Wei et al., 2013).

The *Clostridium* genus, along with *Oscillospira* and *Coprococcus*, also encompasses bacteria capable of producing butyrate (Yang et al., 2017). Butyrate has been demonstrated to have a decisive role on growth performance, intestinal villus structure, and pathogen control, as well as anti-inflammatory properties (Onrust et al., 2015). Furthermore, *Ruminococcus* genus can also produce other short-chain fatty acids, such as acetic and succinic acid (Flint et al., 2008). It is well known that short chain fatty acids are an essential source of energy for enterocytes and are vital for intestinal health (Biasato et al., 2018). The large intestine is abundant in Clostridium clusters IV and XIVa of the phylum Firmicutes that produce butyric acid (Onrust et al., 2015). Some strategies are available to stimulate butyrate production in the distal gut. These include delivery of prebiotic, probiotic, or symbiotic products (Tellez et al., 2006; Plöger et al., 2012). Members of *Clostridium* clusters XIVa and IV such as Lachnospiraceae, *Ruminococcus*, and *Roseburia* are depleted continuously in humans with intestinal inflammation disorders, suggesting that these organisms play a vital role in preserving gastrointestinal homeostasis (Kabeerdoss et al., 2013). Hence, it is essential to distinguish the presence of beneficial clostridial groups from opportunistic pathogenic strains such as *Clostridium perfringens* and *Clostridium difficile* (Honneffer et al., 2014). Clostridiales-OTUs in cecal digesta may have triggered a higher mucosal tolerance towards the commensal microbiota by increasing the expression levels of IL10 and TGFB1 at the cecal mucosa (Wahl et al., 2004). The results of the present study suggest that *in ovo* administration of a BBP can reduce the severity of virulent *E. coli* horizontal transmission and infection of broiler chickens in the hatching cabinet. The reduction in the severity of the transmission and infection were associated with significant changes in beta diversity induced by the BBP, suggesting that the BBP treatment may drive large-scale changes in the microbial community structure and composition, which in turn provided protection against the pathogenic effects of the *E. coli* infection. This has been shown previously; for example treatment with *Bifidobacterium* protected against the virulence of *E. coli* toxins through the production of butyrate in the mouse gut (Fukuda et al., 2011, 2012). We hypothesize that a similar mechanism may explain the results presented here, with the consequent improvement in the health and productivity of broiler chickens.

SUPPLEMENTARY DATA

Supplementary data are available at *Poultry Science* online.

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