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In ovo and Dietary Supplementation of Probiotics Affects Post-Hatch Expression of Immune-Related Genes in Broiler Chicks

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Abstract

During the first week post-hatch, the neonatal chick is immunologically vulnerable and subject to infectious threats found in the environment. Probiotics are live, non-pathogenic microorganisms known to have a positive effect on the host by improving the natural balance of gut microbiota and promoting animal health. The objective of this study was to determine the effects of administering probiotics *in ovo* and in the diet on broiler chick hatchability, and post-hatch immune organ weights and ileal immune-related gene expression. At embryonic day 18, 1584 eggs were injected with nothing (Dry), 1×10^6, or 1×10^7 (P1 and P2 respectively) probiotic bacteria. The remaining 393 eggs were left non-injected to serve as a negative control. Immune organ weights and tissue samples were taken on DOH and d4, 6, 8, 14, and 20. No differences were observed for hatchability or relative bursa weights. Only on d6, the P2 birds receiving the probiotic-supplemented diet had larger spleens as a result of a 2-way interaction between *in ovo* treatment and post-hatch diet. Both *in ovo* and dietary administration of probiotics were able to modulate the expression of the immune-related genes in the ileum; however, expression patterns differed based on the gene, treatment, and time point evaluated. In conclusion, these results indicate that *in ovo* supplementation of this commercial probiotic product does not influence hatchability and is capable of differentially modulating expression of certain genes in the ileum. Furthermore, *in ovo* administration of probiotics has an effect similar to that of dietary supplementation endorsing its usage to potentially promote early colonization of beneficial bacteria to stimulate intestinal and immune system development.

Keywords: *In ovo*; Probiotic; Chickens; Immunity; Hatchability

Introduction

The immune system of the neonatal chick is immature and inefficient during the first week of life, rendering the bird extremely vulnerable to infectious threats found in the environment [1]. Immunomodulators are currently being studied and sought after to counteract the inadequacies of the chick immune system and promote host defense during this immunologically liable time. Furthermore, changes in the poultry industry, such as the banning of sub-therapeutic use of certain antimicrobials in early 2017, have created an impetus for finding alternatives capable of maintaining animal health without having negative consequences on performance and profit margins. Probiotics have received increasing attention as an alternative with their potential to stimulate the immune system and reduce the rate and severity of enteric infections in poultry [2-4]. There are several microbial species commonly utilized as probiotics including those of *Lactobacillus*, *Bifidobacterium*, *Enterococcus*, *Bacillus*, and *Pediococcus* [5,6]. Probiotic products may contain one or several different bacterial species.

The primary function of the gastrointestinal tract is to digest and absorb nutrients in order to meet metabolic demands for maintenance, normal growth, and development; additionally, it acts as a vital barrier preventing the entry of several potentially harmful pathogens [7,8]. The introduction of 'commensal' microbiota to the gastrointestinal tract is critical to the development of the gut-associated lymphoid tissues (GALT). Interactions between the microbiota, the host intestinal tract and its associated immune tissues are necessary for the complete and robust development of the gastrointestinal system [9]. The gut microbial profile can be manipulated with probiotics in order to create conditions favourable to enhancing growth and health. In poultry, probiotics can enhance performance [10], promote a healthy microbial balance, and enhance host defenses against several enteric pathogens through stimulation of the mucosal immune system [11,12]. Oral administration of probiotics results in enhanced heterophil oxidative burst and degranulation as well as augmented phagocytic capacity of macrophages [1,13,14]. Probiotics also influenced humoral and cell-mediated immune responses by increasing antibody production and upregulating T lymphocyte numbers and associated responses [15-17]. Probiotics have proven their ability to enhance the immune response by promoting the clearance of several economically important pathogens such as *Eimeria spp.*, *Salmonella spp.*, *Escherichia coli* and *Clostridium perfringens*, further asserting their potential use as an antibiotic alternative [6,18-20].

Traditionally, probiotics are administered in the feed or water supply to 1-day-old birds. However, as soon as the chick hatches and is exposed to the external environment, it quickly begins to establish the microbial community in the intestine [21]. In order to promote early establishment of beneficial strains, employing *in ovo* technology may be the solution. *In ovo* technology represents a means to take advantage of this crucial time and promote early colonization of beneficial bacteria in order to stimulate intestinal and immune system development [4]. Few researchers have entertained the idea of administering probiotics *in ovo*, which recently has been gaining more interest [22,23]. The earliest attempts of connecting these concepts

demonstrated promising results where *Salmonella typhimurium* colonization was reduced in chicks administered an undefined cecal culture of bacteria *in ovo* [24]. Unfortunately, negative results in terms of hatchability, performance, and mortality have been noted, though these consequences may be attributed to the probiotic strain used and delivery site [22,24-26]. Edens et al. demonstrated positive results administering *Lactobacillus reuteri in ovo* in hatching chicks and turkey poults [27]. They found no differences among treatment groups when comparing the hatchability of embryos injected with *L. reuteri* in either the air cell or the amniotic fluid to non-inoculated controls. Similar results were also seen in turkey embryos. More recently, administration of a commercial (lactobacilli- and bifidobacteria-based) probiotic as early as day 18 embryonic age was shown not to impact hatchability and could enhance early performance [22] and reduce disease severity in broiler chickens challenged post-hatch [26]. The objective of this study was to determine the effects of administering a *Lactobacillus*-based probiotic (PrimaLac) *in ovo* and in the diet on broiler chick hatchability, post-hatch immune organ weights, and developmental expression of intestinal immune-related genes.

Materials and Methods

Birds and treatments

This study was approved and conducted under the guidelines of the Virginia Tech Institutional Animal Care and Use Committee. At embryonic day (d) 18, 1980 fertile Cobb 500 eggs were obtained from a commercial hatchery (Pilgrim's Pride, Broadway, VA) and transported to the Virginia Tech Turkey Research Center. Prior to injection, all eggs were candled to determine position of the air cell. Eggs were sanitized by swabbing the large end (outside of the air cell) with 0.5% sodium hypochlorite and once dried, they were sprayed with 70% isopropyl alcohol. To create a guide and avoid cracking, a pilot hole was made in the center of the air cell of those eggs receiving injections using an 18-gauge needle fitted with a rubber stopper to prevent the needle from piercing the air cell membrane. Needles were disinfected in between each injection by dipping in 0.5% sodium hypochlorite. Next, 1584 eggs were evenly divided and injected with either nothing (dry), or 1×10^6 or 1×10^7 (P1 and P2 respectively) probiotic bacteria dissolved in sterile water (PrimaLac W/S, Star-Labs Inc., containing *Lactobacillus acidophilus*, *Lactobacillus casei*, *Enterococcus faecium*, and *Bifidobacterium bifidum*). Injections were performed using a 1 mL syringe equipped with a 22 gauge 2.5 cm short bevel needle. A new syringe and needle were used for each injection. The remaining 396 eggs were not injected and served as a negative control. Eggs were placed into one of 6 replicate hatching trays (66 eggs/tray). On day of hatch (DOH), birds were individually tagged, divided in half and placed into floor pens relative to treatment group. Half of the pens (6 pens/*in ovo* treatment) received supplemental probiotic (Primalac 454 FG, Star-Labs Inc.) in the feed at 1 kg/ton fed continuously throughout the trial. The basal diet consisted of a standard non-medicated broiler starter feed in crumble form provided from DOH to d19 and a standard non-medicated broiler grower feed in pellet form provided from d19 to d42. Feed and water were offered ad libitum throughout the study.

Hatchability and post-hatch sample collection

On DOH, percent hatchability was recorded, and birds were weighed prior to placement and on sampling days. On DOH, d4, 6, 8, 14, 20 and 42, six birds per treatment were randomly selected and euthanized by cervical dislocation. The bursa of Fabricius and spleen were excised and weighed, and relative organ weights were expressed as a percentage of live BW. The same six birds per treatment were sampled for gene expression analysis. The ileum, defined as the area posterior to the Meckel's diverticulum to the ileo-cecal junction, was sampled, rinsed in cold PBS, and placed in RNA*later* (Qiagen, Germantown, MD) for subsequent gene expression analysis. The samples were stored at -80°C until analysis.

Ileal gene expression

Intestinal samples were removed from -80°C and a 20-30 mg aliquot of each sample was weighed, placed into a 2 mL microcentrifuge tube along with a 5 mm stainless steel bead and 600 µL lysis buffer, and homogenized using the TissueLyser II system (Qiagen) according to manufacturer's recommendation. Total RNA was extracted from individual intestinal tissues using the RNeasy mini kit following the animal tissue protocol (Qiagen). Following extraction, RNA was eluted by rinsing the column membrane twice with 25 µL of RNase-free water. Total RNA concentration was determined at OD 260 (NanoDrop-1000, Thermo Fisher Scientific, Waltham, MA) and RNA purity was verified by evaluating the ratio of OD 260 to OD 280. Total RNA was diluted to 0.2 µg/µL in nuclease-free water. Reverse transcription was accomplished using the high capacity cDNA Reverse Transcription kit (Applied Biosystems, Carlsbad, CA) following the manufacturer's protocol, and the cDNA was stored at -20°C.

Quantitative real-time PCR (qRT-PCR) was performed using an ABI 7500 FAST Real-Time PCR System (Applied Biosystems) as described [26]. The cDNA was diluted 1:30 in nuclease-free water then 1 µL of the diluted cDNA was added to each well of a 96-well plate. Next, 9 µL of real-time PCR master mix containing 5 µL FAST SYBR Green Master Mix (Applied Biosystems), 0.5 µL each of 2 µM forward and reverse primers and 3 µL of sterile nuclease-free water per reaction were added to each well for a final volume of 10 µL. During the PCR reaction, samples were subjected to an initial denaturation phase of 20 sec at 95°C followed by 40 cycles of denaturation at 95°C for 3 sec and annealing and extension at 60°C for 30 sec. Gene expression for interferon (IFN)-γ, interleukin (IL)-4, IL-13, inducible nitric oxide synthase (iNOS), lipopolysaccharide-induced tumor necrosis factor-α (LITAF), mucin (Muc)-2, trefoil family factor (TFF)-2, Toll-like receptor (TLR)-2 and TLR-4 was analyzed using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an endogenous control. Each reaction was run in duplicate. Primers were designed (Table 1) using the Primer Express 3.0 software (Applied Biosystems) and synthesized by MWG Operon (Huntsville, AL). Results from qRT-PCR were analyzed using the 7500 Real-Time PCR software (Applied Biosystems). Average gene expression relative to the *GAPDH* endogenous control for each sample was calculated using the $2^{-\Delta\Delta Ct}$ method [28]. The calibrator for each gene was the average ΔCt value from the negative control group for each sampling day.

Statistical analysis

Results were analyzed as a 4 × 2 factorial using the Fit Model platform in JMP 9.0 (SAS Institute Inc., Cary, NC). Percent hatchability was arc sine transformed prior to analysis. Differences in experimental treatments with individual birds as replicates within group were tested using Tukey-HSD following ANOVA. Values were considered statistically different at $P \leq 0.05$. Results are reported as least squares means with standard errors.

Target	Accession No	Nucleotide sequence (5'→3')
GAPDH_F	NM_204305	CCTAGGATACACAGAGGACCAGGTT
GAPDH_R		GGTGGAGGAATGGCTGTCA
IL-4_F	NM_001007079	GCTCTCAGTGCCGCTGATG
IL-4_R		GAAACCTCTCCCTGGATGTCAT
IL-13_F	NM_001007085	CATGACCGACTGCAAGAAGGA
IL-13_R		CCGTGCAGGCTCTTCAGACT
IFN-γ_F	NM_205149	GCTCCCGATGAACGACTTGA
IFN-γ_R		TGTAAGATGCTGAAGAGTTCATTCG
iNOS_F	D85422	CCTGTACTGAAGGTGGCTATTGG
iNOS_R		AGGCCTGTGAGAGTGTGCAA
LITAF_F	AY765397	TGTTCTATGACCGCCCAGTTC
LITAF_R		AGACGTGTCACGATCATCTGGTTA
Muc-2_F	NM_001318434.1	TTCATGATGCCTGCTCTTGTG
Muc-2_R		CCTGAGCCTTGGTACATTCTTGT
TFF-2_F	XM_416743.4	TGGTCCCCCAGGAATCTCA
TFF-2_R		CACCGACGCATTGAAGCA
TLR-2_F	NM_204278	GCGAGCCCCCACGAA
TLR-2_R		GGAGTCGTTCTCACTGTAGGAGACA
TLR-4_F	NM_001030693	CCACACACCTGCCTACATGAA
TLR-4_R		GGATGGCAAGAGGACATATCAAA

Table 1: Primers used for relative real-time PCR (Primers designed by Primer Express software (Applied Biosystems, Foster City, CA)).

Results

Hatchability and post-hatch immune organ weights

No significant differences were observed for hatchability or BW during this study where hatchability ranged from 89% to 93%. Neither *in ovo* delivery nor diet supplementation of the probiotic had an effect on bursa weights at any time point during the study (data not shown). On d6, *in ovo* treatment and diet resulted in a 2-way interaction on relative spleen weights with the P2 birds receiving the probiotic-supplemented diet having a larger spleen (0.093%) than all other groups except P1 birds given the control diet (0.083%) (P=0.02).

Ileal gene expression

On d14, *in ovo* treatment and diet resulted in a 2-way interaction on *TLR-2* expression (P=0.048) (Table 2). Expression of *TLR-2* was downregulated in P1 birds given the non-supplemented diet when compared to the birds receiving the dry punch and the same diet. When given probiotic supplementation in the diet, P1 birds displayed increased *TLR-2* expression. There was a main effect of *in ovo* treatment (P=0.01) and diet (P<0.001) on *TLR-4* expression on d8 (Table 3). *TLR-4* levels were higher in P2 than the negative control and dry punch groups, while P1 had higher levels than the dry punch group. Additionally, chicks given the probiotic supplemented diet had augmented *TLR-4* expression. On d14, *in ovo* treatment and diet presented a 2-way interaction on *TLR-4* gene expression (P=0.01). Upon examining the groups given the basal diet, P1 birds displayed downregulated expression of *TLR-4* when compared to the dry punch group. In those groups given the probiotic supplemented diet, however, P1 birds demonstrated an increase in *TLR-4* expression when compared to the dry punch and P2 groups. Dietary supplementation resulted in a decrease in *TLR-4* expression in the dry punch and P2 birds.

Age	DOH	d4	d6	d8	d14	d20
Main Effects						
***In Ovo* Treatment**						
Neg	1	1	1	1	1	1
Dry	0.94	1.12	0.76	1.15	1.1	1.2
P1	1.29	1.06	1.28	1.26	1.07	1.73
P2	0.93	0.87	1.2	1.43	1.11	1.45
Dietary Treatment						
Not Supplemented	--	1.00	1.00	1.00	1.00	1.00
Supplemented	--	0.91	0.93	1.28	0.98	0.91
Interactions						
Neg/Not Supp	--	1.00	1.00	1.00	1.00^{ab}	1.00
Dry/Not Supp	--	0.98	0.62	1.40	1.21^{a}	1.27
P1/Not Supp	--	1.37	1.35	1.59	0.81^{b}	2.12
P2/Not Supp	--	0.69	0.84	1.61	1.04^{ab}	1.08
Neg/Supp	--	0.86	0.72	1.68	0.87^{ab}	0.90
Dry/Supp	--	1.11	0.67	1.60	0.86^{ab}	1.02
P1/Supp	--	0.71	0.88	1.68	1.22^{a}	1.27
P2/Supp	--	0.95	1.23	2.15	1.04^{ab}	1.75
Statistical Effects (P-Value)						
In Ovo Treatment	0.59	0.74	0.12	0.34	0.85	0.10
Dietary Treatment	--	0.60	0.67	0.08	0.85	0.57
In Ovo * Diet	--	0.20	0.28	0.66	0.048	0.17

Table 2: Effect of *in ovo* and dietary probiotic supplementation on ileal *TLR-2* gene expression (fold change). Different letters within a column indicate significant difference among treatment groups. [1]Neg=negative control; [2]Dry=dry punch; [3]P1=1 × 10^6 probiotic bacteria; [4]P2=1 × 10^7 probiotic bacteria; [5]Not supp=not supplemented; [6]Supp=supplemented.

On d20, there was a main effect of dietary treatment on *iNOS* gene expression where levels were decreased due to probiotic supplementation (P=0.04) (Table 4). Neither in ovo treatment nor diet altered *TFF-2* expression during this study (data not shown). On d4, there was a main effect of diet on *Muc-2* expression in the ileum where

levels of *Muc-2* were elevated due to probiotic supplementation (P<0.0001) (Table 5). A similar pattern was observed on d14 (P=0.003) and d20 (P<0.0001). There was a main effect of *in ovo* treatment on *Muc-2* expression on d6 where levels in P1 were significantly higher than the negative control and dry punch groups (P=0.01).

Age	DOH	d4	d6	d8	d14	d20
Main Effects						
***In Ovo* Treatment**						
Neg	1.00	1.00	1.00	1.00^{bc}	1.00	1.00
Dry	1.14	0.95	0.90	0.87^{c}	1.14	1.10
P1	1.38	1.15	1.11	1.29^{ab}	1.12	1.37
P2	1.09	0.86	1.30	1.59^{a}	0.85	1.48
Dietary Treatment						
Not Supplemented	--	1.00	1.00	1.00^{b}	1.00^{a}	1.00
Supplemented	--	1.00	1.01	1.77^{a}	0.62^{b}	0.95
Interactions						
Neg/Not Supp	--	1.00	1.00	1.00	1.00^{abc}	1.00
Dry/Not Supp	--	0.83	0.67	1.21	1.39^{a}	0.97
P1/Not Supp	--	1.34	1.12	1.27	0.90^{bcd}	1.18
P2/Not Supp	--	0.78	1.04	1.44	1.10^{ab}	1.14
Neg/Supp	--	0.97	0.78	1.98	0.69^{cd}	0.73
Dry/Supp	--	1.04	0.94	1.22	0.64^{de}	0.91
P1/Supp	--	0.96	0.87	2.59	0.97^{abc}	1.17
P2/Supp	--	0.91	1.28	3.46	0.46^{e}	1.40
Statistical Effects (P-Value)						
In Ovo Treatment	0.59	0.45	0.28	0.01	0.15	0.20
Dietary Treatment	--	0.98	0.94	< 0.001	< 0.001	0.75
In Ovo * Diet	--	0.42	0.33	0.080	0.01	0.66

Table 3: Effect of *in ovo* and dietary probiotic supplementation on ileal *TLR-4* gene expression (fold change). Different letters within a column indicate significant difference among treatment groups. [1]Neg=negative control; [2]Dry=dry punch; [3]P1=1 × 10^6 probiotic bacteria; [4]P2=1 × 10^7 probiotic bacteria; [5]Not supp=not supplemented; [6]Supp=supplemented.

Expression of *IFN-γ* was downregulated on d4 due to dietary probiotic supplementation (P=0.0002) (Table 6). On d14, *in ovo* treatment and diet resulted in a 2-way interaction for *IFN-γ* expression (P = 0.0002). Within the groups receiving the basal diet, P1 and P2 exhibited a downregulation of *IFN-γ* expression. In the groups receiving the supplemented diet, however, P1 displayed greater *IFN-γ* expression than all other groups. Dietary supplementation of probiotics resulted in decreased *IFN-γ* levels in the negative control and dry punch birds.

Age	DOH	d4	d6	d8	d14	d20
Main Effects						
***In Ovo* Treatment**						
Neg	1.00	1.00	1.00	1.00	1.00	1.00
Dry	0.85	1.04	0.86	1.00	0.96	1.00
P1	1.01	0.85	1.10	0.94	1.16	1.06
P2	0.83	0.75	0.90	1.25	0.90	1.07
Dietary Treatment						
Not Supplemented	--	1.00	1.00	1.00	1.00	1.00^{a}
Supplemented	--	0.97	0.94	0.99	0.99	0.79^{b}
Interactions						
Neg/Not Supp	--	1.00	1.00	1.00	1.00	1.00
Dry/Not Supp	--	0.98	0.77	1.2	0.89	1.19
P1/Not Supp	--	0.76	1.39	1.09	0.93	0.99
P2/Not Supp	--	0.58	0.83	1.33	0.98	1.04
Neg/Supp	--	0.78	0.95	1.21	0.89	0.83
Dry/Supp	--	0.86	0.92	1.01	0.93	0.69
P1/Supp	--	0.75	0.83	0.98	1.29	0.94
P2/Supp	--	0.76	0.93	1.42	0.75	0.91
Statistical Effects (P-Value)						
In Ovo Treatment	0.33	0.32	0.54	0.31	0.38	0.96
Dietary Treatment	--	0.85	0.62	0.94	0.96	0.04
In Ovo * Diet	--	0.56	0.23	0.66	0.22	0.40

Table 4: Effect of *in ovo* and dietary probiotic supplementation on ileal *iNOS* gene expression (fold change). Different letters within a column indicate significant difference among treatment groups. [1]Neg=negative control; [2]Dry=dry punch; [3]P1=1 × 10^6 probiotic bacteria; [4]P2=1 × 10^7 probiotic bacteria; [5]Not supp=not supplemented; [6]Supp=supplemented.

In ovo treatment and diet presented a 2-way interaction on *LITAF* expression on d20 (P=0.02) (Table 7). Expression of *LITAF* was significantly downregulated in P2 birds given the non-supplemented diet when compared to negative control and P1 birds given the same diet. No differences were noted among groups given the supplemented diets, but dietary supplementation did reduce *LITAF* levels in the negative control and P1 chicks.

There was a main effect of dietary treatment on *IL-4* gene expression on d4 (P=0.001) and d20 (P=0.02) where *IL-4* levels were decreased due to probiotic supplementation (Table 8). Neither *in ovo* treatment nor diet altered *IL-13* expression during this study (data not shown).

Discussion

The aim of this study was to explore the effects of *in ovo* administration of the probiotic product PrimaLac W/S in broiler

chicks and evaluate those effects with and without dietary probiotic supplementation. In this experiment, *in ovo* supplementation of probiotics had no effect on hatchability further corroborating our previous findings [22,26]. Very few studies have been published regarding the concept of administrating probiotics via the *in ovo* route. Edens et al. compared the hatchability of broiler embryos injected with *Lactobacillus reuteri* in either the air cell or the amniotic fluid to non-inoculated controls and found no differences among the treatment groups [27]. Similar results were also seen in turkey embryos [27]. Alternatively, other researchers have found that *in ovo* injection of some probiotic strains can negatively impact hatchability [24,25]. Our findings suggest that the probiotic bacteria in PrimaLac can be safely administered *in ovo* without negatively affecting hatchability.

Age	DOH	d4	d6	d8	d14	d20
Main Effects						
***In Ovo* Treatment**						
Neg	1.00	1.00	1.00^{b}	1.00	1.00	1.00
Dry	1.22	1.31	0.85^{b}	0.98	1.06	1.09
P1	0.94	0.84	1.79^{a}	0.66	1.37	1.03
P2	1.65	1.15	1.22^{ab}	0.64	1.17	0.69
Dietary Treatment						
Not Supplemented	--	1.00^{b}	1.00	1.00	1.00^{b}	1.00^{b}
Supplemented	--	2.05^{a}	1.22	1.08	1.42^{a}	1.92^{a}
Interactions						
Neg/Not Supp	--	1.00	1.00	1.00	1.00	1.00
Dry/Not Supp	--	1.52	0.64	0.95	1.24	0.97
P1/Not Supp	--	0.95	1.45	0.85	1.68	0.98
P2/Not Supp	--	1.43	0.83	1.28	1.55	0.76
Neg/Supp	--	2.62	0.79	1.58	1.96	1.85
Dry/Supp	--	2.95	0.89	1.62	1.77	2.25
P1/Supp	--	1.94	1.74	1.13	2.20	2.00
P2/Supp	--	2.44	1.41	0.71	1.73	1.17
Statistical Effects (P-Value)						
In Ovo Treatment	0.1	0.18	0.01	0.45	0.22	0.12
Dietary Treatment	--	< 0.0001	0.19	0.29	0.003	< 0.0001
In Ovo * Diet	--	0.81	0.29	0.06	0.33	0.77

Table 5: Effect of *in ovo* and dietary probiotic supplementation on ileal *Muc-2* gene expression (fold change). Different letters within a column indicate significant difference among treatment groups. [1]Neg=negative control; [2]Dry=dry punch; [3]P1=1 × 10^{6} probiotic bacteria; [4]P2=1 × 10^{7} probiotic bacteria; [5]Not supp=not supplemented; [6]Supp=supplemented.

The relative size of the bursa was not affected by *in ovo* or dietary supplementation of probiotics during this study. *In ovo* treatment and diet presented a 2-way interaction for relative spleen weight only on d6 with P2 birds given the supplemented diet having larger spleens than all other groups, except P1 birds given the control diet. Larger spleens have also been observed in studies where probiotics were added to the diets of broilers, suggesting that probiotics have an effect on the systemic immune system [10,29,30]. There are conflicting reports, however, suggesting that probiotics do not affect immune organ weights [31,32].

Age	DOH	d4	d6	d8	d14	d20
Main Effects						
***In Ovo* Treatment**						
Neg	1.00	1.00	1.00	1.00	1.00^{a}	1.00
Dry	1.12	1.05	0.90	0.98	0.91^{a}	0.83
P1	1.22	0.83	1.30	1.01	0.95^{a}	1.00
P2	1.01	0.91	1.37	1.21	0.62^{b}	0.94
Dietary Treatment						
Not Supplemented	--	1.00^{a}	1.00	1.00	1.00	1.00
Supplemented	--	0.55^{b}	1.26	0.94	0.86	1.07
Interactions						
Neg/Not Supp	--	1.00	1.00	1.00	1.00^{a}	1.00
Dry/Not Supp	--	1.67	0.68	1.03	0.96^{a}	0.88
P1/Not Supp	--	1.02	1.32	1.07	0.54^{b}	1.25
P2/Not Supp	--	1.09	1.68	1.33	0.40^{b}	0.93
Neg/Supp	--	0.83	1.22	1.04	0.53^{b}	1.34
Dry/Supp	--	0.55	1.46	0.98	0.45^{b}	1.04
P1/Supp	--	0.57	1.57	0.99	0.89^{a}	0.91
P2/Supp	--	0.63	1.37	1.15	0.52^{b}	1.03
Statistical Effects (P-Value)						
In Ovo Treatment	0.58	0.66	0.20	0.79	0.01	0.61
Dietary Treatment	--	0.0002	0.15	0.72	0.18	0.60
In Ovo * Diet	--	0.19	0.22	0.98	0.0002	0.26

Table 6: Effect of *in ovo* and dietary probiotic supplementation on ileal *IFN-γ* gene expression (fold change). Different letters within a column indicate significant difference among treatment groups. [1]Neg=negative control; [2]Dry=dry punch; [3]P1=1 × 10^{6} probiotic bacteria; [4]P2=1 × 10^{7} probiotic bacteria [5]Not supp=not supplemented; [6]Supp=supplemented.

In spite of the considerable amount of published data regarding the efficacy of probiotics in poultry, the exact mechanism of how probiotics alter the immune system is still not fully understood. Our goal was to identify patterns of gene expression underlying the effects of probiotic supplementation on the immune system, particularly at the gut level. To observe the effects of probiotics on innate immunity, we evaluated the expression of *TLR-2*, *TLR-4*, *iNOS*, *Muc-2* and *TFF-2* in the ileum. The TLR family is a highly conserved group of proteins that act as pathogen recognition receptors (PRR), recognizing microbe-associated molecular patterns (MAMPs) that are expressed

on infectious agents. They play a fundamental role in pathogen detection and are responsible for the initiation and regulation of the innate response.

Age	DOH	d4	d6	d8	d14	d20
Main Effects						
***In Ovo* Treatment**						
Neg	1.00	1.00	1.00	1.00	1.00	1.00
Dry	0.88	0.91	0.94	0.97	1.10	1.04
P1	0.97	1.06	1.00	0.91	1.11	1.00
P2	1.04	0.98	0.88	0.9	1.11	0.90
Dietary Treatment						
Not Supplemented	--	1.00	1.00	1.00	1.00	1.00^{a}
Supplemented	--	0.90	0.95	1.08	0.89	0.80^{b}
Interactions						
Neg/Not Supp	--	1.00	1.00	1.00	1.00	1.00^{a}
Dry/Not Supp	--	0.80	1.05	1.07	1.08	0.83^{abc}
P1/Not Supp	--	1.11	1.08	0.90	1.14	0.89^{ab}
P2/Not Supp	--	0.87	1.02	0.91	1.04	0.66^{cd}
Neg/Supp	--	0.81	1.13	1.00	0.87	0.58^{d}
Dry/Supp	--	0.85	0.94	1.14	0.98	0.75^{abcd}
P1/Supp	--	0.83	1.04	1.04	0.93	0.65^{cd}
P2/Supp	--	0.89	0.86	1.02	1.03	0.72^{bcd}
Statistical Effects (P-Value)						
In Ovo Treatment	0.78	0.55	0.60	0.73	0.66	0.56
Dietary Treatment	--	0.15	0.53	0.29	0.11	0.001
In Ovo * Diet	--	0.30	0.55	0.7	0.79	0.020

Table 7: Effect of *in ovo* and dietary probiotic supplementation on ileal *LITAF* gene expression (fold change). Different letters within a column indicate significant difference among treatment groups. [1]Neg=negative control; [2]Dry=dry punch; [3]P1=1 $\times 10^{6}$ probiotic bacteria; [4]P2=1 $\times 10^{7}$ probiotic bacteria; [5]Not supp=not supplemented; [6]Supp=supplemented.

Though it is evident that probiotic supplementation resulted in differential expression patterns of TLRs, and thus the innate immune system, it cannot be conclusively determined how probiotics influence localized innate responses under healthy conditions. As such, the presence of an enteric challenge might shed some light on the mechanistic functions of probiotic bacteria, and help us determine their impact on TLR pathways consistent with appropriate responses to those challenges. To this end, we reported reduced disease severity in birds inoculated with this probiotic at embryonic day 18 followed by a coccidia challenge post-hatch [26].

When exposed to antigens or chemotactic agents, macrophages will begin to produce *iNOS*. This enzyme leads to the production of nitric oxide, which will subsequently react with superoxide anions to generate toxic derivatives, allowing macrophages to proficiently kill numerous types of pathogens [33]. Though no effects were observed in the *in ovo* treatment groups, dietary administration of probiotics resulted in a downregulation of *iNOS* gene expression on d20, suggesting PrimaLac may possess an anti-inflammatory function. The *Muc-2* gene is responsible for encoding mucin production, which is mediated by T lymphocytes and Th2 cytokines [34].

Age	DOH	d4	d6	d8	d14	d20
Main Effects						
***In Ovo* Treatment**						
Neg	1.00	1.00	1.00	1.00	1.00	1.00
Dry	1.17	1.18	0.96	0.93	1.26	1.11
P1	1.12	0.82	1.36	1.12	1.20	1.02
P2	1.33	0.75	1.04	1.02	1.13	1.35
Dietary Treatment						
Not Supplemented	--	1.00^{a}	1.00	1.00	1.00	1.00^{a}
Supplemented	--	0.65^{b}	1.13	1.07	1.19	0.71^{b}
Interactions						
Neg/Not Supp	--	1.00	1.00	1.00	1.00	1.00
Dry/Not Supp	--	1.47	1.00	1.19	1.47	0.97
P1/Not Supp	--	0.86	1.60	1.58	1.04	1.02
P2/Not Supp	--	0.80	1.40	1.45	1.04	1.57
Neg/Supp	--	0.76	1.44	1.71	1.15	0.72
Dry/Supp	--	0.72	1.35	1.25	1.23	0.91
P1/Supp	--	0.60	1.66	1.36	1.60	0.73
P2/Supp	--	0.54	1.12	1.23	1.40	0.83
Statistical Effects (P-Value)						
In Ovo Treatment	0.40	0.05	0.11	0.56	0.53	0.44
Dietary Treatment	--	0.001	0.26	0.48	0.15	0.02
In Ovo * Diet	--	0.62	0.20	0.07	0.31	0.55

Table 8: Effect of *in ovo* and dietary probiotic supplementation on ileal *IL-4* gene expression (fold change). Different letters within a column indicate significant difference among treatment groups. [1]Neg=negative control; [2]Dry=dry punch; [3]P1=1 $\times 10^{6}$ probiotic bacteria; [4]P2=1 $\times 10^{7}$ probiotic bacteria; [5]Not supp=not supplemented; [6]Supp=supplemented.

Mucin is made up of glycoproteins and serves a protective function by binding to pathogens, thus preventing their adhesion to the intestinal surface. Trefoil factor-2 is a stable secretory protein expressed in gastrointestinal mucosa responsible for protecting the mucosa from insults, stabilizing the mucus layer and promoting the healing of the epithelium [35]. While no differences were noted for *TFF-2* expression,

Muc-2 levels were increased due to *in ovo* treatment and dietary supplementation. Previous studies have shown that PrimaLac is able to modulate the processes of mucin synthesis by altering the intestinal bacterial populations [36]. PrimaLac also increased the goblet cell number and mucus secretion in the small intestine of turkeys, which may protect intestinal epithelia from adverse factors including pathogens [37]. The upregulation of *Muc-2* also suggests the favoring of a Th2 mediated response.

To evaluate effects on the adaptive immune response, we analyzed gene expression of *IFN-γ*, *LITAF*, *IL-4* and *IL-13*. *IFN-γ* is a vital cytokine, secreted mostly by Th1 cells, that plays a central role in regulating the innate and adaptive immune responses, and is responsible for promoting Th1 cell differentiation, suppressing Th2 cell activity, and enhancing innate immune cell activation and function [38]. Expression of *IFN-γ* was differentially downregulated due to *in ovo* and dietary adminsitration. Expression of *LITAF* is principally in the spleen of chickens, as well as in intestinal intraepithelial lymphocytes. The *LITAF* protein is a transcription factor that mediates the expression of members of the tumor necrosis factor ligand superfamily [39]. On d20, expression of *LITAF* was reduced due to both probiotic administration methods; when coupled with the decrease in *IFN-γ* levels, it further supports the thought that probiotics may promote an anti-inflammatory environment, suppress Th1 activity, and promote a Th2 mediated response. Interleukin-4, a representative of Th2 cytokines, plays a fundamental role in the stimulation of B lymphocytes, T lymphocyte proliferation, and the differentiation of CD4+ T cells into Th2 cells [40]. The functions of *IL-13*, also characterized as a Th2 cytokine, overlap considerably with those of *IL-4*. Both *IL-4* and IL-13 function by inhibiting the production of pro-inflammatory modulators. Dietary-administered probiotic, but not *in ovo* administration, resulted in decreased *IL-4* expression while no differences were observed in *IL-13* expression levels.

Other than *Muc-2*, the general trend appears to be a downregulation of host immune-related genes. The decreased transcription of these genes in the probiotic-treated groups may be a response to the inhibitory effects of probiotic bacteria on pathogen colonization. A reduction in intestinal colonization by pathogenic bacteria may have eliminated the need for the induction of these genes. Several studies have demonstrated the ability of probiotics to modulate the levels of several cytokines; however, discrepancies have been noted due to differences in the bacterial strains, combinations of probiotic strains, and presence or absence of a challenge [15-17,22]. Further supporting our findings, many reports have noted decreases in immune-related factors. Akbari et al. reported a downregulation in the expression of antimicrobial peptides in the cecal tonsils of broilers due to probiotic supplementation during a *Salmonella* infection, attributing the outcome to a reduced *Salmonella* load in the intestine [41]. Mountzouris et al. found that avilamycin and probiotic treatment result in reduced levels of plasma IgA and IgG and intestinal IgA against *Salmonella enteritidis* when compared to the challenged control and that those levels were similar to the non-challenged controls [42]. Dalloul et al. observed similar results when they evaluated antibody secretion during a coccidiosis infection between control and probiotic fed birds [43]. When evaluating the effects of probiotic treatment on gene expression in the cecal tonsils of chicks challenged *Salmonella*, Haghighi et al. found that IL-12 and *IFN-γ* levels were suppressed by probiotic treatment, which correlated with reduced intestinal *Salmonella* colonization [44]. The immunosuppressive effects seen in these studies could be a result of the reduced colonization capacity of pathogenic bacteria, enhanced clearance, and accelerated recovery caused by the probiotic treatments.

Based on the results presented in this study, the effect of *in ovo* administration of probiotics on the immune response appears to be similar to that of dietary probiotic supplementation, reinforcing its potential usage to promote early colonization of beneficial bacteria to stimulate intestinal and immune system development. Immunomodulation by *in ovo* supplementation of probiotics in poultry, and early establishment of beneficial microbiota, may lead to increased overall health and well-being, while decreasing the need for prophylactic antibiotic use due to reduced infection rates. To conclude, these data demonstrate that *in ovo* supplementation of the commercial probiotic product PrimaLac does not influence hatchability and can alter the expression of several immune-related genes within the ileum. The mode by which the probiotic bacteria may impact the immune system is multi-faceted with effects on both innate and adaptive immunity. Excluding few exceptions, these results support our previous findings in terms of enhancing performance and general downregulation of immune-related genes [22]. This study further elucidates the immunoregulatory effect of probiotics on intestinal immunity in poultry, which may be more pronounced under more challenging conditions [26] as may be encountered in field settings. Moreover, it provides justification for further research to investigate the beneficial effects of probiotics in poultry and the use of *in ovo* technology as a means of promoting early establishment of beneficial bacteria, development of the local immune system, gut health, and animal well-being.

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References

1. Farnell MB, Donoghue AM, de Los Santos FS, Blore PJ, Hargis BM, et al. (2006) Upregulation of oxidative burst and degranulation in chicken heterophils stimulated with probiotic bacteria. Poult Sci 85: 1900-1906.
2. Patterson JA, Burkholder KM (2003) Application of prebiotics and probiotics in poultry production. Poult Sci 82: 627-631.
3. Kogut MH, Swaggerty CL (2012) Effects of prebiotics and probiotics on the host immune response in Direct-Fed microbials and prebiotics for animals: science and mechanisms of action. Callaway TR, Ricke SC edn Springer, Berlin, Germany.
4. Cox CM, Dalloul RA (2015) Immunomodulatory role of probiotics in poultry and potential in ovo application. Benef Microbes 6: 45-52.
5. Gaggìa F, Mattarelli P, Biavati B (2010) Probiotics and prebiotics in animal feeding for safe food production. Int J Food Microbiol 141: S15-S28.
6. Ritzi MM, Abdelrahman W, van-Heerden K, Mohnl M, Barrett NW, et al. (2016) Combination of probiotics and coccidiosis vaccine enhances protection against an Eimeria challenge. Vet Res 47: 111.
7. Beal RK, Powers C, Davison TF, Smith AL (2006) Immunological development of the avian gut. CABI, Wallingford, UK, Cambridge, MA.
8. Smith AL, Powers C, Beal RK (2014) The avian enteric immune system in health and disease. Pages 227-250 in Avian Immunology. Schat KA, Kaspers B, Kaiser P edn. Elsevier Ltd., London, UK.
9. Dibner JJ, Richards JD, Knight CD (2008) Microbial imprinting in gut development and health. J Appl Poult Res 17: 174-188.

10. Kabir SML, Rahman MM, Rahman MB, Rahman MM, Ahmed SU (2004) The dynamics of probiotics on growth performance and immune response in broilers. Int J Poult Sci 3: 361-364.
11. Nava GM, Bielke LR, Callaway TR, Castañeda MP (2005) Probiotic alternatives to reduce gastrointestinal infections: the poultry experience. Anim. Health Res Rev 6: 105-118.
12. Getachew T (2016) A review on the effects of probiotic supplementation in poultry performance and cholesterol levels of egg and meat. J World Poult Res 6: 31-36.
13. Higgins SE, Erf GF, Higgins JP, Henderson SN, Wolfenden AD, et al. (2007) Effect of probiotic treatment in broiler chicks on intestinal macrophage numbers and phagocytosis of Salmonella Enteritidis by abdominal exudate cells. Poult Sci 86: 2315-2321.
14. Stringfellow K, Caldwell D, Lee J, Mohnl M, Beltran R, et al. (2011) Evaluation of probiotic administration on the immune response of coccidiosis vaccinated broilers. Poult Sci 90: 1652-1658.
15. Dalloul RA, Lillehoj HS, Tamim NM, Shellem TA, Doerr JA (2005) Induction of local protective immunity to Eimeria acervulina by a Lactobacillus-based probiotic. Comp Immunol Microbiol Infect Dis 28: 351-361.
16. Brisbin JT, Gong J, Parvizi P, Sharif S (2010) Effects of Lactobacilli on cytokine expression by chicken spleen and cecal tonsil cells. Clin Vaccine Immunol 17: 1337-1343.
17. Lee KW, Lee SH, Lillehoj HS, Li GX, Jang SI, et al. (2010) Effects of direct-fed microbials on growth performance, gut morphometry, and immune characteristics in broiler chickens. Poult Sci 89: 203-216.
18. Dalloul RA, Lillehoj HS (2005) Recent advances in immunomodulation and vaccination strategies against coccidiosis. Avian Dis 49: 1-8.
19. Knap I, Lund B, Kehlet AB, Hofacre C, Mathis G (2010) Bacillus licheniformis prevents necrotic enteritis in broiler chickens. Avian Dis 54: 931-935.
20. Ritzi MM, Abdelrahman W, Mohnl M, Dalloul RA (2014) Effects of probiotics and application methods on performance and response of broiler chickens to an Eimeria challenge. Poult Sci 93: 2772-2778.
21. Pedroso AA, Menten JFM, Lambais MR (2005) The structure of bacterial community in the intestines of newly hatched chicks. J Appl Poult Res 14: 232-237.
22. Pender CM, Kim S, Potter TD, Ritzi MM, Young M, et al. (2017). In ovo supplementation of probiotics and its effects on performance and immune-related gene expression in broiler chicks. Poult Sci 96: 1052-1062.
23. Silva IGO, Vellano IHB, Moraes AC, Lee IM, Alvarenga B, et al. (2017) Evaluation of a probiotic and a competitive exclusion product inoculated in ovo on broiler chickens challenged with Salmonella Heidelberg. Braz J Poultry Sci 19: 19-26.
24. Cox NA, Bailey JS, Blankenship LC, Gildersleeve RP (1992) Research note: in ovo administration of a competitive exclusion culture treatment to broiler embryos. Poult Sci 71: 1781-1784.
25. Meijerhof R, Hulet RM (1997) In ovo injection of competitive exclusion culture in broiler hatching eggs. J Appl Poult Res 6: 260-266.
26. Pender CM, Kim S, Potter TD, Ritzi MM, Young M, et al. (2016) Effects of in ovo supplementation of probiotics on performance and immunocompetence of broiler chicks to an Eimeria challenge. Benef Microbes 7: 699-705.
27. Edens FW, Parkhurst CR, Casas IA, Dobrogosz WJ (1997) Principles of ex ovo competitive exclusion and in ovo administration of Lactobacillus reuteri Poult Sci 76: 179-196.
28. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25: 402-408.
29. Ahmadi F (2011) The effect of Saccharomyces cervisiae (Thepax) on performance, blood parameters and relative weight of lymphoid organs of broiler chicks. Global Vet 6: 471-475.
30. Sadeghi AA, Shawrang P, Shakorzadeh S (2015) Immune response of Salmonella challenged broiler chickens fed diets containing Gallipro®, a Bacillus subtilis probiotic. Probiotics Antimicrob Proteins 7: 24-30.
31. Al-Barwary LT, Shahin MG, Tayeb IT (2012) Effect of probiotic supplementation on broiler performance. Int J App Poult Res 1: 27-29.
32. Naseem S, Rahman SU, Shafee M, Sheikh AA, Khan A (2012) Immunomodulatory and growth-promoting effect of a probiotic supplemented in the feed of broiler chicks vaccinated against infectious bursal disease. Rev Bras Cienc Avic 14: 109-113.
33. Tizard IR (2009) Veterinary immunology: an introduction. 8th edn Saunders Elsevier, St. Louis, MO.
34. Beum PV, Basma H, Bastola DR, Cheng PW (2005) Mucin biosynthesis: upregulation of core 2 beta 1,6 N-acetylglucosaminyltransferase by retinoic acid and Th2 cytokines in a human airway epithelial cell line. Am J Physiol Lung Cell Mol Physiol 288: L116-L124.
35. Jiang Z, Lossie AC, Applegate TJ (2011) Evolution of trefoil factor(s): genetic and spatio-temporal expression of trefoil factor 2 in the chicken (Gallus gallus domesticus). PLoS One 6: e22691.
36. Smirnov A, Perez R, Amit-Romach E, Sklan D, Uni Z (2005) Mucin dynamics and microbial populations in chicken small intestine are changed by dietary probiotic and antibiotic growth promoter supplementation. J Nutr 135: 187-192.
37. Rahimi S, Grimes JL, Fletcher O, Oviedo E, Sheldon BW (2009). Effect of a direct-fed microbial (Primalac) on structure and ultrastructure of small intestine in turkey poults. Poult Sci 88: 491-503.
38. Kaiser P, Staheli P (2014) Avian cytokines and chemokines. Pages 189-204 in Avian Immunology. Elsevier, London, UK.
39. Hong YH, Lillehoj HS, Lee SH, Park D, Lillehoj EP (2006) Molecular cloning and characterization of chicken lipopolysaccharide-induced TNF-alpha factor (LITAF). Dev Comp Immunol 30: 919-929.
40. Fietta P, Delsante G (2009) The effector T helper cell triade. Riv Biol 102: 61-74.
41. Akbari MR, Haghighi HR, Chambers JR, Brisbin J, Read LR, et al. (2008) Expression of antimicrobial peptides in cecal tonsils of chickens treated with probiotics and infected with Salmonella enterica serovar Typhimurium. Clin Vaccine Immunol 15: 1689-1693.
42. Mountzouris KC, Balaskas C, Xanthakos I, Tzivinikou A, Fegeros K (2009) Effects of a multi-species probiotic on biomarkers of competitive exclusion efficacy in broilers challenged with Salmonella Enteritidis. Br Poult Sci 50: 467-478.
43. Dalloul RA, Lillehoj HS, Shellem TA, Doerr JA (2003) Enhanced mucosal immunity against Eimeria acervulina in broilers fed a Lactobacillus-based probiotic. Poult Sci 82: 62-66.
44. Haghighi HR, Abdul-Careem MF, Dara RA, Chambers JR, Sharif S (2008) Cytokine gene expression in chicken cecal tonsils following treatment with probiotics and Salmonella infection. Vet Microbiol 126: 225-233.