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In Ovo Evaluation of FloraMax-B11 on Marek's disease HVT Vaccine Protective Efficacy, Hatchability, Microbiota Composition, Morphometric Analysis, and Salmonella Enteritidis Infection in Broiler Chickens

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In Ovo Evaluation of FloraMax-B11 on Marek's disease HVT Vaccine Protective Efficacy, Hatchability, Microbiota Composition, Morphometric Analysis, and *Salmonella* Enteritidis Infection in Broiler Chickens

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Abstract

It has previously been shown that effective probiotics can accelerate gut maturation and the development of a normal microflora in poultry. This results in increased resistance to enteric pathogens encountered by chicks early in life. Our objective in experiments 1 and 2 was to evaluate the effect of *in ovo* administration of FloraMax®-B11 (FM) on Marek's disease (MD) herpesvirus of turkeys (HVT) vaccine protective efficacy. In Exp. 1, day 18 White Leghorn embryos were randomly distributed in four groups, 1) HVT vaccinated, no MDV challenge, 2) HVT + FM vaccinated, no MDV challenge, 3) HVT vaccinated, challenge with virulent MDV, 4) HVT+ FM vaccinated, challenge with virulent MDV. Exp. 2 was designed the same as Exp. 1, except chicks were challenged with a very virulent MDV strain. There was no significant difference ($P > 0.05$) in MD incidence between birds vaccinated with and without FM in the HVT vaccine. In Exp. 3 and 4, day 18 commercial broiler embryos were *in ovo* injected with either saline or FM to measure hatchability, microbiota composition, morphometric analysis, and *Salmonella enterica* serovar Enteritidis (SE) infection in chickens. The administration of the probiotic did not negatively affect hatchability, but significantly reduced ($P<0.05$) coliforms within the gut. In Exp. 4, the FM treated group showed significantly increased ($P<0.05$) BW at 7 days when compared to the controls. This is associated with the higher villi surface area observed in the FM group and reduced ($P<0.001$) SE incidence and ($P<0.05$) CFU recovery. The results of these studies suggest that the *in ovo* administration of FM into the amnion at 18 days of embryogenesis does not impact the protection of the HVT vaccine against MD or negatively affect hatchability. It also reduces the recovery of gram negative bacteria, improves BW during the first 7 days, and decreases SE recovery in broiler chickens.

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"I can do all things through Christ, who strengthens me." Phillipians 4:13

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Chapter I. Introduction

Until recently, antibiotics have long been used in the poultry industry. They have been used to stimulate growth rate and feed efficiency and to control pathogenic bacteria. The growing concern of antibiotic resistant bacterial strains and residues in food have caused regulatory agencies and producers to reduce and even abandon the use of antibiotics. This pressure has stimulated a need for viable alternatives to the antibiotics. A very promising alternative is the use of probiotics. Due to this, there has been a recent surge in probiotic development with encouraging results. However, in a commercial setting, chicks will not be able to receive the beneficial bacteria until being placed in the chicken houses, where they can receive it in the water or feed. Prior to this, chicks are exposed to the contaminated air within the hatch cabinets and usually long, stressful transportation to their destinations. This allows pathogenic bacteria to colonize and delay gut maturation and health within the newly hatched chick. In poultry, there is a way to administer the probiotic before the chicks hatch. At Day 18 of embryogenesis, almost all broilers are vaccinated for Marek's Disease virus (MDV). This thesis addresses the testing of a commercial probiotic and its ability to be administered with the Marek's vaccine, its effect on hatchability, and gut development and composition.

Chapter II. Literature Review

Transfer of Microflora

The gut microbiota plays an important role in health by providing a barrier for colonization of pathogens, by utilizing important metabolic functions (fermentation of nondigestible fibers, production of short-chain fatty acids, and vitamin supplementation), and by stimulating the development of the immune system (Guarner and Malagelada, 2003). At birth, animals receive a natural inoculation of microbes, which establish themselves in the intestines. This colonization of beneficial microflora allows the animal to resist potential environmental challenges. In mammals, the inoculation occurs during parturition, with bacteria living in the vaginal mucus, and through breast feeding (De Oliveira et al., 2014). Neonate gastrointestinal tracts are sterile at birth, but the microorganisms near the vagina or anus of the mother rapidly colonize the neonate after birth (Phillips et al., 2004). Within a few hours, bacteria begins to appear in the feces. It is suggested that the gastrointestinal tract is first colonized by facultative aerobes due to the intestinal environment showing a positive reduction potential at birth. As these microbes consume oxygen, the environment transfers to a negative reduction potential and allows the growth of strict anaerobes (Bezirtzoglou, 1997).

As stated earlier, during the first days of life, the bacteria colonizing the infant gastrointestinal tract come primarily from the mother and the environment. For this reason, one of the biggest determinants of the pioneer colonizers is the mode of delivery (Penders et al., 2006). Infants born vaginally, are first colonized by the fecal and vaginal bacteria of the mother, whereas cesarean section born infants are first exposed to bacteria in the hospital environment and health care workers (Bezirtzoglou, 1997; Gronlund et al., 1999). Studies have shown vaginal delivery at home resulted in higher colonization rates and counts of *Bifidobacteria* and

Bacteroides fragilis-group species and reduced incidence and counts of *Clostridium difficile* and *Escherichia coli* when compared with cesarean section (Penders et al., 2006). The timing of colonization and difference of bacterial populations between the two delivery methods can persist for months or even the whole life of the individuals (Schultz et al., 2004; Rao et al., 2009). Unfortunately, cesarean born infants are also usually more susceptible to intestinal disorders (Gronlund et al., 1999; Penders et al., 2006; Cochetiere et al., 2007).

In birds, colonization occurs in nests through contact with brooding hens and nesting materials (Mills et al., 1999). In modern poultry production, there is no contact between chicks and hens, and chicks are first exposed to bacteria in the hatchery and chicken house environment. Artificial incubation of poultry eggs has been shown to delay the colonization of beneficial microflora due to the lack of interaction with adult birds (Hashemzadeh et al., 2010).

Gastrointestinal Tract

The GIT serves as the interface between the diet and the metabolic events that sustain life. It is also the largest immune organ in the body, and serves as a barrier for prevention of infection (Abreu-Martin and Targan, 1996; Mayer, 2000). The avian mucosal immune system contains M cells, plasma cells, T cells, macrophages, intraepithelial lymphocytes, and heterophils (Erickson and Hubbard, 2000). In poultry, a crucial factor in digestion and absorption of nutrients are the intestinal villi, which are underdeveloped at hatch (Uni et al., 1995) and not fully developed until 10 days of age (Noy and Sklan, 1997). As birds hatch, they must transition from energy supplied via endogenous nutrients of the yolk to exogenous carbohydrate rich feed. During this transition, significant changes occur both in morphology and intestinal size (Uni et al., 1995). This maturation of the gut also affects the epithelial cell membranes, which are a major mechanical barrier between the internal environment of the host and the external foreign

material in the lumen (Rozze et al., 1982). By dietary means, it is possible to affect the development of the gut and the competitiveness of both beneficial and harmful bacteria, which can alter not only gut dynamics, but also many physiological processes due to the end products metabolized by symbiotic gut microflora. Within the mucous layer, tight junctions between epithelial cells and gut-associated lymphoid tissue help to maintain a homeostasis between dietary antigens, in addition to enteric pathogens and beneficial microorganisms (Vicuna et al., 2015). Dysbiosis within the gut, opens tight junctions which reduces barrier function, resulting in nonselective permeability. This could lead to malabsorption of nutrients and translocation of possible pathogenic enteric bacteria to various internal organs, which could result in disease and reduced growth performance (Quinteiro-Filho et al., 2012). Recent poultry research has shown that leakage of enteric bacteria into circulation results in non-gastrointestinal diseases (Tellez et al., 2009; Borst et al., 2012; Wideman, 2013).

Microflora of the Gastrointestinal Tract

Naturally, animals are colonized by microorganisms that form a specific ecological community of commensal, symbiotic, and pathogenic microorganisms or microbiota. Within this microbiota, there are both permanently colonizing species and temporary colonizing species that are characterized by a number of environmental microorganisms (Fiebiger et al., 2016). Warm blooded vertebrates gastrointestinal (GI) tract constitutes one of the most densely populated and diverse ecosystems known. The human GI tract's microflora surpasses the number of cells in the body ten-fold and the total mucosal surface area is up to 300 m^2. This makes it the largest area of the body interacting with the environment and it is colonized with over 10^{14} micro-organisms (Lu et al., 2003; Bjorksten et al., 2006). For this reason, the gut microbiota are the most important source of microbial stimulation and the driving force behind the postnatal maturation

of the immune system (Bjorksten et al., 2006). Since the GI tract contains such a large number of diverse microbes that can be commensal or pathogenic, they affect the host's nutrient utilization and intestinal development either positively or negatively (Dumonceaux et al., 2006).

A more complete understanding of the microbial ecology of the chicken intestinal microbiome is necessary to reduce enteric disease and pathogens of public health concern. Studies have shown that bacterial diversity and numbers vary throughout different sections of the GI tract (Yegani and Korver, 2008). According to Donoghue et al (2007), it is estimated that more than 500 bacterial species inhabit the poultry GI tract, however it is believed only 20% to 60% have been recovered by traditional culture methods (Lu et al., 2003). In the cecum, the most abundant 16s rDNA sequences were homologous to *Clostridiaceae* at 65.6%, according to Lu et al (2003). Conversely, *Streptococci* (Barnes et al., 1972) and *Eubacterium* (Salanitro et al., 1974) have also been described as the most abundant sequences of bacteria in the cecum of broiler chickens. In more recent studies, *Ruminococcus*, *Lactobacillus*, and *Bacteroides* were the most predominant genera in the global sequence data set and in two 454 pyrosequencing studies (Qu et al., 2008; Stanley et al., 2012a). However, Callaway et al. (2009) found *Bacteroides* and *Prevotella* to be the most predominant genera in the chicken cecum and Nordentoft et al. (2011) found *Butyricimonas* and *Fecalibacterium* were predominant. It is believed that differences in host, feed, and analysis techniques might all contribute to the differences observed (Wei et al., 2013).

There are few studies on the microbiome of populations within the small intestine of the chicken gastrointestinal tract, as most have focused on the cecum. When Lu et al (2003) sequenced microbiota of the small intestine, they found that the dominant bacteria in the ileum was *Lactobacillus.* As chickens mature, microbial populations do change slightly, but of the 16s

RNA sequences 70% belonged to *Lactobacillus*. It has also been shown that lactobacilli were the main bacteria present in the duodenum and small intestine, and were also the only bacteria present at a level above 10^4 CFU per gram by culture techniques (Barnes et al., 1972). Another study that compared conventional or organic production conditions, also found that *Lactobacillus* was the predominant bacteria present in the ileum of 42 day old broilers regardless of the production setting (Bjerrum et al., 2006). Cressman et al. (2010) discovered that at day 7, fresh litter chicks ileal mucosa was comprised of mainly *Lactobacillus*, followed by *Lachnospiraceae* and *Enterococcus*. Whereas the ileal mucosa of chicks reared on reused-litter was primarily colonized by bacteria within the order *Clostridiales.* Interestingly, in the reused litter, *Lactobacillus* was more predominant in the ileum than in the fresh litter chicks. By day 42, the fresh-litter birds had a microbiota similar to the reused-litter birds, which suggests diminishing environmental effects, as intestinal bacteria from the excreta accumulates in the litter.

Concept of Competitive Exclusion

Metchnikoff (1908) first introduced the idea that intestinal microflora played a role in the maintenance of health, when he studied lactic acid bacteria from fermented milk products. He observed that Bulgarians who ate significant amounts of yogurt were more resistant to enteric infections (Patterson and Burkholder, 2003). He hypothesized that the lactic acid bacteria in the yogurt was providing this protection, and that the beneficial nature of these bacteria led to prolonged life. The term Competitive Exclusion (CE) was first used by Greenberg (1969), where he observed a species of bacteria outcompete another species for receptor sites in the intestinal tract, in an article on the exclusion of *Salmonella typhimurium* from maggots of blow flies. He discovered that without the reduction or elimination of normal intestinal microflora the *S.*

typhimurium would not survive. Later, in 1971, van der Waaij and investigators while studying pathogen colonization in mice, coined a synonymous term "colonization resistance".

Competitive Exclusion

Competitive Exclusion has proven to be the best alternative route to control disturbances within the intestines in poultry. Research worldwide was stimulated by an article in Nature in 1973 by Nurmi and Rantala, where they tried to control a *Salmonella* Infantis outbreak in broiler flocks (reviewed by Schneitz, 2005). They revealed chicks were most susceptible to *Salmonella* infections during the 1st week of life. They suggested this was due to the delayed establishment of normal gut microflora in chicks raised in modern industry production methods. When a *Lactobacillus* strain offered no protection, they decided to assess the effectiveness of CE, a population of intestinal bacteria from adult chickens that were resistant to the *S. infantis.* They administered the mixed culture orally and were able to achieve adult type resistance to *Salmonella* (reviewed by Edens et al., 1997). Rantala and Nurmi (1973) recognized that inoculating young chickens with unidentified GI tract contents could possibly cause a pathogen to be introduced. So, cecal contents from healthy adult chickens were grown anaerobically and passed three times prior to inoculating the chicks. The passages were intended to allow time to check for specific pathogens and also to minimize the risk of pathogens in the culture. In their experiment, day old chicks were treated with the passaged cecal content, diluted rumen content, diluted fresh horse feces, or diluted crop and cecal contents of healthy adult chickens. The chicks were then challenged 24 h post treatment with 10^3 *Salmonella* Infantis. All chicks receiving chicken microflora tested negative for *Salmonella,* while all chicks receiving different species bacteria were positive for *Salmonella*. From this study, it was demonstrated that the passaged culture was as efficacious as the unpassaged and that there is host specificity because only the

chicken cecal microflora were able to protect the chickens, unlike the horse or cattle feces (Rantala and Nurmi, 1973).

Mechanism of CE

There are four major proposed mechanisms of CE: 1) creation of microecology that is hostile to other bacterial species, 2) elimination of available bacterial receptor sites, 3) production and secretion of antimicrobial metabolites, and 4) selective and competitive depletion of essential nutrients (Rolfe, 1991).

The determining factor in the viability of microorganisms is the microecology of the intestinal tract. Meynell (1963) determined that the production of volatile fatty acids at a pH below 6.0 will decrease populations of *Salmonella* and *Enterobacteriacea*. The mechanism of CE can be eliminated by use of antibiotics causing disruptions within the normal intestinal microbial populations. This causes concentrations of volatile fatty acids produced by intestinal bacteria to decrease, allowing for gut pH to increase to a more alkaline state (Edens et al., 1997). It has been shown, in newly hatched chicks that the volatile fatty acid concentration and pH are not adequate enough to chemically exclude pathogens (Barnes et al., 1979, 1980a,b).

In order for the host to be at risk to pathogens attaching to the intestinal epithelium, it is required for there to be accessible sites for adhesion. According to Soerjadi et al. (1982), this attachment is facilitated through the polysaccharide-containing components attached to the cell wall. This component blocks all receptor sites by binding bacteria to each other and the epithelium.

The antimicrobial substances produced and secreted by the endogenous bacteria of the intestinal tract can either kill or inhibit growth of pathogens (Rolfe, 1991). Research has shown that *Lactobacillus* as a group, produced significant amounts of bacterial growth inhibitory

substances (Edens et al., 1997). Talarico et al. (1988), revealed that the secretion of reuterin, *by L. reuteri* had broad-spectrum killing capabilities within the intestinal tract of chickens.

The fourth mechanism, competition for available nutrients as a means to control intestinal bacteria, is probably not the most effective means for CE. There are many environmental factors that enhanced the availability of nutrients from the diet of the host or through manipulation of dietary ingredients, favored the growth of certain populations (Rolfe, 1991). This could result in the exclusion of other bacterial species. Casas et al. (1993) showed that influencing the lactose concentration in the diet of chicks and poults, we can selectively provide an advantage for the enhancement of *L. reuteri*.

Probiotics

The use of selected beneficial lactic acid bacteria as probiotics has been suggested for many years due to their ability to prevent various enteric diseases and improve overall health (Tellez et al., 2006). In 2006, the European Union banned the use of antibiotics as feed supplements and recent concern in the United States has resulted in consumer's requesting antibiotic free chicken. This pressure has generated the need to find alternative options to the use of antibiotics in the feed. A very promising alternative is the use of probiotics (Higgins et al., 2005; Wolfenden et al., 2010; Barbosa et al., 2005).

The definition of probiotics according to the FAO/WHO (2001), is "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host." Probiotics have been used to treat intestinal disorders and improve intestinal health (Aureli et al., 2010; Nicholson, 2002). In poultry, probiotics are used to supplement beneficial colonization of the intestines in chicks after hatch (Fuller, 2001). They are also used to increase

broiler performance (Lutful Kabir, 2009) and control the incidence of pathogens (La Ragione & Woodward, 2003).

Patterson & Burkholder (2003) described ideal probiotics as, non-pathogenic, of host origin, resistant to gastric and bile acids, ability to persist in the gastrointestinal tract, produce antimicrobial substances, adhere to epithelium, modulate immune response, and tolerate processing and storage. The most common types of probiotic bacteria are lactic acid bacteria, such as *Bifidobacterium* spp., *Lactobacillus* spp., and *Enterococcus* spp. (Ljungh & Wadstrom, 2006). Lactic acid bacteria are Gram-positive bacteria that ferment carbohydrates into lactic acid and energy. These bacteria are normally found within the gastrointestinal tract of humans and animals. The most common LAB used as a probiotic is the genus *Lactobacillus*, a Gram-positive, catalase negative, nonspore-forming, and facultative anaerobe. According to Hori (2010), there are currently more than 125 identified *Lactobacillus* species.

Immunomodulation

Normal microflora of the GI tract is crucial for priming the immune system (Gaboriau-Routhiau, 2001), possibly due to beneficial, probiotic-type bacteria that are believed to modulate the immune response (Christensen et al., 2003). Animals lacking normal microflora experience a diminished degree of response to immune stimulus. It has been shown that germ-free animals produce as little as one third of the normal antibody-producing B cells as conventional animals (Mitsuoka, 1978). Intestinal bacteria are essential for the development of gut-associated lymphoid tissue (GALT), which is important in the immune functions of the epithelium and the development of the normal antibody repertoire. Commensal bacteria can be killed by enteric macrophages, but survive within underlying dendritic cells of the gastrointestinal tract (Macpherson and Uhr, 2004). These bacteria are presented to B cells in the mesenteric lymph

nodes which allows for an immune response of IgA. These dendritic cells also respond differently to the normal commensal bacteria as opposed to potentially pathogenic ones. They secrete different cytokines or are unresponsive, likely due to the expression of different toll-like receptors on the differentiated dendritic cells (Christensen et al., 2003; Karlsson et al., 2004).

Stimulation of the innate immune system allows for improved acquired immune responses through better antigen presentation (Chin and Mullbacher, 2003). Clinical studies have reported a negative correlation between infectious disease and the presence of lactobacilli (Alvarez-Olmos et al., 2004) and probiotic bacteria have been shown to increase the humoral and cellular immune response against *E. Coli* (Miettinen et al., 1998) and *Salmonella enterica* serovar Enteritidis (Koenen et al., 2004). It is possible that effects *Lactobacillus* have on the immune system is contributed to secreted signaling molecules and not necessarily the presence of the bacteria, as Lee et al (2004) showed an increase in T, NK, and MHC-II cells after several administrations of cytoplasmic lactobacilli extracts.

It has been shown that inflammatory responses in the GIT are reduced by some commensal, non-pathogenic bacteria (Drakes et al., 2004). This is explained by probiotic bacteria inducing the expression of anti-inflammatory cytokines such as IL-10 and reducing the expression of pro-inflammatory IL-1 (Lammers et al., 2003). With the increased expression of cytokines that do not induce inflammation and the increased expression of MHC-II, it is possible for the immune response to have a higher secretion of antibodies (Abbas et al., 2000).

Studies have shown that probiotics significantly increased antibody production in broilers (Kabir et al., 2004) and that antibody titer in probiotic treated birds was significantly higher post immunization of SRBC when compared to the controls (Khaksefidi and Ghoorchi, 2006). It has also been demonstrated, that administration of probiotics enhances serum and intestinal natural

antibodies to several foreign antigens in chickens (Haghighi et al., 2005). Dalloul et al. (2005) studied the effects of the intestinal immune response to an *Eimeria* Acervulina infection when *Lactobacillus*-based probiotics were supplemented in the feed. It was reported that the probiotic afforded some measure of protection through immune modulation. The early immune response was stimulated by the probiotic, characterized by early IFN-γ and IL-2 secretions which improved local immune defenses against coccidiosis.

FloraMax

The commercial probiotic FM-B11 (FloraMax®) is a defined 11-isolate LAB culture of the genus, or related to, *Lactobacillus*. This product was used in a field trial with 234,105 broilers in Mexico (Vicente et al., 2007). The birds treated with FM-B11 showed a 2.06% improvement in bodyweight, a 3.5% improvement in feed conversion, and a .9% reduction in mortality when compared to the controls. The same probiotic was used in a commercial turkey field trial where they reported increases in average daily gain and body weight of 1.63g and 190g respectively over untreated controls. The costs of production were also compared between the treated and untreated groups and the cost per kilogram of meat was reduced by $0.0153 in the probiotic treated group (Torres-Rodriguez et al., 2007).

A study was performed to evaluate the efficacy of typical prophylactic antibiotic use compared with the use of probiotics, or a combination of both in turkey brooding houses deemed likely to experience an outbreak of idiopathic diarrhea (Higgins et al., 2005). In the experiment, the poults receiving FM-B11 periodically in the drinking water had significantly higher mean body weights than the control group and numerically higher weights than the other probiotic group and antibiotic groups. In another experiment, the poults were experiencing a severe

Salmonella Senftenberg infection and were administered FM-B11 or antibiotics plus another probiotic. Between d 12 and 47 the poults receiving the antibiotic and other probiotic gained significantly more weight than FM-B11 alone or the untreated controls. However, weight gain at the end of the experiment between d 29 and 47 no differences in BWG across all treatments were reported. (Higgins et al., 2005).

Higgins et al. (2007) evaluated the ability of FM-B11 to reduce the amount of recoverable *Salmonella* from the ceca of broiler chicks. In their first 3 experiments, the administration of the probiotic 1 hour post *Salmonella enterica* serovar Enteritidis or *Salmonella* Typhimurium challenge significantly reduced the incidence of Salmonella recovery, 60-70% and 89-95% respectively, from the cecal tonsils when compared to the control group, 24 hours post treatment. They also observed a 2.9 log10 reduction in *Salmonella enterica* serovar Enteritidis recovery from probiotic treated birds when compared to the controls, 24 hours post treatment. In the next 4 experiments, FM-B11 again significantly reduced cfu recovery 24 hours post treatment, the probiotic also reduced cfu recovery at 12 hours post treatment, even though there was no difference in incidence. FM-B11 was also shown to reduce *Salmonella enterica* serovar Enteritidis. The encouraging data collected from FM-B11 administration resulted in a commercial product that is currently used in the poultry industry.

Marek's Disease

Marek's disease (MD) is one of the most widespread avian diseases and can be found in chicken flocks worldwide. The disease was first recognized by Jozsef Marek, a Hungarian veterinarian in 1907 (Sluis, 1997). It is a highly contagious disease caused by a herpes virus and is identified by the presence of lymphoid tumors in various organs (Okazaki et al., 1970). This airborne pathogen costs the poultry industry $1-2 billion annually (Morrow and Fehler, 2004), by

causing paralysis, condemnations and high mortality due to T cell lymphomas and peripheral nerve damage (Reddy et al., 1996). The disease may survive for months or years in the litter or poultry dust. Since the virus is inhaled, infection occurs through the respiratory tract and infected birds continue to be carriers long after their infection. Chickens are usually infected at an early age, however lesions are normally not seen until 8-24 weeks of age. At first, all attempts of isolation, sanitation, and genetic resistance to protect flocks from the disease were mostly unsuccessful.

Over the past 40 years, the poultry industry has depended on a series of avirulent or attenuated live virus vaccines to provide protection against field strains of Marek's disease in chickens (Calnek, 2001). These vaccines are the first effective practical means for the control of any neoplastic disease in man or animals. Marek's disease virus-1 (MDV1) causes the condition known as MD in chickens (Churchill and Biggs, 1967). Herpes virus of turkeys (HVT/MDV3) and MDV2 are naturally occurring, infectious viruses that are apathagenic and non-oncogenic in chickens, and are used as vaccines against MD either separately or in combination (Witter et al., 1970). Viruses from all three serotypes have been used as cell-associated live vaccines. In the 1970s, the HVT vaccine provided tremendous protection for a short period of time, until there was a decline in the efficacy of monovalent HVT vaccine due to interference from homologous maternal antibodies and to the development of MDV field strains of increased virulence. By the 1980s, a bivalent vaccine composed of a mixture of HVT and the serotype 2 strain SB-1 was introduced. This vaccine offered better protection than either of the individual components used alone, a well-known phenomenon protective synergism (Witter and Lee, 1984). As field viruses continued to increase in virulence, the attenuated serotype 1 strain CVI988 known as Rispens vaccine was implemented for extensive use in the 1990s (Rispens et al., 1972). The CVI988 is

still the most widely used vaccine and is the most protective due to being a serotype 1 and the most closely antigenically related to field strains (Baignet, 2006).

The vaccines establish a persistent infection which helps to reduce early viremia, after exposure to pathogenic strains. It also protects against tumor formation and mortality which takes away economic consequences due to infection (Morimura et al., 1998). MDV vaccines however, do not prevent super-infection due to challenge viruses. The challenge virus still multiplies and sheds from feather tissues and is oncogenic to non-vaccinated birds. The selection pressure forced on these virulent viruses in vaccinated birds is instigating evolution of field viruses towards pathotypes of greater virulence (Witter, 1997). Even though current vaccines are effective, an ideal vaccine would prevent replication of the virus or shedding.

Inoculation of the vaccine into day-old chicks is followed by replication of the virus a few days later in the lymphoid organs. Then, the vaccine infected lymphocytes are released into the peripheral blood. For optimal protection, the vaccine needs 1-2 weeks between vaccination and exposure. This allows the vaccine virus to enter into the latency stage of infection. It is believed that there is a two-step mechanism of protection. First, the antigens of the vaccine virus are similar to those of the virulent strains and these antigens stimulate an immune response to the virulent virus. This results in decreased viremia, viral replication, malignant transformation, and immunosuppression (Baignet et al., 2006). The second step is MDV tumor antigens stimulating the immunological rejection of tumor cells by cytotoxic T cells (Powell, 1978).

Vaccines used to be administered to day-old chicks subcutaneously in the neck or intramuscularly in the leg using a semi-automated device. This machine relies on the operator to correctly press the chicken against the needle to receive the full dose and could only vaccinate up to 3,000 birds per hour. Due to this laborious task that cost a lot of time and money, now

hatcheries use an *in ovo* delivery system to administer the vaccine to embryos at transfer from the setters to the hatchers (day 18). These machines are equipped with individual floating injectors that can adjust automatically for uniform needle depth in eggs of all sizes and administer a precise dose to 30,000 eggs per hour. The machine also sanitizes the needles after each injection and accurately distinguishes live from infertile or dead embryos and delivers vaccine only to viable eggs. With this method, 100% of chicks are correctly vaccinated against MDV and the timely administration allows for early and effective stimulation of the immune response (Baignet et al., 2006) In 2006, Baignet et al. (2006) reported that a total of 4.2 billion layers, 0.5 billion breeders, and 17 billion broilers are vaccinated annually worldwide.

In Ovo Technology

The agriculture industry is reducing and eliminating drug use as growth promoters in animal diets. These antibiotic growth promoters were used to control poor intestinal conditions caused by dysbacteriosis or parasites, by adding lows doses of the AGP and coccidiostats to commercial poultry diets (De Oliveira et al., 2014). Therefore, many alternatives to growth promoters are now available (Buchanan et al., 2008; Kim et al., 2011). Preferably, usage of drugs in production would be avoided by prevention of diseases rather than treatment. This could be achieved by prophylactic administration of probiotics that prevent colonization of pathogens (Cukrowska et al., 2002). In poultry, colonization is thought to take place after hatching, however there is some evidence from Pedroso (2009) and Bohorquez (2010) that before hatch, small numbers of live bacteria can be found in the intestines. Even though probiotics (DFM) are often used in the pre-starter and starter diets, chicks can be exposed to pathogenic bacteria within the hatchery long before they ever consume any feed (De Oliveira et al., 2014).

Fertile eggs from breeder farms can be contaminated with many bacteria that are on the shells surface or that have penetrated beneath the shell. This can be from contaminated nesting material or the simple fact that a freshly laid, warm, wet egg is highly susceptible to contamination from microorganisms (Cox et al., 1991). At hatch, chicks have an unestablished microflora in their gut and are highly susceptible to intestinal colonization of enteric pathogens within the hatching environment (Cox et al., 1992). Bacteria such as *Salmonella* and *E. coli* can also penetrate the eggshell and proliferate as incubation conditions similarly favor these microorganisms. These bacteria do not usually affect the chick from hatching and consequently create widespread bacterial reservoirs within commercial hatcheries (Cox et al., 1990). Therefore, the presence and persistence of bacterial contamination within the hatchery implies that the susceptible day-of-hatch chicks could be at a greater risk of colonization by pathogenic bacteria in the hatchery than during grow-out (Cox et al., 1990). With this knowledge that the first microbes the chicks may come into contact with could be pathogenic, it seems intuitive that we should not leave pioneer intestinal colonization to chance when we can intentionally inoculate with beneficial bacteria (Cukrowska et al., 2002).

The concept of *in ovo* CE was first put forth in the 1990s when chicken embryos were inoculated into the air cell membrane (Cox et al. 1992). However, previous attempts of this method resulted in practical issues such as higher post hatch mortality and reduced hatchability (Cox et al., 1992; Meijerhof and Hulet, 1997). Cox et al. (1992) administered a CE culture from 1 year old, *Salmonella*-free caged layers that was grown anaerobically and passed once. Embryos were injected onto the air cell membrane or below the air cell membrane. Only the 1:1,000 and 1:1,000,000 dilutions administered into the air cell resulted in higher hatchability numbers that still were not acceptable, 81% and 78% respectively compared to the controls at 96%. The

1:1,000 dilution group also acquired resistance to an oral challenge of *S. typhimurium* (10^6) at day of hatch when compared to the controls. A subsequent experiment demonstrated that dose and delivery depth had an effect on chick hatchability (air cell and amnionic fluid).Doses ranging from undiluted to 1:1,000,000 of CE culture had a significant negative effect on hatchability. Air cell administration reduced hatchability, but chicks showed *Salmonella* resistance at hatch. However, when the CE mixture was administered into the amnionic fluid, the undiluted and 1:1,000 dilution prevented any hatching, and the 1:1,000,000 dilution hatched less than 50%.

Edens et al. (1997) were able to successfully apply *in ovo Lactobacillus reuterin* in the air cell and amnion without having negative effects on hatchability. All studies reported since Edens (1997) paper have concentrated on *in ovo* inoculation of different bacteria into the air cell due to the work of Cox et al. (1992), however those studies have all had a negative impact on hatchability (Meijerhof and Hulet, 1997; Hashemzadeh et al., 2010; Hosseini-Mansoub et al., 2011; Yamawaki et al., 2013). The reasons these studies saw differences could be due to factors such as delivery technique, site of injection (air cell vs. amnion), type of bacteria used, and inoculated dose (De Oliveira et al., 2014). These are all vital parameters that must be considered in order to successfully inoculate bacteria *in ovo*. This was demonstrated by De Oliveira et al. (2014), where 14 different bacterial isolates were *in ovo* injected and it was found that some strains were lethal regardless of dose and some lethal due to dose. This confirmed results of Edens et al. (1997), that there is no reason to avoid amnion inoculation based on hatchability if strains are tested for negative effects and adjusted to avoid this issue. Amnion injections are a preferred method because it eliminates the complications of other techniques such as, imprecise dose reaching the chick by air cell and spraying delivery and practical issues of individual bird inoculation of oral gavage and vent lip application (De Oliveira et al., 2014). Due to current *in*

ovo technology, the administration of vaccines, probiotics, and other components should be combined into a single dose, given together for individual and precise delivery.

In ovo vaccination is currently the standard procedure for the application of Marek's disease virus and infectious bursal disease vaccines in the hatchery for broiler chickens in the United States (Williams and Zedek, 2010). Newly hatched chicks encounter many antigens during the first few days of life, while their immune system is still developing. The highest mortality in commercial chickens usually occurs within the first 7 days of life.

Summary

Due to the intense pressure on the poultry industry and the scientific community to find alternatives to antibiotics for food producing animals, the industry is in need of a replacement therapy for fighting enteric disease in poultry. In commercial poultry, the chicks do not receive beneficial microflora from the hen. Instead, the first exposure they have is the contaminated air inside the hatch cabinets. This allows for pathogens such as *Salmonella* or *E. coli* to have an opportunity to be the pioneer colonizers of the developing embryo's gut. With the surge in probiotic development and the promising results that have been observed, if a proper candidate probiotic could be administered before hatching, the embryo could be colonized with beneficial bacteria before leaving the egg. In poultry, this is possible due to *in ovo* technology and the administration of the Marek's disease vaccine at day 18 of embryogenesis. This is a promising method with commercial application if the right probiotic could be mixed with the vaccine and administered together in one package.

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Chapter III

EFFECTS OF IN OVO ADMINISTRATION OF FLORAMAX®-B11

In ovo evaluation of FloraMax®-B11 on Marek´s disease HVT vaccine protective efficacy, hatchability, microbiota composition, morphometric analysis, and *Salmonella enteritidis* infection in broiler chickens

HEALTH AND DISEASE

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ABSTRACT

Four experiments were conducted to evaluate the effect of *in ovo* administration of FloraMax®-B11 (FM) on Marek´s disease (MD) herpesvirus of turkeys（HVT) vaccine protective efficacy, hatchability, microbiota composition, morphometric analysis and *Salmonella enteritidis* (SE) infection in chickens. In Exp. 1, day 18 White Leghorn embryos were randomly distributed in four groups: 1) HVT vaccinated *in ovo* and no Marek's disease virus (MDV) challenge; 2), HVT + FM vaccinated *in ovo* and no MDV challenge; 3) HVT vaccinated *in ovo* and challenge with virulent MDV (vMDV; strain 583A); 4), HVT + FM vaccinated *in ovo* and challenge with vMDV. Exp. 2 was designed exactly the same as Exp. 1 but chicks were challenged with very virulent MDV (vvMDV; strains Md5 and 612). In both experiments, birds were monitored until 8 wk of age, and tested for MD incidence. Exp. 3 and 4, day 18 commercial broiler embryos were injected *in ovo* with either saline or FM to measure hatchability and gastrointestinal composition. In addition, in Exp. 4, all chickens that hatched were then orally gavaged with SE at hatch and kept for 7 d to monitor post hatch BW. In Exp. 1 and 2, there was no significant difference ($P > 0.05$) between % MD in birds vaccinated with HVT alone or HVT + FM. In Exp. 3 and 4, administration of the probiotic did not negatively affect hatchability, but did reduce coliforms. Further, increase in BW was associated with higher villi surface area in ileum in chickens that received the probiotic as well as a significant reduction in the SE incidence. These results study suggest that *in ovo* administration of FM does not negatively impact the ability of HVT to protect against MD or hatchability of chickens, and improves BW during the first 7 d of life and decreases SE recovery in broiler chickens.

Key words: *In ovo,* Marek's disease vaccine, probiotic, chickens, hatchability

INTRODUCTION

Bacterial communities living and colonizing in the gastrointestinal tract of animals outnumber total somatic cells of metazoans by an estimated 10-fold (Neish, 2009). Today, the microbiome is recognized as the 'forgotten organ,' operating like an organ within the host and orchestrating numerous physiological and biological functions that have a profound impact on the balance between health and disease (O'Hara and Shanahan, 2006; Tellez, 2014). Early establishment of the microbiome have been reported to improve the assembly of the gut-associated lymphoid tissue (Martin et al., 2010), intervene in the development of the immune system (McFall-Ngai, 2007), maintain mucosal barrier integrity (Duerkop et al., 2009), modulate proliferation of enterocytes (Moran, 2007), adjust blood flow (Sekirov et al., 2010), regulate the enteric nervous system (Tlaskalová-Hogenová et al., 2011), and improve digestion of nutrients (Dass et al., 2007; Walter et al., 2011; Qiu et al., 2012). Essential colonization of these bacterial populations starts at birth/hatch, and is followed by progressive assembly of a complex and dynamic microbial society (Di Mauro et al., 2013).

Under commercial conditions, millions of chickens and turkeys hatch in a hostile environment, and are exposed for several hours to heat stress and potential pathogenic bacteria in the hatcheries. Increased stress along with the potential abundance of pathogens in the hatching cabinet leads to ideal conditions for pathogen colonization. It is generally accepted that the natural route of transmission of zoonotic pathogens such as *Salmonella,* is fecal-oral (White et al., 1997; Galanis et al., 2006). However, published studies have also suggested that airborne transmission of *Salmonella* in poultry is possible (Wathes et al., 1988; Baskerville et al., 1992; Leach et al., 1999; Fallschissel et al., 2009). Understanding the anatomical and immunological defenses of the avian respiratory tract helps to clarify this issue. Architecture of the avian respiratory tract is an

important component to susceptibility and resistance to infectious agents. In day old chickens and turkeys, no or very few infiltrating lymphocytes are seen in the primary bronchi region (Fagerland and Arp, 1990; Smialek et al., 2011) and it isn't until 3-4 weeks of age the lymphoid nodules are developed at these locations (Fagerland and Arp, 1993; Drolet et al., 2010). During the following week, the number of IgG, IgA or IgM-producing cells continues to increase, however, the bronchial-associated lymphoid tissue (**BALT**) is not mature until chickens are 6–8 weeks old (Bienenstock, 1980; Bienenstock and McDermott, 2005; De Geus, 2012). Hence, commercial neonate poultry are extremely susceptible to airborne pathogens, regardless of respiratory or enteric bacteria (Arshad et al., 1998). In support of these findings, our laboratory has recently showed that transmission by the fecal-respiratory route is a viable portal of entry for *Salmonella* (Kallapura et al., 2014a,b,c). This mode of infection could explain some clinical expression of relatively low-dose infectivity under field conditions in relation to the high oral challenge dose that is typically required for infection through the oral route in laboratory studies. This also supports previous studies demonstrating fan driven spread of *Salmonella* within the hatching cabinet and hatchery incubators (Hashemzadeh et al., 2010).

Over a century ago, Eli Metchnikoff proposed the ground-breaking idea to ingest viable bacteria to endorse health (Metchnikoff, 1908). This concept is more appealing today, since antimicrobial resistant bacteria have become a problem in many countries (Kiser, 1976; Dahiya et al., 2006; Teillant and Laxminarayan, 2015). The imminent ban of antibiotics in animal feed creates a challenging scenario for expansion of alternative prophylactics (Parker, 1990; Dahiya et al., 2006; You and Silbergeld, 2014). Probiotics and direct-fed microbials are becoming accepted as one of the best tools on keeping gastrointestinal health and promoting performance in poultry raised without antibiotics (Dominguez-Bello and Blaser, 2008). In addition to improving intestinal

microbial balance, metabolism, and gut integrity (Isolauri et al., 2002; Salminen and Isolauri, 2006), studies have also shown that probiotics have anti-inflammatory (Borchers et al., 2009; Lyte, 2011), anti-oxidant (Farnell et al., 2006; Tao et al., 2006; Zareie et al., 2006; Segawa et al., 2011; Howarth and Wang, 2013), and enhacing barrier integrity properties (Yu et al., 2012). Furthermore, several researchers have confirmed benefits of probiotics on innate immunity (Alvarez-Olmos and Oberhelman, 2001; Vanderpool et al., 2008; Molinaro et al., 2012) as well as humoral immunity (Arvola et al., 1999; Haghighi et al., 2006; Howarth and Wang, 2013).

FloraMax®-B11 is a defined lactic acid bacteria (**LAB**) probiotic culture that has demonstrated an accelerated development of normal microflora in chickens and turkeys. It provides increased resistance to *Salmonella* spp. infections (Farnell et al., 2006; Higgins et al., 2007, 2008, 2010; Vicente et al., 2007; Menconi et al., 2011, 2013; Tellez et al., 2012; Biloni et al., 2013; Delgado et al., 2014), reduces idiopathic diarrhea in commercial turkey brooding houses (Higgins et al., 2005), as well as increased performance and reduced costs in poultry (Torres-Rodriguez et al., 2007; Vicente et al., 2008). However, no studies have been evaluated for administration of FloraMax®-B11 *in ovo,* and the only practical and reliable way to evaluate this route of administration, would be mixing it with the diluent of the Marek's disease (**MD**) vaccine. Hence, the objective of the present study was to evaluate the effect of the *in ovo* administration of FloraMax®-B11 on MD vaccine herpesvirus of turkeys (**HVT**) protective efficacy, hatchability, microbiota composition, morphometric analysis, and SE infection in chickens.

MATERIAL AND METHODS

Probiotic Culture

FloraMax®-B11 (Pacific Vet Group USA Inc., Fayetteville, AR) is a defined probiotic culture derived from poultry gastrointestinal origin that contains proprietary strains of LAB.

In ovo *evaluation of FloraMax®-B11 on* Marek´s disease HVT vaccine

Chickens and Viruses. Maternal-antibody-negative, White Leghorn $15I_5x7_1$ chickens were used in these experiments (Bacon et al., 2000). These MD-susceptible chickens were from an SPF breeding flock with no MD vaccinations or exposure that tested negative for MDV antibodies, exogenous avian leukosis virus, and reticuloendotheliosis virus by routine surveillance testing. All birds were housed in negative-pressure Horsfall-Bauer isolators, and experiments were conducted following approval by the USDA Avian Disease and Oncology Laboratory (**ADOL**) Animal Care and Use Committee. Viruses were propagated on primary duck embryo fibroblasts (**DEF**) maintained in Leibovitz L-15 medium plus McCoy 5A medium (1:1), supplemented with 2.5% bovine serum and antibiotics (Witter et al., 1980). In experiment 1, chickens were challenged with the MDV strain 583, a virulent (v) strain. In experiment 2, chickens were challenged with MDV strains Md5 or 612, both very virulent (vv). HVT is a commercial vaccine, and was prepared and utilized as recommended by the manufacturer.

Experimental Design

Experiment 1. Chicks were randomly distributed into four groups (each with 17 birds) in two independent trials: 1) HVT vaccinated *in ovo* and no MDV challenge; 2) HVT + FloraMax®-B11 vaccinated *in ovo* and no MDV challenge; 3) HVT vaccinated *in ovo* and challenged with MDV; 4) HVT + FloraMax®-B11 vaccinated *in ovo* and challenged with MDV. MD vaccine was

administered *in ovo* at the manufacturer recommended dosage either alone or with FloraMax®-B11 (10^4 cfu). Birds were monitored until 8 wk of age, then humanely euthanized and evaluated for MD incidence. Chickens were considered MD positive if peripheral nerve enlargements, tumors, or both were present at necropsy. When enlarged nerves or gross tumors were in question, tissue samples were collected and processed for microscopic evaluation. Chicks that died during the first wk of placement were considered nonspecific chick mortalities and were excluded from the experiment.

Experiment 2. The identical conditions were used as described for experiment 1 except that MDV strains Md5 and 612 were used instead of strain 583A and the experiment included only one trial.

Effect of* in Ovo *Application of FloraMax®-B11 on Hatchability and Microbiota Composition.. Experiment 3 consisted of three independent trials. Eighteen-day-old embryos were obtained from Cobb-Vantress (Siloam Springs, AR). In each trial, eggs were candled and inoculated with either saline or 10^4 cfu of FloraMax®-B11 via *in ovo* injection into the amnion. The two treatment groups were placed in separate hatchers to avoid cross contamination. On d 21, chicks were pulled from hatchers and hatchability was determined. In each trial, 12 chickens from each group were humanely euthanized to evaluate gastrointestinal composition on selective media as describe below.

Enumeration of Bacteria. For trial 1, the whole gut (ventriculus to cecum) was aseptically removed. For trials 2 and 3, the fore gut (ventriculus to Meckel's diverticulum) and hind gut (Meckel's diverticulum to cecum) were removed separately. Sections were collected into sterile bags and homogenized. Samples were weighed and 1:4 wt/vol dilutions were made with sterile 0.9% saline. Ten-fold dilutions of each sample, from each group were made in a sterile 96 well

Bacti flat bottom plate and the diluted samples were plated on two different culture media; to evaluate total number of LAB in Man Rogosa Sharpe (Difco™ Lactobacilli MRS Agar VWR cat. no. 90004-084, Suwanee, GA 30024); total coliforms in MacConkey (VWR cat. no. 89429–342, Suwanee, GA 30024).

Evaluation of* in Ovo *Administration of FloraMax®-B11 on Body Weight, Salmonella enteritidis Recovery, and Morphometric Analysis in Broiler Chickens. In experiment 4, the challenge organism used in all experiments was a poultry isolate of *Salmonella enterica* (SE) serovar, Enteritidis, bacteriophage type 13A, obtained from the USDA National Veterinary Services Laboratory, Ames, IA. This isolate was resistant to 25 µg/mL of novobiocin (**NO**, cat. no. N-1628, Sigma, St. Louis, MO 63103) and was selected for resistance to 20 µg/mL of nalidixic acid (**NA**, cat. no. N-4382, Sigma) in our laboratory. For the present studies, 100 µL of SE from a frozen aliquot was added to 10 mL of tryptic soy broth (cat. no. 22092, Sigma) and incubated at 37°C for 8 h, and passed three times every 8 h to ensure that all bacteria were in log phase. Post-incubation, bacterial cells were washed 3 times with sterile 0.9% saline by centrifugation at 1,800 × *g* for 10 minutes, reconstituted in saline, quantified by densitometry with a spectrophotometer (Spectronic 20D+, Spectronic Instruments Thermo Scientific, Waltham, MA 02451), and diluted to an approximate concentration of 10^8 cfu/ml. Concentrations of SE were further verified by serial dilution and plating on brilliant green agar (**BGA,** cat. no. 70134, Sigma) with NO and NA for enumeration of actual cfu used to challenge the chickens.

In this trial, 300 eighteen-day-old embryos were received from Cobb-Vantress. At d 18, eggs were candled and inoculated with either saline or 10^4 cfu FloraMax®-B11 via *in ovo* injection into the amnion. The two treatment groups were placed in separate hatch cabinets placed in

separate rooms to avoid cross contamination. On d 21, chicks were pulled from hatchers to measure hatchability. All chickens were then orally gavaged with SE on d of hatch (~10^4 cfu/chick). Twenty-four hours post inoculation (**PI**), twenty chickens were euthanized with carbon dioxide asphyxiation to determine SE intestinal colonization as described below. From these chickens, 5 samples were also taken to determine intestinal morphometric analysis as described below. BW was determined at d 1, 3, and 7. Chickens were provided *ad libitum* access to water and a balanced unmedicated corn-soybean diet meeting the nutrition requirements of poultry recommended by NRC (1994). All animal handling procedures were in compliance with Institutional Animal Care and Use Committee at the University of Arkansas.

Salmonella Recovery

Ceca-cecal tonsils (**CCT**) were homogenized and diluted with saline (1:4 by wt/vol) and tenfold dilutions were plated on BGA with NO and NA, incubated at 37°C for 24 h to enumerate total SE colony forming units. Following plating to enumerate total SE, the CCT samples were enriched in double strength tetrathionate enrichment broth and further incubated at 37°C for 24 h to enrich. Following this, enrichment samples were plated on BGA with NO and NA and incubated at 37°C for 24 h to confirm presence/absence of typical lactose-negative colonies of *Salmonella*.

Intestinal Morphological Analysis

For enteric morphometric analysis ileum and duodenum samples were collected (n = 5). A 1-cm segment of the midpoint of the duodenum and the distal end of the lower ileum from each bird was removed and fixed in 10% buffered formaldehyde for 48 h. Each of these intestinal segments was embedded in paraffin, and a 5-μm section of each sample was placed on a glass slide and stained

with hematoxylin and eosin for examination under a light microscope. All morphological parameters were measured using the ImageJ software package (http://rsb.info.nih.gov/ij/). Ten replicate measurements for each variable studied were taken from each sample, and the average values were used in statistical analysis. Villus length (**VL**) was measured from the top of the villus to the top of the lamina propria (Yitbarek et al., 2013). Crypt depth was measured from the base upward to the region of transition between the crypt and villus (Biloni et al., 2013). Villus width (**VW**) was measured at the widest area of each villus, whereas the villus:crypt ratio was determined as the ratio of villus height (**VH**) to crypt depth. Villus surface area (**VSA**) was calculated using the formula $(2\pi)(VW/2)(VL)$, (Sakamoto et al., 2000).

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 is expressed as mean ± standard error. Significant differences among the means were determined using Duncan's multiple-range test at $P < 0.05$. MDV as well as SE incidence data were expressed as positive/total chickens (%), and the percent recovery of SE was compared using the chi-squared test of independence, testing all possible combinations to determine the significance ($P \leq 0.001$) for these studies (Zar, 1984).

RESULTS

This study addressed three major concerns: 1) whether *in* ovo administration of FloraMax®-B11 mixed with MD vaccine would negatively impact vaccine efficacy, 2) the effect of *in ovo* administration on hatchability and microbiota composition and 3) the impact on *Salmonella* infections in broiler chickens. Experiment 1 consisted of two independent replicates to determine if there was any difference when birds were vaccinated *in ovo* with HVT only or with HVT + FloraMax®-B11 followed by challenge with vMDV. The results of the *in ovo e*valuation of FloraMax®-B11 on HVT vaccine efficacy in experiments 1 and 2 are summarized in Table 1. In both experiments, there was no significant difference between % MD in birds vaccinated with HVT alone or HVT + FloraMax®-B11, although numerical differences between treatment suggest that probiotics may have slightly improved protection immunity in birds challenged with MDV strain 583. This benefit was not apparent when we used vvMDV strains (Md5 and 612) in experiment 2 (Table 1).

The effect of *in ovo* administration of the probiotic FloraMax®-B11 on hatchability in experiment 3 is displayed in Table 2. There was no significant difference in hatchability between embryos administered probiotics or the controls. The results of the effect of *in ovo* application of FloraMax-B11® on microbial composition in the gastrointestinal tract of hatching broiler chickens in experiment 3 are summarized in Table 3. In trials 1 and 3, chickens treated with FloraMax®-B11 showed a significant reduction in coliforms recovery when compared with saline control group at d of hatch. In trial 2, the treated group had numerically lower recovery than the control group and in fact had reduced gram negatives to non-recoverable numbers. With the exception of hindgut in trial 2, a significant increase in the total number of LAB was observed in probiotic group when compared with saline treated group (Table 3).

The results of *in ovo* administration of FloraMax®-B11 on hatchability, BW and SE recovery in broiler chickens of Experiment 4 are summarized in Table 4. In this experiment, no significant changes were observed in hatchability or the BW of the neonates when they were removed from the hatching cabinets; however, a significant increase in BW was observed in chickens that received the probiotic when compared with saline control groups on d 3 and 7 (Table 4). Interestingly, chickens that received the probiotic, showed a significant reduction in the incidence and total SE cfu numbers recovered from CCT when compared with saline control chickens (Table 4).

The results of the effect of *in ovo* application of FloraMax®-B11 on morphometric analysis of the gastrointestinal tract of hatching broiler chickens of experiment 4 are summarized in Table 5. A numerical increase in VH, VW, and VSA was observed in the treated group when compared to the controls for the duodenum. Nevertheless, embryos that received the probiotic showed a significant increase in the villus:crypt depth ratio when compared with saline control group. In the ileum, there was a significant increase in VH, VSA, and crypt depth in the probiotic treated group when compared to the control group.

DISCUSSION

MMD is a lymphoproliferative disease of domestic chickens caused by an oncogenic α-herpesvirus (Churchill and Biggs, 1967; Calnek, 2001). The disease is associated with lymphomas, neurologic manifestations, and immune suppression (Calnek, 2001). Without a question, MD has been a major concern to the poultry industry for over half a century (Nair, 2005), and the modern poultry industry as we know it today, would not exist without the development of MD vaccines (Baigent et al., 2006; Gimeno, 2008; Parvizi et al., 2010; Silva et al., 2010; Dunn and Silva, 2012). The virus is so abundant and stable in the environment, that vaccination at the hatchery is the only effective method to control MD in commercial flocks (Witter et al., 1980, 2005; Baigent et al., 2006; Dunn et al., 2010). Due to the significant economic and immunosuppression impact, modern commercial chickens are vaccinated before they leave the hatchery.

Although, we have reported the benefits of spray application of FloraMax®-B11 in the hatcheries (Wolfenden et al., 2007), this is the first report of *in ovo* application of this defined probiotic, mixed with HVT vaccine simultaneously. One of the two major concerns we addressed in this study was whether *in* ovo administration of FloraMax®-B11 mixed would negatively affect MD vaccine protective efficacy. The results of experiments 1 and 2, demonstrated that there was no negative impact and even possibly a small improvement of the probiotic depending on the MDV challenge strain. As far as we are aware, this is the first report showing the possibility of combining a probiotic with an *in ovo* MD vaccine showing no negative effect. The other major concern with *in ovo* application of FM was on broiler hatchability, but in every trial conducted the probiotic also showed no negative effects on hatchability.

In the present study, it was remarkable to observe that embryos, which received the probiotic before hatch, had a significant reduction in coliforms when compared with saline treated

chickens (Table 3). Although there is extensive evidence demonstrating that this particular probiotic is able to control *Salmonellae* infections in poultry in both, laboratory or commercial conditions (Farnell et al., 2006; Higgins et al., 2007, 2008, 2010; Vicente et al., 2007; Menconi et al., 2011, 2013; Tellez et al., 2012; Biloni et al., 2013; Delgado et al., 2014). This current study further validated the probiotics efficacy via *in ovo* administration by reducing the recovery of SE when chickens were challenged at d of hatch and cultured 24 h later (Table 4). These results are in agreement with the work of De Oliveira et al. (2014) who demonstrated that *in ovo* colonization with probiotic could become an important method to reduce *Salmonella* and other intestinal bacterial infections in poultry.

In experiment 4, the significant increase in BW in treated chickens at d 3 and 7 (Table 4), were associated with significant morphometric changes in the duodenum and ileum observed at d 1 (Table 5). It is likely that the higher BW in the probiotic treated group was due to the increase VH, leading to more VSA leading to better nutrient absorption. These results are quite impressive, when a newly hatched modern d broiler chick increases its BW by 25% overnight and 5000% by 5 wk, to 2kg (Choct, 2009). Similarly, it is also important to consider the productive life of broiler chickens. The full genetic potential of modern chickens starts at conception and the first 21 d of embryo development. During this period, variables as temperature or oxygen are important and any problem related to them could cause a big impact later in life. Hence, the 21 d of embryogenesis plus the first 7 d of life of the chicken could potentially represent between 50% to 74% of the life of a commercial broiler chicken, depending on the time they are slaughtered (56 or 77 d) (Cherian, 2011). Therefore, earlier administration of probiotics to embryos can have a profound impact on growth and overall health of the birds.

In summary, the results of the present study suggest *in ovo* administration of FloraMax®-B11 does not negatively affect HVT vaccine efficacy or hatchability of the chickens, and improves BW and intestinal integrity during the first 7 d of life while decreasing SE intestinal load in broiler chickens. Studies to evaluate these effects under commercial conditions are currently underway.

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Table 1.
*In ovo e*valuation of FloraMax®-B11 on HVT Marek´s vaccine virus stability and incidence of disease.

	HVT only	HVT + FloraMax®-B11
Experiment 1 (Trial 1)		
Unchallenged	0/15 (0 %)	0/17 (0 %)
vMDV challenge (583)	3/17 (18 %)	0/17 (0 %)
Experiment 1 (Trial 2)		
Unchallenged	1/16 (6 %)	0/17 (0 %)
vMDV challenge (583)	4/15 (27 %)	3/16 (19 %)
Experiment 2		
Unchallenged	0/17 (0 %)	0/17 (0 %)
vvMDV challenge (Md5)	10/17 (59 %)	9/17 (53 %)
vvMDV challenge (612)	11/17 (65 %)	13/17 (76 %)

Marek's disease HVT vaccine was administered *in ovo* at manufacturer labeled dosage alone or with FloraMax®-B11 (10^4 cfu/g). MDV challenge was administered at 5 d of age using 500 pfu vMDV strain 583 in experiment 1, or 500 pfu vvMDV strains Md5 or 612, respectively. Birds were monitored until 8 wk of age, then euthanized and measured for MD incidence. $P > 0.05$

Table 2.
Effect of *in ovo* application of FloraMax®-B11 on hatchability.

Treatment	Trial 1	Trial 2	Trial 3
Saline	137/140 (97.8%)	46/48 (95.8%)	144/145 (99.3%)
FloraMax®-B11	121/121 (100%)	47/48 (97.9%)	142/150 (94.6%)

At d 18 eggs were candled and inoculated with either 0.9% saline or FloraMax®-B11 via *in ovo* injection into the amnion. On d 21, chicks were pulled from hatchers and hatchability was determined, $P > 0.05$.

Table 3.
Effect of *in ovo* application of FloraMax®-B11 on microbial composition in the gastrointestinal tract of hatching broiler chickens.

Selective media and experimental groups	Trial 1	Trial 2		Trial 3	
	Whole gut	Fore gut	Hind gut	Fore gut	Hind gut
Total coliforms/g [1]					
Saline	8.24 ± 0.27	0.8 ± 0.5	1.6 ± 0.8	4.06 ± 0.52	8.54 ± 0.24
FloraMax®-B11	0.92 ± 0.48 *	0.0 ± 0.0	0.0 ± 0.0	0.05 ± 0.01 *	0.0 ± 0.0 *
Total LAB/g [2]					
Saline	8.70 ± 0.26	4.90 ± 0.5	7.7 ± 0.40	0.00 ± 0.0	0.84 ± 0.60
FloraMax®-B11	6.43 ± 0.94 *	6.20 ± 0.50 *	7.9 ± 0.40	4.33 ± 0.50 *	6.00 ± 0.31 *

At d 18 eggs were candled and inoculated with either saline or FloraMax®-B11 via *in ovo* injection into the amnion. On d 21, chicks were pulled from the hatchers and for experiment 1, the whole gut (ventriculus to cecum) was aseptically removed. For experiment 2 and 3 the fore gut (ventriculus to Meckel's diverticulum) and hind gut (Meckel's diverticulum to cecum) were removed separately.

1 Samples were plated on MacConkey agar to evaluate total coliforms.

2 Samples were plated on MRS agar to evaluate total lactic acid bacteria.

Data is expressed as Log_{10} CFU/gram

Data is expressed as mean ± standard error. *Superscripts within columns for each plate indicate significant difference at $P < 0.05$, n = 12.

Table 4.
Evaluation of *in ovo* administration of FloraMax®-B11 on hatchability, body weight, and *Salmonella enteritidis* recovery in broiler chickens.

Treatment	Hatchability	Day 1 BW (g)	Day 3 BW (g)	Day 7 BW (g)	SE incidence Ceca–cecal tonsils 24 h PI	Log_{10} SE/g of ceca content 24 h PI
Saline	148/150 (98.6 %)	49.13 ± 0.30 [a]	62.53 ± 0.81 [b]	132.89 ± 3.06 [b]	20/20 (100 %)	7.13 ± 1.01 [a]
FloraMax®-B11	142/150 (94.6 %)	49.72 ± 0.36 [a]	65.42 ± 0.77 [a]	144.98 ± 3.02 [a]	9/20 (45 %) *	5.45 ± 1.25 [b]

At d 18 eggs were candled and inoculated with either saline or FloraMax®-B11via *in ovo* injection into the amnion. On d 21, chicks were pulled from the hatchers and were challenged with *Salmonella* Enteritidis (SE) on d of hatch ~10^4 cfu/chick. Incidence data is expressed as positive/total chickens (%) at 24 h post inoculation (PI), asterisk indicate significant differences $P < 0.001$, n = 20/group. Log_{10} SE/g of ceca content is expressed as mean ± standard error. [ab]Superscripts within columns indicate significant differences $P < 0.05$, n = 12/group.

Table 5.
Evaluation of in ovo administration of FloraMax®-B11 on morphometric analysis of the gastrointestinal tract of hatching broiler chickens**.**

	Villus height (µm)	Villus width (µm)	Villus surface area (mm^2)*	Crypt depth (µm)	VH:CD ratio**
Duodenum					
Control	223.39 ± 3.55 [a]	36.01 ± 0.72 [a]	25.39 ± 0.69 [a]	49.92 ± 1.15 [a]	4.74 ± 0.14 [b]
FloraMax®-B11	234.58 ± 5.19 [a]	36.14 ± 0.60 [a]	26.87 ± 0.86 [a]	39.93 ± 0.88 [b]	6.09 ± 0.19 [a]
Ileum					
Control	148.09 ± 4.26 [b]	27.42 ± 0.86 [a]	13.10 ± 0.67 [b]	36.70 ± 1.04 [b]	4.16 ± 0.12 [a]
FloraMax®-B11	176.77 ± 5.50 [a]	29.01 ± 0.78 [a]	16.47 ± 0.80 [a]	40.55 ± 1.19 [a]	4.59 ± 0.21 [a]

[a,b] Means with different superscripts within the same column differ significantly ($P < 0.05$).

* Villus surface area: [$2\pi \times$ (villus width/2) $\times$ (villus height)]

** Villus height (VH) to crypt depth (CD) ratio

Chapter IV

Conclusion

This study evaluated the early administration of a probiotic, which could potentially be an alternative to antibiotics, for use in the poultry industry. In the study discussed in Chapter III, a commercial probiotic was administered *in ovo* and evaluated. We assessed if the mixture of the probiotic and the Marek's vaccine had an effect on the ability of the vaccine to protect against Marek's Disease. The results showed that the administration of the mixture of the vaccine and the probiotic had no negative effect on the protectiveness of the vaccine when compared with birds administered the vaccine only. In this study, we also evaluated hatchability and bacterial recovery from the gastrointestinal tract. The *in ovo* administration of the probiotic had no effect on hatchability and significantly reduced coliforms within the gastrointestinal tract at hatch. Lastly, we evaluated the ability of the probiotic to reduce *Salmonella* recovery and increase BW post *in ovo* inoculation. 24 h post *Salmonella* challenge we observed a significant reduction in *Salmonella* recovery and incidence, along with a significant increase in BW at days 3 and 7. Which could be a result of the increased villus surface area observed in the ileum of the probiotic treated group. This study shows a favorable administration technique and probiotic that could allow for early colonization of beneficial microflora and increased efficiency.

Appendix

UNIVERSITY OF ARKANSAS

Office of Research Compliance

MEMORANDUM

TO: Billy Hargis

FROM: Craig N. Coon, Chairman

DATE: May 16, 2016

SUBJECT: IACUC Approval

Expiration Date: September 14, 2017

The Institutional Animal Care and Use Committee (IACUC) has APPROVED your modification to add Kyle Teague and Lucas Graham to protocol # 15006: "Development of enteric inflammation models for investigation of antibiotic alternatives in poultry".

In granting its approval, the IACUC has approved only the information provided. Should there be any further changes to the protocol during the research, please notify the IACUC in writing (via the Modification form) prior to initiating the changes. If the study period is expected to extend beyond September 14, 2017 you must submit a new protocol prior to that date to avoid any interruption. By policy the IACUC cannot approve a study for more than 3 years at a time.

The IACUC appreciates your cooperation in complying with University and Federal guidelines involving animal subjects.

CNC/aem

cc: Animal Welfare Veterinarian

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