In ovo inoculation of an *Enterococcus faecium*–based product to enhance broiler hatchability, live performance, and intestinal morphology

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ABSTRACT Previous studies have suggested the use of probiotics, as alternative to antibiotics, to enhance broiler performance. The administration of probiotics in feed has been widely explored; however, few studies have evaluated the in ovo inoculation of probiotics. Therefore, the objective was to evaluate the impact of in ovo inoculation of different concentrations of GalliPro Hatch (**GH**), an *Enterococcus faecium*–based probiotic, on hatchability, live performance, and gastrointestinal parameters. Ross x Ross 708 fertile eggs were incubated, and on day 18, injected with the following treatments: 1) 50 μL of Marek's vaccine (**MV**), 2) MV and 1.4×10^5 cfu GH/50 μL, 3) MV and 1.4×10^6 cfu GH/50 μL, 4) MV and 1.4×10^7 cfu GH/50 μL. On the day of hatch, chicks were weighed, feather sexed, and hatch residue was analyzed. Male birds (640) were randomly assigned to 40 floor pens. On day 0, 7, 14, and 21 of the grow-out phase, performance data were collected. One bird from each pen was used to obtain yolk weight and intestinal segment weight and length. Hatchability was not impacted by any GH treatment ($P = 0.58$). On day 0, yolk weight was lower for all treatments than for MV alone. On day 0 to 7, feed intake was lower for 10^5 and 10^7 GH; the feed conversion ratio (**FCR**) was lower for all treatments than for MV alone ($P = 0.05$; $P = 0.01$, respectively). From day 14 to 21, the 10^7 GH treatment had higher BW gain ($P = 0.05$). For day 0 to 21, 10^7 GH had a lower FCR than MV alone ($P = 0.03$). On day 0, all GH treatments resulted in heavier tissues and longer jejunum, ileum, and ceca lengths than MV alone ($P < 0.05$). Spleen weight was higher for 10^5 and 10^7 GH than for MV alone. In conclusion, GH does not impact hatchability, and some concentrations improved live performance through the first 21 d of the grow-out phase. These improvements could result from the increased yolk absorption and improved intestinal and spleen morphology seen in this study.

Key words: GalliPro Hatch, in ovo inoculation, intestinal morphology, broiler, probiotic, *Enterococcus faecium*

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INTRODUCTION

The use of antibiotics as growth promoters has been banned in the European Union for more than a decade (Phillips, 2007). Even though there is no ban in the United States, consumers are demanding antibiotic-free animal products (Phillips, 2007). The search for probiotics as alternatives to antibiotics has increased over the past years (Fallah et al., 2013). When used as feed supplements, probiotics are advantageous for poultry health and overall performance (Patterson and Burkholder, 2003; Kabir, 2009; Karimi et al., 2010; Eckert et al., 2010; Hashemzadeh et al., 2010; Mountzouris et al., 2010; Youssef et al., 2011; Liu et al., 2012). The most used probiotic species are *Lactobacillus*, *Bacillus*, *Enterococcus* spp., and *Bifidobacterium* (Patterson and Burkholder, 2003; Fontana et al., 2013).

Enterococcus spp. are a group of gram-positive lactic acid bacteria, commonly isolated in the form of single, paired, or short-chain cocci. They are known to be ubiquitous in nature and can be found in foods of animal origin because of their ability to colonize the intestines of both humans and animals (Giraffa, 2003; Cocolin et al., 2007). Although there are many virulent and

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infectious strains of *Enterococcus*, certain *E. faecium* serotypes can reduce pathogens through the production of enterocins to promote a beneficial microbial balance within the gastrointestinal tract of the host (Cleveland et al., 2001; Franz et al., 2011; Hanchi et al., 2018). Some *E. faecium* serotypes are therefore considered to be safe for use in the fermentation of meats and dairy products, as well as a probiotic species to reduce intestinal *Escherichia coli* infections and promote the development of the immune system in broilers (Cao et al., 2013; Franz et al., 2011). The use of *E. faecium*–based probiotics has become more prevalent because of their resistance to bile salts and low pH encountered in digestion, allowing the probiotic strain to reach the small intestine to exert its beneficial effects (Zommiti et al., 2018).

E. faecium has been previously evaluated as a probiotic additive in feed and has resulted in improved growth performance and intestinal morphology in broilers challenged with *E. coli* (Mountzouris et al., 2010). When added into the broiler diet, it has also improved the feed conversion ratio (**FCR**), meat yield, and meat quality (Zheng et al., 2016). This probiotic has also been found to reduce pathogenic bacteria such as *C. perfringens* and *E. coli* within the intestinal microflora (Samli et al., 2010; Huang et al., 2018). Most importantly, beneficial bacteria preexisting in the bird's microbiota, such as *Lactobacillus*, are not affected by the presence of *E. faecium* (Kacániová et al., 2006; Samli et al., 2007; Cao et al., 2013).

The ability of *E. faecium* to improve broiler performance when added to the bird's feed has led to more questions on its applicability to further enhance its beneficial effects. It has become of recent interest to evaluate the delivery of probiotics such as *E. faecium* in ovo and determine its ability to establish a healthy microbiota, earlier within the chick's life. Chr. Hansen developed GalliPro Hatch (**GH**), an *E. faecium*–based product for in ovo inoculation. With this being a relatively new product, it needs to be evaluated to determine its effect on the embryo and consequentially, the hatched chick. For this reason, research within this article used different concentrations of *E. faecium* isolated from the commercial product and in ovo injected them on day 18 of incubation using Inovoject Technology. Although Inovoject technology was originally developed for in ovo delivery of vaccines to the embryo (Sharma and Burmester, 1982; Gildersleeve et al., 1993), it has been recently verified to be effective for the delivery of probiotics (Triplett et al., 2018). Thus, the objective of this study was to determine if the early administration of *E. faecium* at different concentrations, using commercial in ovo inoculation technology, will affect hatchability, broiler performance, and intestinal parameters, as well as immune tissue morphology within broilers.

MATERIALS AND METHODS

Incubation

For this study, all animals were treated in compliance with the Guide for the Care and Uses of Agriculture Animals in Research and Teaching (Federation of Animal Science Societies, 2010) and the Mississippi State University Institutional Animal Care and Use Committee (IACUC Animal Welfare Assurance #A3160-01).

A total of 2,300 Ross x Ross 708 fertilized eggs were obtained from commercial breeder hens at 55 wk of age and stored for 3 d at 20°C before setting. Eggs were labeled according to treatment, flat, and egg number. Simultaneously, excessively dirty and broken eggs were removed. A total of 2,160 eggs were distributed into 18 egg flats for each treatment (540 eggs per treatment) and randomly placed into 2 NatureForm Incubators (Model NMC-1080; Jacksonville, FL). Each treatment was represented on each level within the incubator. The incubators were sanitized with 70% ethanol before egg placement. The dry and wet bulb temperatures were set at 37.5°C ± 0.1°C and 28.9°C ± 0.1°C, respectively. On day 10 of incubation, eggs were candled to discard eggs that were infertile, cracked, contaminated, or presented early dead embryos. On day 18 of incubation, all eggs were inoculated according to treatment. After in ovo inoculation, eggs belonging to each treatment were transferred into 18 previously sanitized hatching baskets that were equally distributed among 3 Georgia Quail Farm hatcher units (6 baskets/hatcher, 3 hatchers/treatment). Eggs for each treatment were set into 3 hatchers to avoid cross contamination (3 hatchers X 4 treatments = 12 total hatchers; GQF MFG, 1502 Digital Sportsman incubator; Savannah, GA) until day 21 of incubation. The hatcher dry and wet bulb temperatures were set at 36.9°C ± 0.1°C and 30°C ± 0.1°C, respectively. Sterile water was added each day at the same time, to maintain the desired humidity level.

Treatments

The commercially available GH product used in this study contained 10^9 cfu/g of *E. faecium*. One gram of the product was reconstituted in Tryptic soy broth (TSB Millipore Sigma, St. Louis, MO) and incubated at 37°C under anaerobic conditions (1535 incubator; VWR International, Cornelius, OR). After 24 h of incubation, the bacterial culture was 10-fold serially diluted, plated onto Bile Esculine agar plates (BEA; Millipore Sigma, St. Louis, MO), and incubated for 24 h at 37°C. To obtain the different concentrations of *E. faecium* desired for each treatment, a 10^9 cfu/mL culture was 10-fold serially diluted and centrifuged at 4,000 rpm for 5 min to obtain a pellet. The supernatant was removed, and the pellet was reconstituted with sterile diluent. All treatments were prepared the day of inoculation and individually distributed into 800-mL bags of a commercial sterile diluent. A standard herpesvirus of turkey vaccine (16,000 doses/800 mL bag; Merial Select, Inc, Gainesville, GA) was aseptically added to each diluent bag. The applied treatments included 1) 50 µL of Marek's vaccine (MV) and no probiotic, 2) MV + ~10^5 cfu GH/

50 μL, 3) MV + ∼10^6 cfu GH/50 μL, 4) 50 μL of MV + ∼10^7 cfu GH/50 μL. The diluent bags containing each treatment were kept on ice until their utilization. During the in ovo inoculation procedure, 50 μL were collected from each treatment and spread onto the appropriate agar plates to confirm that the correct concentration of bacteria was delivered for each treatment.

Inoculation Procedure

On day 18 of incubation, one egg from each flat was set aside for embryo staging. Each flat of developing eggs was injected at a time. Eggs were injected on their large end, into the amniotic sac. The needle punctured each egg at a depth of 2.49 cm to deliver each 50-μL concentration automatically. The different concentrations of the probiotic culture were injected in ascending concentration of bacteria to ensure the correct dosage was applied according to each treatment. However, between each treatment applied, a sanitization cycle was conducted to eliminate any contamination in the Inovoject equipment. After each cycle, 50 μL were collected and spread onto tryptic soy agar (Millipore Sigma, St. Louis, MO) plates to confirm that no bacterial contamination occurred between treatments. After all treatment inoculations, the eggs removed from each flat were in ovo inoculated with 50 μL of a Coomassie blue dye and immediately euthanized via CO_2 asphyxiation. Each embryo was analyzed to confirm that the injected eggs were in the appropriate stage of development for 18 d of incubation. Also, the presence of the dye surrounding the embryo's body through the amniotic fluid confirmed that the inoculation was correctly delivered in the amniotic fluid and did not puncture the embryo's tissue.

Hatch and Grow-Out

On day 21 of incubation, all hatched and unhatched eggs were removed from the hatching baskets. Unhatched eggs were counted and evaluated through hatch residue analysis to determine the developmental stage of the embryo before its death, according to Aviagen's "How to … Break Out and Analyze Hatch Debris" guidelines (Aviagen, 2017). The number, treatment, and stage of each egg were recorded, including early dead, mid-dead, late dead, pipped, and contaminated. Hatched chicks were counted and weighed to determine hatch of fertile eggs and average chick weight. Chicks and embryos were treated in accordance with the Guide for the Care and Uses of Agricultural Animals in Research and Teaching (FASS, 2010).

Hatched chicks were weighed and feather sexed, and 640 male birds were moved to a grow-out facility where they were raised through a 21-d grow-out cycle. Male chicks were assigned to each pen (16 chicks/pen), with a total of 10 pens for each treatment. The treatments were assigned to 10 blocks down the length of the house, skipping a pen to avoid cross-contamination within birds of different treatments. Each floor pen was equipped with one hanging feeder and 3 nipple drinkers and top-dressed with fresh wood shavings litter. The chicks were set at a 23L:1D photoperiod from day 0 to 7 and a 20L:4D photoperiod from day 8 to 21. A commercial temperature program was followed as recommended by Aviagen's Ross Broiler Management Manual (Aviagen, 2009). A regular corn and soybean meal diet was provided in crumble form for the 2 feeding phases: starter feed from day 0 to 14 and grower feed from day 14 to 21 following Ross 708 guidelines (Aviagen, 2014). Water and feed were provided ad libitum. Feed intake (**FI**) and body weight gain (**BW gain**) were recorded on day 7, 14, and 21. Mortality was recorded daily and the FCR calculation was corrected for mortality.

Sampling

On day 0, 7, 14, and 21 of the grow-out phase, a bird from each pen was randomly selected to be weighed, humanely euthanized, and aseptically necropsied to access their digestive tract (10 birds/treatment). The gizzard, duodenum, jejunum, ileum, and cecum were collected to obtain their individual weight and length. The spleen, bursa, and yolk were collected to obtain their weight.

Statistical Analysis

All data were analyzed using SAS version 9.4 (SAS Institute, Cary, NC). Hatch of transfer and hatch residue data were analyzed using a completely randomized design where each flat of eggs served as the experimental unit (18 flats/treatment). BW gain, FCR, FI, intestinal parameters, as well as yolk, spleen, and bursa weight data were analyzed using a randomized complete block design with a split plot over time. Each pen served as an experimental unit, and there was a total of 10 pens for each treatment. Means were separated using Fisher's protected low stocking density and were considered significantly different if the P value was ≤ 0.05 (Steel and Torrie, 1980).

RESULTS

Inoculation Procedure and E. faecium Concentration

The embryo staging analysis conducted on the day of in ovo inoculation demonstrated that the procedure was conducted at the right stage of development. As expected for this day of incubation, the embryos showed a 3-lobed yolk sac, and their intestines were mostly enclosed within the embryos' abdominal cavity. The delivery of the inoculum into the amnion was also confirmed with the presence of coomassie blue dye surrounding the embryo's body and therefore in the amniotic fluid. None of the embryos presented punctures in their bodies. The concentration of each *E. faecium* recovered on the day of inoculation was confirmed to be 1) MV alone, no bacterial growth; 2) for the 10^5 cfu GH/50 μL concentration, 4.5×10^5 cfu GH/

50 μL; 3) for the 10^6 cfu GH/50 μL concentration, 6.5×10^6 cfu GH/50 μL; and 4) for the 10^7 cfu GH/50 μL concentration, 9.4×10^7 cfu GH/50 μL.

Hatch and Growth Performance

For hatch of transferred eggs, there were no differences detected among treatments when compared to the control ($P > 0.05$; Table 1). No differences were detected on early, mid, and late dead embryos, as well as for pipped, contaminated, and culled embryos. Average chick weight was not different among any of the treatments evaluated ($P > 0.05$; Table 1). Differences were seen in growth performance among treatments and the days of the grow-out (Table 2). From day 0 to 7, FI was lower for the 10^5 and 10^7 cfu GH/50 μL concentration when compared to the MV-alone treatment and the 10^6 cfu GH/50 μL concentration ($P = 0.049$). On day 0 to 7, the FCR was on average 12 points lower for all GH-injected birds than that for MV-alone treatment ($P = 0.014$). No differences were detected in BW gain ($P = 0.985$). From day 7 to 14, no differences were detected for any growth performance variables evaluated.

From day 14 to 21 of the grow-out period, some differences were detected, where a higher BW gain was obtained by the highest GH concentration (10^7 cfu GH/50 μL) compared only to the lowest GH concentration (10^5 cfu GH/50 μL) and not to the other treatments ($P = 0.045$). For FCR, there was a trend ($P = 0.068$) where the increasing GH concentrations caused a numerical decrease in FCR, ultimately reducing the FCR numerically by 11 points compared to MV alone.

The overall broiler performance from day 0 to 21 resulted in improvements in FCR. FCR was significantly reduced by the highest concentration of probiotic (10^7 cfu GH/50 μL), resulting in a 9-point difference compared to the MV-alone treatment and the lowest concentration injected (10^5 cfu GH/50 μL) ($P = 0.049$). There was a trend in BW gain ($P = 0.073$) where birds were numerically heavier for the highest concentration of the probiotic injected (10^7 cfu GH/50 μL) when compared to the rest of the treatments, especially the lower concentrations of GH (10^5 cfu GH/50 μL, 10^6 cfu GH/50 μL).

Immune Tissues and Yolk Weight

Treatment effects were detected for yolk weight relative to BW on day 0, where a decrease in yolk weight was observed for all GH treatments ($P = 0.0003$) when compared to MV alone. Differences were also detected for spleen weight relative to BW, which was higher for the 10^5 cfu GH/50 μL and 10^7 cfu GH/50 μL treatments than for the MV-alone treatment ($P = 0.013$). No differences were seen for bursa weight relative to BW ($P = 0.448$) (Table 3).

Intestinal Relative Weight and Length

Intestinal weight relative to chick BW resulted in treatment by day interactions throughout the 21-d grow-out period for the gizzard, duodenum, jejunum, ileum, and ceca ($P = 0.0001$, for all tissues; Table 4). On day 0, the gizzard, duodenum, jejunum, ileum, and ceca weights were higher for all probiotic treatments than for the MV-alone treatment. However, by day 7, this increase in weight was lost, and the jejunum, ileum, and ceca weights were lower than those for the MV-alone treatment, while the gizzard and duodenum were not different. On day 14, the duodenum weight relative to BW was higher for the 10^6 cfu GH/50 μL treatment than that for all other treatments. The weight of the ileum relative to BW was higher for the 10^6 cfu GH/50 μL treatment than for the 10^7 cfu GH/50 μL treatment. No differences were detected among treatments on day 21.

Intestinal length of the jejunum, ileum, and ceca relative to the chicks' BW (cm/100 g) was also influenced by the different GH concentrations, resulting in treatment by day interactions ($P = 0.01$, $P = 0.02$, $P = 0.03$, respectively; Table 5). On day 0 of hatch, all GH-injected treatments resulted in longer relative jejunum and ileum lengths than the MV-alone treatment. While for the ceca, birds injected with 10^6 cfu GH/50 μL demonstrated greater length than those in the other treatments. On day 7, jejunum and ceca relative lengths were similar among all injected treatments. However, ileum length was higher in the 10^7 cfu GH/50 μL treatment than that in the MV-alone treatment. On day 14

Table 1. Effect of the in ovo inoculated MV-alone (Marek's vaccine with not addition of probiotic) and GalliPro Hatch (GH) at 10^5 cfu GH (MV + 10^5 cfu GH/50 μL), 10^6 cfu GH (MV + 10^6 cfu GH/50 μL), and 10^7 cfu GH (MV + 10^7 cfu GH/50 μL) on hatch parameters.[1]

Hatch parameter	MV alone	10^5 cfu GH	10^6 cfu GH	10^7 cfu GH	*P* value	SEM
Hatch of transfer (%)	94.0	94.3	94.4	91.96	0.58	1.399
Infertile embryos (%)	0	0.21	0	1.1	0.22	0.429
Early dead embryos (%)	0	0	0	0.26	0.39	0.132
Mid dead embryos (%)	0.41	0.43	0.35	0.2	0.94	0.287
Late dead embryos (%)	4.54	4.26	4.35	5.46	0.78	0.914
Pipped embryos (%)	0.65	0.62	0.91	1.01	0.92	0.469
Contaminated embryos (%)	0.19	0	0	0.21	0.57	0.143
Culled embryos (%)	0.22	0	0	0	0.39	0.111
Average chick weight (g)	44.22	44.1	43.11	43.68	0.26	0.424

[1]Means are calculated from 18 replicate values using a flat of eggs as the experimental unit.

Table 2. Live performance parameters of broilers in ovo inoculated with different concentrations of GalliPro Hatch (GH) *Enterococcus faecium*–based probiotic on day 18 of incubation: MV alone (Marek's vaccine with not addition of probiotic), 10^5 cfu GH (MV + 10^5 cfu GH/50 µL), 10^6 cfu GH (MV + 10^6 cfu GH/50 µL), and 10^7 cfu GH (MV + 10^7 cfu GH/50 µL).[1]

Day of the grow-out	Performance parameter	MV alone	10^5 cfu GH	10^6 cfu GH	10^7 cfu GH	*P* value	SEM
Day 0–7	Feed intake (kg)	0.153[a]	0.138[b]	0.143[a,b]	0.141[b]	0.049	0.0037
	BW gain (kg)	0.111	0.113	0.113	0.112	0.985	0.0043
	FCR	1.384[a]	1.273[b]	1.265[b]	1.265[b]	0.014	0.0264
Day 7–14	Feed intake (kg)	0.355	0.339	0.340	0.379	0.328	0.0166
	BW gain (kg)	0.238	0.222	0.224	0.266	0.224	0.0165
	FCR	1.548	1.563	1.531	1.438	0.228	0.0469
Day 14–21	Feed intake (kg)	0.576	0.532	0.555	0.593	0.183	0.0202
	BW gain (kg)	0.400[a,b]	0.378[b]	0.400[a,b]	0.446[a]	0.045	0.0173
	FCR	1.324	1.290	1.277	1.213	0.068	0.0300
Day 0–21	Feed intake (kg)	1.083	1.006	1.035	1.114	0.148	0.0356
	BW gain (kg)	0.752	0.706	0.734	0.821	0.073	0.0320
	FCR	1.450[a]	1.433[a]	1.422[a,b]	1.360[b]	0.049	0.0238

Means in a column not sharing a common superscript are different ($P \leq 0.05$).
Abbreviation: FCR, feed conversion ratio.
[1]Means are calculated from 10 replicate values using one randomly chosen bird per pen and each pen as the experimental unit (10 pens/treatment,16 birds/pen; 160 total birds/treatment).

and 21, no other differences were observed for any other tissue ($P > 0.05$).

DISCUSSION

Hatchability and Hatch Performance

In the present study, commercial in ovo inoculation technology was used to administer different concentrations of a commercially available *E. faecium* into the amnion of fertile broiler eggs. It has been previously demonstrated that commercial in ovo injection increases the accuracy of injection from 36.1 to 83.8% compared with manual injection (Wakenell et al., 2002). However, most of the existing literature evaluating in ovo administration used manual procedures for injection, which lacks applicability to commercial settings. Previous studies evaluating the injection of competitive exclusion culture derived from chicken intestinal contents reduced hatchability levels as low as 0 to 5% when manually injecting into the amnion and 56 to 84% when delivered onto the air cell (Cox et al., 1992; Maijerhof and Hulet, 1997). However, it was later shown that the manual in ovo injection of specific probiotic cultures such as *Lactobacillus* or *Bacillus*, whether into the amnion or onto the air cell, does not seem to impact hatchability, thus validating the early use of probiotic cultures (Edens et al., 1997; de Oliveira et al., 2014). More recently, studies have not detected any differences in hatchability between a control and in ovo–injected probiotic treatments (Pender et al., 2017; Teague et al., 2017; Beck et al., 2019).

Triplett et al. (2018) evaluated the use of commercial in ovo injection, and in their study, the percent hatch of transfer of noninjected eggs as well as a *Lactobacillus* and a *Bifidobacterium* injection was approximately 90%. However, different concentrations of a specific *Bacillus subtilis* strain reduced hatchability to as low as 10 to 50%. In their study, the decreased hatch for *B. subtilis* compared to the 2 other strains evaluated was attributed to a bacterial effect and not the in ovo inoculation procedure. However, the negative impact in hatchability obtained by injecting *B. subtilis* was not expected because this probiotic culture has been previously found to be beneficial for broilers when added to their feed (Jeong and Kim, 2014; Bai et al., 2017). Therefore, it is likely

Table 3. Treatment effect for weight of immune tissues and yolk weight relative to body weight of broilers in ovo inoculated with different concentrations of GalliPro Hatch (GH) *Enterococcus faecium*–based probiotic on day 18 of incubation: MV alone (Marek's vaccine with not addition of probiotic), 10^5 cfu GH (MV + 10^5 cfu GH/50 µL), 10^6 cfu GH (MV + 10^6 cfu GH/50 µL), 10^7 cfu GH (MV + 10^7 cfu GH/50 µL).[1]

Tissue (%)	MV alone	10^5 cfu GH	10^6 cfu GH	10^7 cfu GH	*P* value	SEM
Yolk on D 0[2]	10.55[a]	6.408[b]	6.558[b]	6.281[b]	0.0003	0.6957
Spleen	0.093[b]	0.113[a]	0.099[a,b]	0.113[a]	0.013	0.0048
Bursa	0.13	0.135	0.261	0.154	0.448	0.0646

For each tissue, means in a row not sharing a common superscript are different ($P \leq 0.05$).
[1]Means are calculated from 10 replicate values using one randomly chosen bird per pen and each pen as the experimental unit (10 pens/treatment,16 birds/pen; 160 total birds/treatment).
[2]Yolk weight obtained only on D 0. No egg yolk was present for most of the replication units during the remaining days of the grow-out cycle. Spleen and bursa weighed obtained on D 0, 7,14, and 21.

Table 4. Treatment by day interaction for gizzard and small intestine weights relative to BW (%) of broilers in ovo inoculated with different concentrations of the probiotic *Enterococcus faecium* from GalliPro Hatch (GH): MV (Marek's vaccine with not addition of probiotic), 10^5 cfu GH (MV + 10^5 cfu GH/50 µL), 10^6 cfu GH (MV + 10^6 cfu GH/50 µL), and 10^7 cfu GH (MV + 10^7 cfu GH/50 µL).[1]

Day of grow-out	Gizzard				Duodenum				Jejunum			
	MV alone	10^5 cfu GH	10^6 cfu GH	10^7 cfu GH	MV alone	10^5 cfu GH	10^6 cfu GH	10^7 cfu GH	MV alone	10^5 cfu GH	10^6 cfu GH	10^7 cfu GH
Day 0	5.793^{b}	8.955^{a}	8.839^{a}	8.867^{a}	0.955^{g}	$1.752^{a,b}$	1.845^{a}	1.848^{a}	1.269^{i}	$2.409^{b,c,d}$	$2.333^{c,d,e}$	2.658^{b}
Day 7	4.062^{c}	4.189^{c}	4.161^{c}	4.113^{c}	$1.606^{b,c,d}$	$1.522^{c,d,e}$	$1.593^{b,c,d}$	$1.449^{c,d,e}$	2.959^{a}	2.621^{b}	$2.577^{b,c}$	$2.554^{b,c}$
Day 14	3.028^{d}	3.277^{d}	3.063^{d}	2.985^{d}	$1.343^{e,f}$	$1.401^{d,e}$	$1.643^{a,b,c}$	1.350^{e}	$2.207^{d,e,f}$	$2.106^{e,f,g}$	$2.440^{b,c,d}$	$2.004^{f,g,h}$
Day 21	2.328^{e}	2.231^{e}	2.155^{e}	2.007^{e}	1.094^{g}	0.985^{g}	$1.123^{f,g}$	1.108^{g}	1.813^{h}	$1.838^{g,h}$	$2.067^{e,f,g,h}$	$1.934^{f,g,h}$
P value	0.0001				0.0001				0.0001			
SEM	0.1593				0.0803				0.0984			

Day of grow-out	Ileum				Ceca			
	MV Alone	10^5 cfu GH	10^6 cfu GH	10^7 cfu GH	MV Alone	10^5 cfu GH	10^6 cfu GH	10^7 cfu GH
Day 0	0.896^{f}	$2.015^{b,c}$	2.140^{b}	2.083^{b}	$0.606^{g,h}$	$0.78^{b,c,d,e,f}$	$0.910^{a,b}$	$0.850^{a,b,c,d}$
Day 7	2.476^{a}	2.214^{b}	$1.993^{b,c}$	$1.991^{b,c}$	0.974^{a}	$0.790^{b,c,d,e,f}$	$0.893^{a,b,c}$	$0.796^{b,c,d,e,f}$
Day 14	$1.648^{d,e}$	$1.616^{d,e}$	$1.835^{c,d}$	1.511^{e}	$0.685^{e,f,g,h}$	$0.829^{a,b,c,d,e}$	$0.737^{c,d,e,f,g}$	$0.644^{f,g,h}$
Day 21	1.485^{e}	1.546^{e}	1.452^{e}	1.526^{e}	$0.717^{d,e,f,g,h}$	$0.688^{d,e,f,g,h}$	$0.576^{g,h}$	0.564^{h}
P value	0.0001				0.0001			
SEM	0.0842				0.0583			

For each tissue, means in a row and column not sharing a common superscript are different ($P \leq 0.05$).
[1]Means are calculated from 10 replicate values using one randomly chosen bird per pen and each pen as the experimental unit (10 pens/treatment, 16 birds/pen; 160 total birds/treatment).

that not all probiotic bacteria are suitable for in ovo inoculation, even if they are commonly known to be safe for use in feed. For this reason, the bacteria to be injected, even if it is a well-known probiotic, needs to be evaluated before commercial application.

In the present study, hatch of transfer for all treatments including MV alone, as well as MV with increasing concentrations of the probiotic, was between 91% and 94% and showed no differences among treatments. In addition, on the day of hatch, no differences were observed in contaminated embryos; early, mid, and late dead embryos; or average chick weight among any of the treatments injected. Similar results were obtained by the in ovo inoculation of a noncommercial strain of *E. faecium* (Beck et al., 2019). These results are promising for the use of a commercial in ovo procedure in the administration of probiotics without negatively impacting hatchability. This verifies the commercial in ovo inoculation as a viable method for the delivery of probiotics and this specific serotype of *E. faecium* as a beneficial culture, which is safe for in ovo administration.

Live Performance

E. faecium, as a probiotic culture, has been widely evaluated as a feed supplement in poultry diets (Cao et al., 2013; Samli et al., 2007; Capcarova et al., 2010; Samli et al., 2010; Zhao et al., 2013; Huang et al., 2018). The supplementation of *E. faecium* in broiler feed has shown improved BW gain (Samli et al., 2010). Other studies have demonstrated reduced *E. coli* (Cao et al., 2013; Capcarova et al., 2010; Gheisar et al., 2016; Awad et al., 2009; Huang et al., 2018) and a slight reduction of *Salmonella* concentrations in the ceca (de Oliveira et al., 2014). *E. faecium*, alone and in combination with a prebiotic dried whey, has also been shown to increase BW gain, reduce the FCR, and increase lactic acid bacteria in the birds' ileum and excreta (Samli

Table 5. Treatment by day interaction for small intestine length relative to body weight (cm/100 g) of broilers in ovo inoculated with different concentrations of the probiotic *Enterococcus faecium* from GalliPro Hatch (GH): MV alone (Marek's vaccine with not addition of probiotic), 10^5 cfu GH (MV + 10^5 cfu GH/50 µL), 10^6 cfu GH (MV + 10^6 cfu GH/50 µL), and 10^7 cfu GH (MV + 10^7 cfu GH/50 µL).[1]

Day of grow-out	Jejunum				Ileum				Ceca			
	MV alone	10^5 cfu GH	10^6 cfu GH	10^7 cfu GH	MV alone	10^5 cfu GH	10^6 cfu GH	10^7 cfu GH	MV alone	10^5 cfu GH	10^6 cfu GH	10^7 cfu GH
Day 0	37.2^{b}	42.8^{a}	42.4^{a}	43.7^{a}	31.5^{c}	$38.3^{a,b}$	41.1^{a}	36.7^{b}	8.29^{b}	7.77^{b}	9.16^{a}	8.06^{b}
Day 7	24.1^{c}	23.2^{c}	25.1^{c}	26.1^{c}	22.7^{e}	$24.2^{d,e}$	$24.9^{d,e}$	26.3^{d}	4.22^{c}	4.28^{c}	4.30^{c}	4.64^{c}
Day 14	11.7^{d}	11.3^{d}	11.5^{d}	$9.17^{d,e}$	$10.3^{f,g}$	11.2^{f}	$9.95^{f,g,h}$	$8.72^{f,g,h,i}$	2.29^{d}	2.51^{d}	2.26^{d}	$1.96^{d,e}$
Day 21	6.51^{e}	7.52^{e}	7.48^{e}	6.75^{e}	$6.68^{h,i}$	$7.39^{g,h,i}$	6.59^{i}	$6.71^{h,i}$	1.47^{e}	1.54^{e}	1.46^{e}	1.34^{e}
P value	0.01				0.002				0.03			
SEM	1.107				1.193				0.238			

For each tissue, means in a row and column not sharing a common superscript are different ($P \leq 0.005$).
[1]Means are calculated from 10 replicate values using one randomly chosen bird per pen and each pen as the experimental unit (10 pens/treatment, 16 birds/pen; 160 total birds/treatment).

et al., 2007). However, other studies have shown that *E. faecium* alone does not cause changes in BW, FCR, or FI through a 42-d grow-out period (Zhao et al., 2013). Although many improvements have been seen with the use of this probiotic in feed, little research has been conducted to evaluate the use of *E. faecium* in ovo to evaluate its effect on hatch and growth performance.

In previous research, Majidi-Mosleh et al. (2017) evaluated the manual in ovo injection of a 10^7 cfu dose of *B. subtilis*, *E. faecium*, and *Pediococcus acidilactici* individually into the amnion of fertile eggs and found no differences in growth performance. Coskun et al. (2015) evaluated the in ovo delivery of *E. faecium* and dried whey, and although they also used an automated machine for in ovo injection, the probiotic concentration was delivered onto the air cell. In their study, no differences were seen in growth performance for any *E. faecium*–injected treatments through a 21-d grow-out period. In the present study, the probiotic *E. faecium* was delivered into the amnion, and differences in growth performance were observed throughout most of the grow-out cycle, most prominent from day 0 to 7 and day 0 to 21. From day 0 to 7, the FCR was reduced by the different GH treatments compared to MV-alone treatment because of a reduction in FI; however, no differences were detected in BW gain. The improvement in FCR and the trend in BW gain, especially by the highest GH concentration (10^7 cfu GH/50 μL), were carried through day 21 of the grow-out period. This could mean that this serotype of *E. faecium* at a higher concentration can colonize and multiply in the chicken's gastrointestinal tract, thus exerting its beneficial effects for a longer period (Skjøt-Rasmussen et al., 2019). The delivery of the probiotic into the amnion, as compared to the air cell (Coskun et al., 2015), possibly made the probiotic available earlier for the embryo to absorb as suggested by Castañeda et al. (2019). The earlier availability of the probiotic concentration within the bird's gastrointestinal tract and its ability to remain within may have led to the improvements seen in performance characteristics throughout the grow-out cycle.

Intestinal Morphology and Yolk Weight: Effects on Performance

The different probiotic concentrations seemed to alter the morphology of the chicks' intestine. The probiotic doses increased gizzard, duodenum, jejunum, ileum, and ceca weight especially on day 0 to 7 after hatch. Similarly, all probiotic doses increased jejunum, ileum, and ceca length compared to the control, except for the lowest *E. faecium* concentration used (10^5 cfu GH/50 μL). These increases in small intestine weight have also been previously detected with the supplementation of *E. faecium* in poultry and piglet feed (Awad et al., 2009; Ciro et al., 2015). The in ovo administration of other serotypes of *E. faecium* alone or in combination with dried whey has also demonstrated an increased jejunum and ileum weight (Coskun et al., 2015) and length (Beck et al., 2019). The small intestine's ability to digest and absorb nutrients is highly related to intestinal structure, such as its weight and length (de Verdal et al., 2010; Moghaddam and Alizadeh, 2013). In this study, the in ovo delivery of *E. faecium* resulted in heavier and longer segments of the small intestine, mostly during the first days of the grow-out phase. It is believed that these early modulations in intestinal morphology resulted in an efficient nutrient absorption, which could be responsible for the improvements obtained in growth performance parameters. Other studies had also shown modulations in some segments of the small intestine, such as increased ileum villus height (Coskun et al., 2015) and increased jejunum and ileum weights on day 14 and 21 of the grow-out phase because of the in ovo probiotic inclusion (Beck et al., 2019). However, their changes in intestinal morphology were not enough to elicit a significant improvement in growth performance as compared to the ones obtained in this study.

The modulations obtained in intestinal morphology, especially in the first 7 d of the grow-out period, could be related with the increased absorption of egg yolk caused by all probiotic inoculated treatments. The eggs yolk is known to be the main nutrient supply for growth of the embryos and a major source of energy for the hatching bird during its first days (Nangusuay et al., 2011; Sahan et al., 2014). During the first 48 h after hatch, the yolk is the main source of energy for intestinal development, thus preparing the chick for its transition to the consumption of a regular basal diet (Jamroz et al., 2004; Yegani and Korver, 2008). In the present study, all *E. faecium* injected concentrations resulted in a more rapid yolk utilization than the MV-alone treatment on day 0 after hatch. The in ovo inoculation of *E. faecium* seemed to stimulate a faster consumption of these nutrients to be used not only for hatching energy but also for an enhanced intestinal development. Previous studies demonstrated that the uptake of yolk by the small intestine can be enhanced through the in ovo injection of exogenous nutrients into the amniotic fluid (Uni et al., 1998; Geyra et al., 2001; Noy and Sklan, 2001; Noy et al., 2001; Tako et al., 2004). However, it is exceptional that the in ovo administration of a probiotic culture has the potential to elicit and improve yolk absorption that could lead to further improvements in gut morphology and broiler performance.

Treatment Effect on Spleen Weight

The effect of the in ovo inoculation of *E. faecium* on immune organ development was evaluated in this study. It was observed that all in ovo inoculated concentrations of *E. faecium* yielded increased spleen weight compared to the MV-alone treatment. These results are in agreement with previous research stating that the administration of probiotics in ovo can stimulate important immune tissues (Castañeda et al., 2019) as previously seen in probiotic-fed broilers (Kabir et al., 2004; Willis et al., 2007). The spleen is a secondary lymphoid structure characterized by aggregated lymphocytes and

antigen presenting cells. It has been previously demonstrated that there is a strong correlation between the weight of immune tissues such as the spleen and bursa and their immune competence through the increased level of antibody expression (Kabir et al., 2004; Slawinska et al., 2014). The detection of bacteria, whether pathogenic or probiotic, seems to stimulate an immune response in chickens (Hughes, 2005). The early detection of probiotic bacteria could therefore result in an earlier "maturation" of the immune system. Although no differences were seen in bursa weight, the increased spleen weight could be promising for an earlier protection against diseases within the first week after hatch as opposed to a 3-wk posthatch immune maturation (Fagerland and Arp, 1993). However, it still needs to be determined if these immunomodulations are strong enough to suppress an *E. coli*, *Salmonella*, or coccidiosis challenge in chicks during a full grow-out period.

CONCLUSIONS

The results of this study indicate that none of the concentrations of *E. faecium* had a negative impact on hatchability. Although the lower GH concentrations evaluated resulted in some modulations, the 10^7 cfu GH/50 µL concentration of *E. faecium* resulted in numerical improvement in BW gain and significant improvements in FCR. However, all GH concentrations increased intestinal weight and lengths, particularly 1 wk after hatch. The intestinal modulations obtained are believed to be a result of a faster yolk absorption by *E. faecium*–treated embryos. These changes in intestinal morphology may lead to better nutrient absorption, resulting in an improved growth performance. Increases in spleen weight were also seen on the day of hatch for all *E. faecium* concentrations evaluated. This modulation may have great implications for an earlier development of a "mature" immune system even before the embryo hatches, which could become more efficient as the chicks grow.

The 9-point improvement in FCR seen in this study could yield great economic margins in industrial production systems. These improvements, as well as the possible boosting of the immune system, have a great potential to establish in ovo injected probiotics, such as *E. faecium*, as viable alternatives to antibiotics. However, further research is needed to determine if these improvements will be carried through a 49-d grow-out phase and if these modulations, especially of the spleen, are enough to confer protection against parasitic and pathogenic challenges. Most importantly, additional research should evaluate if the modulations obtained can reduce the overall incidence of unwanted bacteria in the broiler house and ultimately in the processing plant, while maintaining an improved growth performance.

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