THE EFFECT OF *IN OVO* PROBIOTICS ON HATCHABILITY AND DEVELOPMENT OF POULTS WITH OR WITHOUT DIETARY RESTRICTIONS

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ABSTRACT

THE EFFECT OF *IN OVO* PROBIOTICS ON HATCHABILITY AND DEVELOPMENT OF POULT WITH OR WITHOUT DIETARY RESTRICTIONS

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Turkeys have to overcome several challenges early in life, including exposure to pathogens and difficulty with the transition from yolk as a nutrient source to CHO dietary sources. Probiotics are thought to help alleviate these symptoms in the post-hatch poult, but any beneficial effects, if given prior to hatch are unknown. Additionally, the optimal level of probiotic that can be injected into the egg must be established. Once optimal levels of hatch are established, the effects post hatch must be known. A series of experiments were performed to determine the benefits of *in ovo* probiotics. The overall objective was to evaluate different probiotic concentrations injected into turkey eggs prior to hatch on hatchability and intestinal bacterial concentrations. Secondary objectives were to determine the impact of *in ovo* probiotics on poult hatchability and first week mortality. Finally, whether a probiotic injection could mitigate poult growth ramifications due to delayed access to feed or a 20% dietary reduction in Ca and P. To study these objectives a series of experiments were conducted. The general design of the study was for groups of fertile turkey eggs to be incubated. The eggs were candled on d25 and allocated to different treatment groups: (1) Non-injected eggs hatched in a separate hatcher (Negative Control), (2) eggs injected with 1ml of saline and placed in the hatcher with probiotic injected eggs (Sham Control), (3) eggs injected with various amounts of probiotic solution (Probiotic). The most commonly used concentration was 10^6cfu/ml. An additional experimental factor of delayed placement on feed was

added to experiment 2. Placement of eggs in the incubator was offset by 24h, and the hatched poults were all placed on feed at the same time. Hatch counts were taken for each replicate and cecal contents collected from two birds per replicate to determine bacterial counts on d28. Cecal contents were plated on media plates with LB agar, MRS agar, Bifidobacteria agar or Enterococcusel agar. Intestinal contents were collected to measure nutrient transporters and intestinal morphology in trial two. Birds from experiments two and three were placed on feed for one (experiment 2) or three (experiment 3) wks. At DOH of exp3, half of the hatched poults were given access to a diet with a 20% reduction in calcium and phosphorus. Tibia and femur samples were collected for morphology and ash content from birds during exp3. The injection of 10^6cfu of bacteria or lower into the egg did not alter hatchability of eggs from trials one and two. Overall, hatchability was reduced by almost 20% in exp3. Bacteria load in the intestine was two to three logs higher in probiotic injected birds compared to control for every trial. The first week livability of probiotic injected birds was not significantly different than controls in exp2, but was almost double in exp3. Sham control poults from exp2 had the highest BW for the entire trial. Probiotic injected birds had improved FCR through d3 in exp2 but the effect on FCR was lost by d7. Probiotic injected birds had lower BW and had shorter, narrower tibias and femurs at d7 and d21 compared to controls. The results indicate that *in ovo* probiotics can negatively influence poult growth and performance in the first 21d of life, if the bacteria reduce hatchability. When *in ovo* probiotics are injected into eggs and increase bacterial load in the intestine without altering hatchability, the benefits are improved FCR through the first 3d of life. The results from the series of experiments are a promising insight into the effects *in ovo* bacteria can have on the early post hatch poult.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LITERATURE REVIEW

The early development sets the poult up for optimal performance later in life. Problems at the breeder farm or hatchery can lead to problems shortly after hatch, which further leads to either early mortality or low performance numbers. If optimal conditions are met before hatch, then enhanced performance and reduced mortality can be seen early in life. Producers have used many different types of dietary additives over the years to improve performance. The increasing complexity of dietary additives recently has resulted in the need for producers to better understand intestinal development and the role these additives play.

Incubation

The time difference between the first and last hatched chick or poult is called the hatch window (HW). The variability in HW is approximately 24 to 48h (Decuypere et al., 2001). Hatch window times and the ability of the embryo to hatch are affected by the air quality and temperature. One reason for varying incubation length relates to the ability of the egg shell to diffuse oxygen (Rahn et al., 1974). The levels of 1 to 3% CO_2 during the first 8d of incubation and over 6% for the 9 to 12d of incubation can adversely affect hatch (Tona et al., 2007). Concentration of CO_2 during the last few days of incubation helps stimulate hatching. When the egg cannot meet the oxygen demands of the embryo, pipping will begin. Excessive CO_2 during development can be lethal to the embryo (Tona et al., 2007).

Temperature plays an important role as well. High temperatures early in incubation causes embryonic mortality (French, 2000). Overheating in the 2nd and

3rd quarters of incubation causes a head malposition, which reduces hatchability. Turkey eggs incubated at a consistent reduced temperature hatched later than those incubated at industry recommended temperature (French et al., 1994). Individual eggs will experience different temperatures in the incubator (French et al., 1997). The slight difference in incubation temperature for each egg contributes to the variability of the HW.

Fasting or delayed access to feed: effects on the intestine

The main issue with HW is that early hatched chicks often have no access to feed or water up to 72h after hatch by considering the spread of the HW, chick handling, and transport time (Romanini et al., 2013). Simulation of shipping times as short as 4 or 10h reduced BW compared to 0h shipping times (Bergoug et al., 2013). This reduction in BW was maintained for the first 21d in broilers. Fasting at any age for a period of 48h reduces the BW of birds (Geyra et al., 2001b). Additionally, fasting during the first 48h post-hatch retards intestinal growth and reduces the number of total cells and percentage of proliferating cells in the crypt (Geyra et al., 2001a). In turkeys, a 48h delayed access to feed at the start of life reduces BW by over 10% (Pchavov and Noy, 1993). Poults with a 48h delayed access to feed have fewer bacteria in the digesta after 24h on feed (Potturi et al., 2005). In poults that had a 54h delayed access to feed, the pancreatic amylase and trypsin activities were reduced (Corless and Sell, 1999). The mucin layer thickens in chickens whose feed is withheld for 72h (Smirnov et al., 2004). Additionally, the overall weight of the intestine is reduced by 30%, and the villus surface area is decreased by over 25%

after 72h of fasting. In contrast, feeding in the hatcher or later in transport boxes improves intestinal development (Noy et al., 2001).

In ovo technologies

The recent development of *in ovo* technologies has allowed researchers and producers to introduce medicinal and nutritional factors into the egg prior to hatch. *In ovo* vaccination is a practice used in over 90% of broiler production (Williams and Zedek, 2009). Vaccines are injected into both the air cell and the amnion without reducing hatchability. The vaccine technologies could be used for the introduction of probiotics before hatch, but it is unclear if *in ovo* probiotics would reduce hatchability. Hatcheries strive to keep the egg as clean as possible to prevent pathogenic bacterial contamination that would reduce hatchability. One milligram of competitive exclusion cultures injected into the air cell decreased hatchability (Meijerhof and Hulet, 1997). Inoculation of diluted bacteria (10^3 cfu) from adult cecal droppings into the air cell reduced hatchability as well, but the birds that did hatch were more resistant to Salmonella (Cox et al., 1992). Injection into other parts of the egg may not yield the same results. Currently, industry belief is that any bacterial contamination of the egg could reduce hatchability.

Previous studies have focused on *in ovo* nutrition as a way to optimize the start in life in chicks or poults. *In ovo* nutrition can come in several forms such as injectable carbohydrates, amino acids, etc. The injectable carbohydrates could be glucosamine, glucose, or other simple sugars. The exogenous injection of carbohydrates into chicken eggs increased BW and intestinal villi area due to increased villus height (Tako et al., 2004). There was an increased number of goblet

cells in the intestine of chickens given maltose, dextrose and dextrin at 17.5d of incubation (Smirnov et al., 2006). *In ovo* injection of 19 different amino acids did not increase serum amino acid levels in E19 embryos (Ohta et al., 2001). The use of mannanoligosaccarides in chicken eggs at 17d of incubation increased villi area 20 to 32% (Cheled-Shoval et al., 2011). Goblet cells in the villi were increased 20 to 50% higher. Brush border peptidases and isomaltase activity was also increased. These nutritive additives have demonstrated that intestinal development can be increased prior to hatch.

Bacterial colonization of the poultry intestine

The egg has several antibacterial defenses. Ovotransferrin is a an Fe binding protein that has antibacterial properties (Giansanti et al., 2005). Lysozyme, the other major defensive protein, exhibits antibacterial properties against gram-positive and gram-negative bacteria (Pellegrini et al., 1997). After the bird hatches, these defenses are lost and the bird must rely on passive immunity before developing adaptive. As a result, the bacterial profile of the intestine is in flux as the bird ages (Lu et al., 2003). Diet, age of host, disease challenge, and antibiotic administration are factors that can influence the intestinal community.

Born without a mature immune system, chickens must rely on maternal antibodies for protection against pathogens early in life. Exposure to pathogens begins at the hatchery, where birds consume debris and dust that contain *E. coli*. and other disease causing bacteria (Cortés et al., 2004). Once exposed to harmful bacteria, a young bird's growth is reduced (Vila et al., 2009). Antibiotic use, the traditional method of control, is being phased out due to consumer preferences and

concern of disease resistant bacteria (Vila et al., 2009). An alternative to mitigate the effects of pathogenic bacteria is the use of competitive exclusion (CE) cultures. The CE cultures are often called probiotic bacteria or direct fed microbials. Research shows that not all bacteria are harmful to birds and that beneficial bacteria can increase energy utilization from the diet (Muramatsu et al., 1991; Muramatsu et al., 1994). Birds raised in sterile conditions, thus reducing the amount of microflora in the gut, have a reduced intestinal weight and villus length (Maisonnier et al., 2003). Intestinal development is not the only beneficial effect of early establishment of bacteria, as immune function is upregulated as well (Kelly et al., 2007).

The effect of age on bacterial colonization

The intestine of a newly hatched chick or poult is thought to be sterile, but by 24h post hatch, a bacterial population is present in the intestinal tract (Naqi and Lewis, 1970). Traditionally, the amount of bacteria is determined by plating. However, modern molecular techniques have demonstrated that not all bacteria species can be plated (McCracken, 2001). The use of PCR and other modern methods of molecular sampling have demonstrated that a greater diversity of bacteria species exists in the intestine than previously thought (Apajalahti et al., 2004). These modern methods have shown conflicting results as to whether there are bacteria in the intestine of chicks at hatch (Pedroso et al., 2005) or not (Wielen et al., 2002). Though the population diversity and density is unclear at hatch, as the bird ages, the bacterial load increases. Bacterial species diversity reaches a peak, in the poult, between week 9 and 18 (Lu and Domingo, 2008). In chickens, bacterial density peaks at 35d with a concentration of approximately 10^{11} cfu/g of digesta in

the ceca and ileum (Apajalahti et al., 2004). In younger birds, there are predominate bacterial species throughout the duodenum, jejunum, and ileum (Wielen et al., 2002). As the bird ages, these bacteria become more section specific because the microenvironments throughout the gastrointestinal tract are different. These environments affect the bacterial load in each section. The diversity and density of bacteria increases toward the distal parts of the gastrointestinal tract (Brisbin et al., 2008). In the proximal end of the small intestine, the bacterial load is 10^3 to 10^5 cfu/g while the distal portion of the intestine has 10^8 to 10^9 cfu/g. The ceca has bacteria as high as 10^{12} cfu/g (Gong et al., 2002).

Non-host factors that effect intestinal microbiota

There are several factors beyond the host or intestinal section that can influence the bacterial community in the intestine. The diet provides nutrients not only to the host, but to the intestinal bacteria as well. The passage rate of the diet will effect the bacterial community composition (Rozee et al., 1982). When 0 to 21d chickens from organic or conventional production settings were compared, the gastrointestinal tract microbes were not different suggesting that the nutrient intake was more important than management practices (Wise and Siragusa, 2007).

Antibiotic growth promoters have been used in the poultry industry to reduce pathogenic bacteria in the intestine. Virginiamycin increases the number of commensal bacteria in the proximal small intestine, whereas there is a large population shift in the distal intestine (Dumonceaux et al., 2006). Antibiotics increase the percentage of commensal population mostly influencing gram-positive bacteria (Knarreborg et al., 2002). Several broad spectrum antibiotics increase the

total amount of *Lactobacillus* in the intestine and alter the overall bacteria population in the ileum (Torok et al., 2011). Antibiotic use not only reduces the pathogenic bacteria load, but may also select for bacteria that are able to confer a health benefit to the host. With these subtherapeutic uses of antibiotics being phased out, viable alternatives need to be identified.

Probiotics in a non-challenge setting

Lactobacilli and *Enterococci* are often used in probiotics (Patterson and Burkholder, 2003) with some strains having been shown to improve BW and FCR in poultry (Cavazzoni et al., 1998; Jin et al., 1998; Khan et al., 2007). Day of hatch broiler chicks given a probiotic containing *Lactobacillus acidophilus*, *Lactobacillus bifidus*, and *Enterococcus faecalis,* had significantly increased BW compared to those not given a probiotic by 42d although FCR was not different (O'Dea et al., 2006). In a comparison study when chicks were given either *Lactobacilli* or *Enterococci*, both improved BW and FCR compared to control birds, but feed conversion in birds given *Enterococci* was significantly better than the *Lactobacilli* fed birds (Awad et al., 2009). The performance improvements with probiotics may be due to improved intestinal health. Villus height, crypt depth, and villus height to crypt depth ratio (VCR) were improved in the jejunum and ileum of broilers fed probiotics (Chichlowski et al., 2007).

The mechanism behind this increased performance may differ. Probiotics increase the amylase activity levels in broilers when fed for the first 40d of life (Jin et al., 2000). Undigested polysaccharides such as cellulose, xylan, and undigested starches are further broken down by the gut microbiota (Resta, 2009). Microbiota

can ferment these carbohydrates and produce SCFA that can be used by the enterocytes as a primary source of energy (Resta, 2009). Broilers supplemented with *Lactobacillus* cultures for 28d, were found to have reduced abdominal fat, LDL, and total serum cholesterol (Kalavathy et al., 2003). Additionally, total carcass cholesterol of the broiler was reduced 13 to 19% by *Lactobacillus* (Kalavathy et al., 2006).

Lactic acid bacteria

Lactic acid bacteria (LAB) are a large group of bacteria across many species that share many of the same characteristics. Lactic acid bacteria are generally accepted to be gram-positive, usually catalase negative, non-spore forming cocci, and coccibacilli or rods with a DNA base composition of less than 55 molar percent G+C (Klein et al., 1998). Bifidiobacteria is an exception to these rules with a G+C content over 60%. All LAB grow anaerobically, but unlike most anaerobes, they grow in the presence of O_2 as "aerotolerant anaerobes". The term lactic acid bacteria comes from the ability of the group to ferment glucose primarily to lactic acid or to a combination of lactic acid, CO_2 and ethanol. Lactic acid production as a result of fermentation is a characteristic of many bacteria, but the term LAB is reserved for genera in the order *Lactobacillales* and *Streptococcus*.

Lactobacillus

Lactobacilli are one of the most frequently used bacteria in probiotics and have demonstrated competitive exclusion properties with pathogenic bacteria. They also attach to epithelia cells in the intestine and enhance the immune response (Heravi and Kermanshahi, 2011). *Lactobacillus* and other bacteria are often species

specific and inhabit a small area in the gastrointestinal. These bacteria colonize in the small intestine and caeca of chickens, a week after hatch (Heravi and Kermanshahi, 2011). Over 10 strains of *Lactobacillus* have been shown to adhere to the crop and the intestinal epithelium (Jin et al., 1996) of chickens. Dietary inclusion of *Lactobacillus* has been shown to have multiple effects in several avian species. In combination with *Enterococcus* and *Bifidobacteria, Lactobacillus* has been shown to increase the villus height in the intestine of broilers (Chichlowski et al., 2007). Whether as a single strain or as a combination of 12 strains, *Lactobacillus* increased amylase activity in the small intestine of broilers without effecting proteolytic and lipolytic activities (Jin et al., 2000). *Lactobacillus* can be as effective as antibiotics from a growth promoter perspective (Kalavathy et al., 2008). *Lactobacillus salivarius* promoted butyric acid producing bacteria in the broiler ceca, which in turn reduced the Salmonella population (Meimandipour et al., 2010).

L. reuteri is of great interest as a probiotic due its production of reuterin, a bacteriocin where it gets its name (Talarico et al., 1988). Purified reuterin reduced *Listeria* and *E. coli* numbers in contaminated meat (El-Ziney et al., 1999). The membrane-anchored ubiquitin-fold protein allow *L. reuteri* to adhere to the mucus layer in the intestine (Roos and Jonsson, 2002). Once adhered, the bacteria can block receptor sites from pathogenic bacteria. *L. reuteri* has developed host specificity, so producers using it as a probiotic must select a strain from the host species or a closely related species to ensure maximum effect (Frese et al., 2011).

Bifidiobacteria

Bifidobacterium are gram-positive anaerobic, non-motile, non-spore forming bacteria (Petr and Rada, 2001). *Bifidobacteria* are found in the intestinal tracts of humans and many other animal species and have history of safe consumption. The ubiquitous nature of the bacteria has led to many companies and researchers to use it as a probiotic (Picard et al., 2005). The bacteria has been proven to be safe for human consumption even in immune-compromised scenarios (Borriello et al., 2003). *Bifidobacteria* can be found in several places throughout the gastrointestinal tract of poultry. In laying hens, *Bifidobacteria* has been found in the crop (Petr and Rada, 2001) and in the ceca of poultry (Rada, 2000; Thitaram et al., 2005b).

Bifidobacteria can become dormant during storage, allowing for better shipping and storage (Saarela et al., 2005). The actions of *Bifidobacteria* are varied. Several researchers have demonstrated that *Bifidobacteria* is capable of adhering to intestinal cells (Crociani et al., 1995; Gopal et al., 2001; Servin, 2004). The adhesion to intestinal cells allows for a barrier to prevent pathogenic bacteria from interacting with intestinal receptor sites. The bacteria may also bind to the mucus secreted from intestinal cells (Servin, 2004). Adhesion comes from several forces including passive, electrostatic interactions, hydrophobic steric forces, lipoteichoic acids; and specific structures, such as lectin-covered external appendages (Servin, 2004). *Bifidobacteria* may alter the immune response of chickens. Depending on the age of bird and route of administration, research has demonstrate that the systemic immune response can be altered by *Bifidiobacteria* inclusion in the diet (Haghighi et al., 2005).

In poultry, the population of *Bifidiobacteria* within the intestinal tract can be altered though several mechanisms. The most effective method is to supplement the diet or drinking water with the bacteria themselves. Dietary additions of certain sugars, such as oligosaccharides and isomaltooligosaccharide increased *Bifidobacteria* in the ceca of chickens (Thitaram et al., 2005a). The bacteria have the ability to ferment and digest these sugars as a food source. Since these carbohydrates are indigestible to the host, breakdown of the sugars is a benefit to both the host and the commensal bacteria (Williams et al., 2009). Certain pathogenic bacteria can utilize indigestible starches as a food source as well, thus by increasing the commensal bacteria load within the intestine of the host, there is a reduction in food sources for pathogenic bacteria.

Enterococcus

Enterococcus has a long history of study and undergone several name changes. The name "enterocoque" was first used by Thiercelinina paper published in 1899; the name was used to emphasize the intestinal origin of this gram-positive diplococcus (Murray, 2003). The name *Streptococcus faecalis* (faecalis, relating to feces) was given by Andrewes and Horder (Murray, 2003). They isolated the organism from the intestine of a patient with endocarditis and renamed the bacteria *Streptococcus faecalis*. More modern DNA techniques have distinguished enterococcus from streptococcus and thus a new genus was formed.

Enterococcus is another lactic acid bacteria which are non-spore forming, but tolerant of a wide variety of temperatures, pH conditions, and high salt concentrations. *Enterococcus* species have been used in several animal species as a

probiotic to offset the effects of several pathogenic bacteria. *Camplyobacter spp.* and *Clostridium spp*. were found in dogs fed *E. faecium* (Vahjen and Männer, 2003). Inclusion in the diet reduced sow mortality during an *E. coli* infection (Taras et al., 2006). *Enterococcus* can be ingested and survive in the gastrointestinal tract of humans (Lund et al., 2002)

In poultry, *Enterococcus* has mainly been used to combat the effect of Salmonella in several species of poultry. *Enterococci* are effective against *Salmonella pullorum* if administered prior to infection but not as a therapeutic agent (Audisio et al., 2000). *E. faecium* produces bacteriocins that inhibit the growth of Salmonella on media (Audisio et al., 1999). When fed to broilers, villus height was increased and FCR was improved (Samli et al., 2007). In turkey poults, dietary supplementation with *Enterococcus* increased the amount of lactic acid bacteria in the small intestine (Vahjen et al., 2002). In combination with other probiotic species, *Enterococcus* improved growth and antibody production (Kabir et al., 2004).

Pediococcus

Pediococcus, another potential probiotic strain, is a gram-positive bacteria, able to grow in various environmental pH ranges, temperatures, and osmotic pressures, and thus able to colonize and inhabit digestive tracts (Klaenhammer, 1993). A common *Pediococcus* strain used is *acidilacti*. This has been demonstrated in several studies to reduce the harmful effect of a coccidial infection (Lee et al., 2007; Taheri et al., 2010). *P. acidilacti* has been used in the production of meat products, such as sausage, to reduce microbial contamination of food (Baccus-Taylor et al., 1993; Yousef et al., 1991). There are several bacteriocins that can be

isolated from *Pediococcus*. The bacteriocin, *Pediococcus acidilacti pediocin* is the most studied, but there are other bacteriocins that not only reduce the growth of listeria, but can cause harm to host cells as well (Villarante et al., 2010). Though bacteriocins harm host cells, the bacteria has been demonstrated to increase survivability of tilapia (Ferguson et al., 2010). In poultry, *P*. *acidilacti* in a combination with other bacteria improved the growth of broilers (Mountzouris et al., 2007). In laying hens, *P. acidilacti* supplementation decreased the amount of broken eggs and reduced yolk cholesterol (Mikulski et al., 2012).

Bone development and probiotics

Understanding the skeletal system's development is important for any poultry operation. Losses from skeletal development cost the industry millions of dollars (Cook, 2000). Each avian species must be managed differently due to age and BW when marketed. The turkey doesn't reach full tibia length until around 130d whereas the broiler reaches full tibia length in under 50d (Lilburn, 1994). Just as bone length is different between species, mineralization can be different as well. The amount of bone mineralization regardless of species or age determines the stiffness and flexibility (Seeman, 2008). Bone mineralization responds to load potential, therefore, each bone mineralizes differently depending on the applied loads. The bones of poultry species are approximately 70% mineral (Rath et al., 2000). A reduction in mineralization or mineralization rates can be the cause of several skeletal problems in poultry. Ossification of bone in poultry species begins *in ovo*, but primarily takes place after hatch (Bain and Watkins, 1993) Mineralization in turkeys, as represented by femoral ash, continues to increase from DOH to 20wk,

with a slight decrease from 12 to 16wk (Zhong et al., 2012). Mineralization of tibiotarsus in meat type chickens increases rapidly between 4 and 11d (Williams et al., 2000). Research suggests that the rate of mineralization throughout life is constantly changing.

There are several factors that influence mineralization and bone development. Genetics play an important role in bone mineralization rates in turkeys and chickens with birds selected for the fastest growth tending to have the highest number of skeletal problems (Dibner et al., 2007; Talaty and Katanbaf, 2009; Zhong et al., 2012). Higher stocking densities increase lameness and tibial dyschondroplasia in broilers (Sanotra et al., 2001; Sorensen et al., 2000). The most important factor in bone development that can be altered by producers is diet. Calcium and P are the two most important minerals in bone development. The ratio of Ca:P is important to monitor as variations in the Ca:P ratio of bone will cause alteration in bone mineral crystal structure and consequently mechanical properties (Thorp and Waddington, 1997). If either Ca or P is too high, the availability of the other is reduced (Williams et al., 2004). Calcium deficient diets lead to osteoporosis, especially in laying hens or older birds (Whitehead and Fleming, 2000). Large particulate Ca increases bone mineralization in laying hens (Saunders-Blades et al., 2009). The form and amount of Ca and P can affect the availability of these nutrients to the bird.

A probiotic bacteria effect on Ca metabolism is important for producers to consider when adding probiotics to the diet. Potential mechanisms for probiotic alterations of Ca metabolism maybe an increase in absorptive surface by

proliferation of enterocytes, increased expression of Ca binding proteins, increased releasing of bone modulating factors like phytoestrogen from food or degrading of phytic acid (Peacuterez et al., 2008). A yogurt containing several *Lactobacillus* species increased apparent Ca absorption and bone mineral content in rats (Scholz-Ahrens et al., 2007). *In vitro* studies with Caco-2 cells demonstrated that *Lactobacillus salivarius* increased transepithelial Ca transport into the cells (Gilman and Cashman, 2006). Feeding a combination of *Clostridium butyricum* and *Bacillus subtilis* in a low Ca diet (75% of recommended) resulted in similar bone health (length, weight, ash percentage, and bone strength of tibia) for broiler chickens fed the control diet (100% of recommended). Thus, ameliorating the effects of a low Ca diet (Houshmand et al., 2011). Further studies with *Bacillus licheniformis* and *subtilis* in broiler diets, resulted in an increase in thickness of lateral and medial wall of the tibia, tibiotarsal index, and ash percentage (Mutus et al., 2006).

Common practice is to use bacteria derived phytase in the diet to improve P digestibility or bioavailability. There are several bacterial sources for microbial phytase that have proven effective and thus usage has increased over the last 20 years (Kiarie et al., 2013). Bacterial phytase improved P retention and bone mineralization in turkeys (Applegate et al., 2003). The addition of pure strains of bacteria could alter P digestion in the same way. In laying hens, diets supplemented with molasses and *Lactobacillus* had increased phytase activity and P retention (Nahashon et al., 1994). Inclusion of phytase-producing *Mitsuokella jalaludinii* from rumen of cattle increased tibial ash in broiler chickens (Lan et al., 2002). Due to the variety of strains and implementation method used, which strains or time of

administration would be ideal to improve bone health in poultry is unclear.

Glucose Transporters

Feed is the highest cost to any livestock operation, and understanding nutrition is key to optimal performance. Understanding the body's ability and capacity to absorb nutrients is a key factor in designing and implementing diets. Nutrient transporters affect the ability of the body to carry nutrients from the lumen into the blood stream. Glucose is an almost ubiquitous energy source for all animals. The poult embryo begins to break down the yolk early in development. Compared to mammals, birds have a higher blood glucose concentration throughout life (Akiba et al., 1999; Tokushima et al., 2003). Glucose can be detected as early as E4, in chickens; therefore, nutrient transporters must exist at that time to allow for absorption into the body (Hazelwood, 1971). Further evidence of the embryo's ability to digest sugars is in the levels of digestive enzymes. Detectable levels of disaccharidases are seen as early as E9. Other digestive enzymes such as carboxypeptidase A and chymotrypsin are seen at E16 (Brisbin et al., 2008; Marchaim and Kulka, 1967). The enzyme activity level of sucrase, maltase and aminopeptidase increases two days prior to hatch in chickens (Uni et al., 2003). As the body develops methods to breakdown simple sugars, the body must also develop nutrient transporters to allow these nutrients into the cell. In chicken embryos, aminopeptidase, sodium-glucose cotransporter-1 (SGLT-1), is found on E19 whereas detectable levels of mRNA expression was detected as early as E15 (Haller et al., 2000; Uni et al., 2003). Though there are several detectable nutrient transporters seen throughout development, the one class that is most essential to

glucose is the GLUT or solute carrier(SGLT) family. Glucose transport is done through several mechanisms but is mostly facilitated by GLUT and SGLT transporters. There are several GLUT isoforms in poultry (GLUT 2, 3, and 8) (Kono et al., 2005). The most common GLUT transporter is GLUT1. The GLUT4 glucose transporter in other species has not been identified in birds.

The diet plays an important role in regulation of GLUT2. The ability of GLUT2 to translocate to the plasma membranes of islet cells is inhibited in rats fed high energy diets for a week (Reimer and Ahrén, 2002). In rats, mice, and sheep, intestinal glucose transport increases in about 1 to 3d after a switch to a high-carbohydrate diet (Ferraris, 2001). Low salt diets reduced the presence of GLUT2 in the enterocyte membranes of White male leghorns (Garriga et al., 2000). Dietary ingredients such as flavonoids in fruit inhibit the expression of GLUT2 *in vitro* (Kwon et al., 2007). Dietary contaminates such as the mycotoxin Cytochalasin B inhibits GLUT2 expression (Ferraris, 2001).

There are several environmental factors that affect GLUT expression. The age of the birds affects expression. The expression of GLUT2 in the intestine of broilers increases linearly from E20 to d14 (Gilbert et al., 2007). The body's physiological reaction to a meal regulates GLUT2, regardless of age. Insulin causes GLUT2 to retreat from the apical and basolateral membrane of enterocytes, thus reducing blood sugar (Kellett et al., 2008). In challenge conditions, expression is altered. Bacterial infection of brush border cells reduces GLUT5 expression *in vitro* (Lievin-Le Moal et al., 2002). The previous studies on glucose transport highlight the understanding of glucose transport in other species, yet there is a need to better

understand transport in turkeys.

Peptide transporters

Protein is a key nutrient in the building of muscle and other body tissues. The source of protein and amino acids mainly comes from the diet. A large portion of the dietary amino nitrogen is absorbed as oligopeptides rather than as free or single amino acids (Ganapathy et al., 1994). A key oligopeptide transporter is PepT1, which is H^+-dependent. PepT1 is the main peptide transporter in the intestine of many animals and potentially can transport all 400 di and 8000 tripeptides that combine to form 20 different dietary AA (Daniel, 2004). In poultry, PepT1 is located in multiple tissues, but distribution is greatest in the small intestine, kidney, and the ceca (Chen et al., 1999).

There are several factors that influence the presence of PepT1 in the intestine. Age is important as the expression of PepT1 is seen as early as E23 in turkeys, and there is roughly a 3-fold increase in expression beginning 5d prior to hatch (Van et al., 2005). Based on PepT1 expression patterns in turkeys, small peptides can be transported across the lumen at hatch (de Oliveira et al., 2009). Expression of intestinal PepT1 increases with enterocyte maturation in mammals (Meredith and Boyd, 2000). Dietary and intestinal environment play an important role as well. PepT1 is pH dependent, and Na and K independent (Van et al., 2005). As crude protein levels increase in the diet, the levels of PepT1 remain unchanged (Frazier et al., 2008). *In vitro* studies demonstrated that specific amino acids or dipeptdies decrease PepT1 expression (Adibi, 2003). Increase in quality of protein or decrease of feed intake, increases the amount of PepT1 in broiler chicks in the

first week (Gilbert et al., 2008). A 24h fast increased the levels of PepT1 in the intestine of rats (Adibi, 2003). These results indicate that the maturing intestine will alter PepT1 regulation depending on both the age and dietary status of the host.

Conclusion

The increased emphasis on early life development of turkeys and the understanding that bacteria play an important role in that development, *in ovo* probiotics need to be explored. Previous work on *in ovo* bacteria has thus far been unable to find a safe and effective bacterial load. There is most likely a bacteria concentration that can be injected to help establish the intestinal microflora without harming the host. The benefits or negative ramifications need to be further studied and understood to determine application to the poultry industry. Therefore, the potential exists for probiotics and commensal bacteria to benefit the host, which could help the bird's transition to feed and resist pathogenic bacteria during the important first week post hatch.

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CHAPTER 1: TURKEY POULT HATCHABILITY IN RESPONSE TO VARIOUS LEVELS OF *IN OVO* PROBIOTICS

ABSTRACT

Turkeys have to overcome several challenges early in life, including exposure to pathogens and failure to transition from yolk nutrition to dietary sources. Probiotics are thought to help alleviate these symptoms in the post-hatch poult, but any beneficial effects, if given prior to hatch are unknown. Additionally, the optimal level of probiotic that can be injected into the egg must be established. The overall objective was to evaluate different probiotic concentrations injected into turkey eggs prior to hatch. Specific objectives were to measure hatchability differences and determine bacterial load in the poult ceca at hatch. Fertile turkey eggs were candled on d25 and allocated to one of six treatment groups: (1) Non-injected eggs hatched in a separate hatcher (positive control), (2) non-injected eggs housed with hatcher with probiotic injected eggs (negative control), (3) eggs injected with 1 ml of saline and placed in the hatcher with probiotic injected eggs (sham control), (4) eggs injected with 10^4 cfu/ml probiotic solution, (5) eggs injected with 10^6 cfu/ml probiotic solution or (6) eggs injected with 10^8 cfu/ml probiotic solution with 9 replicates per treatment and 15 eggs per replicate. All injections were in the amnion. Hatch counts were taken for each replicate and cecal contents collected from two birds per replicate to determine bacterial counts on d28. Cecal contents were plated on media plates with LB agar, MRS agar, Bifidobacteria agar or Enterococcusel agar. Plates were incubated for 24h and colony counts were recorded. Probiotics injected into turkey eggs at 10^8 cfu or lower did not affect hatchability (P<0.05). The

bacterial counts for *Bifidobacteria* were highest among all other bacteria and the injected treatment's bacterial counts were higher compared to the negative control treatment. The bacterial number increased in the negative control birds compared to the positive control suggests that hatchery debris impacts the bacterial load in the intestine. The injection of probiotic bacteria into the egg increased bacterial content in the intestine without reducing hatchability.

INTRODUCTION

The EU implementation of an antibiotic ban has left the poultry industry searching for an efficacious and consumer accepted alternative (Pugh, 2002). Probiotics have demonstrated the ability to improve performance ~~in~~ during a disease challenge and because of their help during a challenge may be a suitable alternative to antibiotics (Grimes et al., 2008; Guarner and Malagelada, 2003; Higgins et al., 2010). Probiotics optimal administration period is reported as a 24 h minimum prior to the onset of the challenge (Audisio et al., 2000). The initial challenge for a newly hatched chick or poult can occur on the day of hatch (Craven et al., 2001; Rodgers et al., 1999); therefore, the probiotic administration should occur *in ovo*.

The poultry industry has used *in ovo* technologies since 1980 with the introduction of Marek's vaccine (Sharma, 1987). The technology is widely used to vaccinate embryos prior to hatch, thereby improving the viability or health status of the chick post-hatch (Goyal and Patnayak, 2004; Johnston et al., 1997; Ricks et al., 1999). More recently, *in ovo* nutrition has improved intestinal development and

energy stores in the first week chick or poult (Uni et al., 2005). Although the studies demonstrate an impact at day of hatch, the effect does not always improve the first week livability.

One main factor in early poult mortality is the transition from a gluconeogenic state relying primarily on lipid based yolk stores to a carbohydrate and protein dense diet post-hatch and an immature intestine that is not equipped to handle it (de Oliveira et al., 2013; 2009). Commensal bacteria have been shown to improve intestinal maturation and are instrumental in the development of the intestine (Grimes et al., 2008; Guarner and Malagelada, 2003; Higgins et al., 2010; Hooper et al., 2002). Probiotic bacteria, which often originate from commensal bacteria, have the ability to improve intestinal maturation post-hatch and alter intestinal colonization (Audisio et al., 2000; Matur and Eraslan, 2012). The early maturing intestine, in turn, could adapt to feed quicker, improving performance. The injection of *in ovo* probiotics could hasten the maturation of the intestine or could overwhelm the embryo and reduce hatchability. The commercial bird's immune system isn't functional until after the first week (Bar-Shira et al., 2003; Craven et al., 2001; Rodgers et al., 1999). The added bacteria, though non-pathogenic, wouldn't be recognized and the host and bacteria interaction could result in death or the bacteria could reduce the pathogenic bacteria in the intestine. Previous work has shown that injection of bacteria or bacterial contamination in the egg, reduce hatchability or increase mortality in the first week post hatch (Cortés et al., 2004; Sharma and Burmester, 1982). Therefore, the study's objective was to determine the effect of a commercial probiotic injected *in ovo* on poult hatchability and cecal

bacterial load at hatch. The first hypothesis is that *in ovo* probiotic injection will maintain or increase hatchability in turkey eggs compared to control eggs. The second hypothesis is that *in ovo* probiotic injection will increase poult cecal bacterial counts at DOH compared to control birds.

MATERIALS AND METHODS

All procedures were approved by Michigan State University Institutional Animal Care and Use Committee. The approval number was AUF # 11/10-190-00.

Probiotic preparation

The microencapsulated probiotic contained 2 strains of *Lactobacillus reuteri*, and a single strain of *Enterococcus faecium*, *Bifidobacteria animalis* and *Pediococcus acidilacti* (Biomin, Herzogenburg, Austria). The probiotic mixture was suspended in sterile PBS, and LB media was plated prior to solutions being made to determine concentration. The probiotic mixture was weighed and added to sterile PBS (25 ml) the day of the sample injection (E25) and diluted to create solutions of 10^8 cfu/ml, 10^6 cfu/ml and 10^4 cfu/ml. Solutions were stored on ice (~4h) and vortexed for 15s immediately prior to egg injection.

Bird incubation and treatments

One thousand turkey fertile eggs were individually weighed, set in an incubator (Petersime Model 5; Petersime Incubator Co., Gettysburg, PA), and incubated for 25d. Egg weights were within 0.5 g average per replicate. Incubator conditions (37.5 °C dry bulb and 29.2°C wet bulb) were monitored throughout the trial using General Tools H10 data loggers (General tools, New York, NY). All eggs

were candled on E24; infertile eggs were removed, and a line was drawn at the lowest point of the air cell. On injection day, E25, externally pipped eggs were removed from the study. The remaining eggs were randomly assigned, using the experimental animal allotment program, to six treatments based on initial egg weight (EAAP, 2009). The treatments were: (1) Non-injected eggs hatched in a separate hatcher (positive control), (2) non-injected eggs housed in the hatcher with probiotic injected eggs (negative control), (3) eggs injected with 1 ml of saline and placed in the hatcher with probiotic injected eggs (sham control), (4) eggs injected with 10^4 cfu/ml probiotic solution, (5) eggs injected with 10^6 cfu/ml probiotic solution or (6) eggs injected with 10^8 cfu/ml probiotic solution with 9 replicates per treatment and 15 eggs per replicate. A treatment replicate was removed from the incubator, and the large/blunt end of each egg wiped with an alcohol disinfectant wipe. A Dremel stylus rotary tool (Model # 1100-01: Dremel, Racine, WI) was used to drill a hole into the lowest point of the egg's air cell as marked on the previous day. An Allflex MR2 repeater syringe (Allflex USA INC) equipped with a 22 ga 1" needle was used to inject each egg with 1 ml of probiotic solution (10^4, 10^6, or 10^8 cfu/ml) or a sham injection of sterile saline. The repeater syringe was rinsed with sterile saline between each replicate and the needle replaced. All drilled holes were sealed with paraffin wax, and each replicate was assigned to one of two hatchers. Two treatments (negative control and sham control) were not drilled or injected but rather each replicate was removed from the incubator, wiped with an alcohol wipe and left at room temperature for the same amount of time as the other replicates. The rest of the treatments were placed in pedigree baskets and randomly placed in

one of two Surepip (Agro Environmental Systems Inc., Dallas, Georgia, USA) hatchers.

Sampling

The hatchers were opened and hatch counts recorded on E28. A poult was considered hatched if the poult had completely cleared the shell; pipped if any portion of the shell was cracked but the poult failed to clear the shell; unhatched if the shell was completely intact. All poults were removed from the hatcher one replicate at a time and euthanized. Body weights were recorded on each hatched poult. For cecal content collection and yolk sac collection, two birds per replicate were euthanized one at a time and the whole body of each bird was dipped in an antiseptic solution. The bird was removed from the antiseptic solution and placed on a clean disinfected cutting board. The abdominal cavity was opened with scissors that had been dipped in alcohol and flame dried. Gloves were changed after the abdominal cavity was opened to prevent contamination from the outside of the bird. Using two sets of forceps, the yolk sac and both ceca were extracted from the bird. The yolk sac was placed in a weigh boat and the difference between yolk weight and total body weight was yolk-free body weight. Cecal contents were collected and pooled by gently squeezing the cecal contents into 1.5 mL microcentrifuge tube~~s~~, using the forceps to prevent contamination from gloves. Sterile forceps and gloves were used for each bird to prevent cross contamination. Samples were immediately chilled on ice until ¼ of the poults had been sampled at which point they were taken back to the lab to make dilution series. This was done to prevent prolonged exposure to the ice and to ensure viability of samples for plating.

Cecal content plating

Each cecal sample was a pool from two birds. For the six serial dilution series, 30 μl of each cecal sample was pipetted into 270 μl of sterile PBS. The mixture was vortexed for 15s, and the pipetting procedure repeated to create the next dilution. Next, 50 μl of each serial dilution and the pure sample were pipetted onto the center of each of four different media plates and spread using a L-shaped glass spreader rod. Rods were flamed in alcohol between each use. The four medias used were: Luria-Bertani agar (Acumedia, Lansing MI) for general gram-negative bacteria, Bifidiobacteria agar (HiMedia Laboratories Ltd. Mumbai, India) for *Bifidobacteria* spp, MRS agar (HiMedia Laboratories Ltd. Mumbai, India) for *Lactobacillus* spp. plus *Pediococcus* spp. and Enterococcsel agar (a modified esculin bile agar; Becton Dickinson and Company, Franklin Lakes, NJ) for *Enterococcus* spp. All medias were made according to manufacturer instructions and stored at 15°C prior to being used. Plates were incubated for 24h at 37°C in an incubator (Precision Industries model# 30M,Chicago, IL), removed, and colony counts were made. Only counts from a single plate from the dilution series with approximately 25 to 300 total colonies was selected for counting. The plate was placed on a lightbox and a marker was used to mark counted colonies, and a lab cell counter was used to keep track of the data counts.

Statistical Analysis

All parameters were analyzed using the PROC MIXED analysis of SAS (v 9.3) with the LSMeans procedure. Differences between means were tested using the pdiff option of the LSMeans statement with significance accepted at $P<0.05$.

RESULTS AND DISCUSSION

In ovo administration of probiotics did not affect poult hatchability except the hatchability of 10^8 cfu injected eggs were lower compared to the saline sham treatment (Tables 1, 2). The hatchability of the saline sham treatment was the highest and significantly higher than the 10^8 treatment ($P<0.05$). This might be a result of the sham poults not being as dehydrated as the control poults. If the poult is dehydrated, and needs liquid to enhance hatchability, this might be a simple solution. There were differences in pipping numbers. The saline treatment had lower pipping rates than all the injected treatments ($P<0.05$). The higher percent of pipping may indicate that the bacteria injection shifts the hatching window slightly compared to saline sham treatment. Reason for this shift and if the injection is involved is unknown. There were no differences in number of unhatched eggs, though there was a trend where 10^4 cfu injected eggs to be lower than 10^8 cfu injected eggs ($P=0.08$).

The hatch results of the current study support previous work involving *in ovo* technology showing that in most chicken eggs hatchability was not altered (Goyal and Patnayak, 2004; Johnston et al., 1997; Ricks et al., 1999). Other studies examining *in ovo* nutrition found the that injection of nutrients into the egg will not alter hatchability (Bailey and Line, 2001; Tako et al., 2004; Uni et al., 2005). The confirmation that probiotic injections does not alter hatchability adds a potential reason to use *in ovo* technology.

The injection site may be more important than the substance injected. Most *in ovo* research has been done with injection of bacteria into the air cell through the top of the egg, rather than into the amnion. Injection with a 19 mm needle compared to a 13 mm needle reduced hatchability of broiler breeder eggs (de Oliveira et al., 2013; 2009; Ohta and Kidd, 2001). Contrary to the current study, competitive exclusion cultures injected into the air cell reduced hatchability and injection into the embryo resulted in nearly a complete loss of hatch (Meijerhof and Hulet, 1997). However, *E. coli* contaminated eggs given a *Lactobacillus reuteri* injection had increased hatchability (60%) over controls (46%) (Edens et al., 1997). A single 10^8cfu dose of *Lactobacillus* into the amniotic fluid did not alter hatchability (89%; (Edens et al., 1997). The current study's hatchability numbers were lower than expected, but much higher than the studies where cultures were injected into the small end of the egg, 76% vs. 50%, respectively. Hatch result differences across the studies where bacteria were injected into the amniotic fluid may be due individual strains of bacteria or combinations used in each study.

Table 3 lists the bacterial counts from the cecal contents. Overall, bacterial counts across all treatments were highest in the *Bifidobacteria*, though individual treatments, such as 10^8, may have had higher counts of other bacteria. *Bifidobacteria* had higher counts than both the *Enterococcus* and *Lactobacillus* ($P<0.05$). The general gram-negative bacteria had an intermediate count between *Bifidobacteria* and *Enterococcus* and were not different from any of the other bacterial types. Studies have found that *Bifidobacteria* is present but not the most predominate species in the intestine shortly after hatch (Lu et al., 2003; Scupham,

2007). The *Lactobacillus* and *Pediococcus* spp. are higher than *Bifidobacteria* in the intestine of normal production birds and were expected to be higher in this study (Scupham, 2007). The higher *Bifidobacteria* numbers in this study indicate that normal succession of bacteria in the poult intestine is altered by probiotic injection. The change in bacterial makeup of the intestinal population and the probiotic bacteria presence in the intestine could be indicative of competitive exclusion properties that would benefit the host during an early challenge setting. Further studies are needed to determine if these properties are useful to the poultry industry.

There were treatment differences in the bacteria counts. Bacterial counts were lowest in the negative control treatment compared to all other treatments with an average count of 6.87 $\log_{10}$cfu/ml ($P<0.001$). All other treatments had an approximate value of 9 $\log_{10}$cfu/ml. The 10^4 and 10^6 injected birds had the highest *Bifidobacteria* counts ($P<0.05$). This was true for all injected bacteria expect *Lactobacillus* where 10^6 and 10^8 had the highest numerical counts. There may have been environmental sources of bacteria, besides the injected bacteria and these bacteria could account for the level of bacteria seen in the control birds. In chickens, bacteria can be as high as 10^6 to 10^8 cfu/g of digesta after 24h (Apajalahti et al., 2004). The current results show that levels of 10^6 cfu may be present at hatch or obtained shortly thereafter as all poults had at least 6 $\log_{10}$cfu/ml bacteria on each of the media. The environment is known to influence bacteria population and colonization in poultry (Apajalahti et al., 2004). One source of environmental bacteria may be the hatcher. The negative control treatment birds, hatched in a

separate hatcher, had bacterial growth though less than the sham saline or probiotic injected birds ($P<0.001$). Sham control treatment birds were in the same hatcher as the probiotic and saline injected birds. The difference in the bacterial levels of birds from these differing types of controls indicates that there was an environmental source of bacteria. Hatchery debris and dander floats throughout the hatcher while the birds wait to be transferred. These particles are inhaled or ingested and might be the source for bacteria. The amount of birds or debris needed to increase bacteria in non-injected birds should be determined with future studies. Producers may want to investigate ways to reduce bacterial loads in the hatcher. Fumigation or other aerosol antibiotic treatments may help control bacteria in the environment. Air filtration may be able to remove hatchery debris as birds hatch, prevent access to the birds.

This study demonstrated that probiotics could be injected into the egg without reducing hatchability while increasing bacterial presence in the intestine. These bacteria have shown to improve the health and intestinal function post-hatch (Ghareeb et al., 2012; Giannenas et al., 2014; Mountzouris et al., 2007; 2010). If an increased amount of bacteria from the current study will benefit performance or health of the host is unclear. Future studies are warranted to monitor the *in ovo* injection's effect on performance. This study's results did not support current convention that all bacteria in the egg are harmful to the embryo. Also, this study further provides a basis for future work to fully elucidate the effect of beneficial bacteria have after hatch.

APPENDIX

Table 1.1: Hatchability of turkey poults administered *in ovo* probiotics

Treatment[1]	Hatch (%)[2]	SE	Pip (%)[3]	SE	Unhatch (%)[4]	SE
Positive Control	79.79[ab]	3.20	4.50[ab]	1.50	14.97	3.16
Negative Control	82.54[ab]	3.20	2.33[ab]	1.50	15.13	3.16
Sham Control	87.06[a]	3.20	0.79[b]	1.50	12.15	3.16
10^4cfu/ml	85.61[ab]	3.20	5.29[a]	1.50	9.10	3.16
10^6cfu/ml	83.51[ab]	3.20	5.10[a]	1.50	11.40	3.16
10^8cfu/ml	76.80[b]	3.20	6.20[a]	1.50	17.00	3.16

[a,b] Means within column for each treatment with no common superscript differ significantly ($P<0.05$)

[1] Mean of 9 replicate groups of 15 eggs. The treatments were: (1) Positive control with non-injected eggs in a separate hatcher; (2) Negative Control with non-injected eggs in the same hatcher as the injected treatments; (3) Sham Control with sterile saline injected eggs placed in the same hatcher as the injected treatments; (4) eggs injected with 10^4cfu/ml of probiotic solution (5) eggs injected with 10^6cfu/ml of probiotic solution or (6) eggs injected with 10^8cfu/ml of probiotic solution

[2] Percentage of poults that had hatched (completely cleared the shell) at time of count.

[3] Percentage of poults that had started to hatch (cracked the shell) at time of count.

[4] Percentage of poults that had not started to hatch (shell completely intact) at time of count.

Table 1.2: The hatch weight of turkey poults administered *in ovo* probiotic

Treatments[1]	BW (g)	SE	Yolk Wt (g)	SE	Yolk Free BW (g)	SE	Yolk (%)	SE
Positive Control	62.59	1.11	7.03	0.48	55.56	0.98	11.24	0.63
Negative Control	64.54	1.11	8.52	0.48	56.02	0.98	13.20	0.63
Sham Control	64.50	1.11	7.75	0.48	56.75	0.98	12.00	0.63
10^4 cfu/ml	63.67	1.11	6.89	0.48	56.78	0.98	10.86	0.63
10^6 cfu/ml	63.53	1.11	7.55	0.48	55.98	0.98	11.87	0.63
10^8 cfu/ml	64.34	1.11	8.09	0.48	56.25	0.98	12.43	0.63

[1] Mean of 9 replicate groups of 15 eggs. The treatments were: (1) Positive control with non-injected eggs in a separate hatcher; (2) Negative Control with non-injected eggs in the same hatcher as the injected treatments; (3) Sham Control with sterile saline injected eggs placed in the same hatcher as the injected treatments; (4) eggs injected with 10^4 cfu/ml of probiotic solution (5) eggs injected with 10^6 cfu/ml of probiotic solution or (6) eggs injected with 10^8 cfu/ml of probiotic solution

Table 1.3: Total cecal bacterial populations (log_{10}cfu/ml) as determined by cultivable plate count on four different medias from poults administered various levels of *in ovo* probiotics

Treatment[1]	BIF[2]	SE	ENT[3]	SE	GN[4]	SE	LAC[5]	SE
Positive Control	6.87[b]	0.23	5.52[b]	0.15	8.09[b]	0.20	5.36[b]	0.15
Negative Control	8.97[a]	0.16	8.45[a]	0.15	8.64[a]	0.16	8.58[a]	0.15
Sham Control	8.85[a]	0.26	8.71[a]	0.16	8.83[a]	0.18	8.73[a]	0.15
10^4 cfu/ml	8.92[a]	0.17	8.75[a]	0.15	8.79[a]	0.17	8.69[a]	0.15
10^6 cfu/ml	9.04[a]	0.16	8.85[a]	0.16	8.84[a]	0.17	8.88[a]	0.15
10^8 cfu/ml	8.65[a]	0.23	8.61[a]	0.15	8.52[a]	0.23	8.68[a]	0.15

[a,b] Means within column for each treatment with no common superscript differ significantly ($P<0.05$)

[1] Mean of 9 replicate groups of 15 eggs The treatments were: (1) Positive control with non-injected eggs in a separate hatcher; (2) Negative Control with non-injected eggs in the same hatcher as the injected treatments; (3) Sham Control with sterile saline injected eggs placed in the same hatcher as the injected treatments; (4) eggs injected with 10^4 cfu/ml of probiotic solution (5) eggs injected with 10^6 cfu/ml of probiotic solution or (6) eggs injected with 10^8 cfu/ml of probiotic solution

[2]BIF- Bifidobacteria

[3]ENT-Enterococcosel

[4]GN- Gram-negative bacteria

[5]LAC-Lactobacillus

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CHAPTER 2: TURKEY POULT HATCHABILITY AND LIVABILITY: THE EFFECT OF *IN OVO* PROBIOTICS

ABSTRACT

The injection of probiotics into the egg could provide advantages to the poult post hatch. The primary objectives of this study were to determine the effect of *in ovo* probiotics on poult hatchability and investigate the ability of a probiotic injection to mitigate the impact of delayed feed access on poult performance. Nine hundred ninety fertile commercial turkey eggs were weighed, assigned to one of three treatments, and incubated under standard conditions for 24 days. The first treatment was placed in the incubator on d0 and treatments 2 and 3 were placed 24 and 48h after treatment 1. At E24 for each treatment, eggs were removed from the incubator and assigned to one of three injection treatments: (1) eggs not injected (Negative Control), (2) eggs injected with 1 ml of saline (Control), (3) eggs injected with 10^6 cfu/ml probiotic solution (Probiotic). At E28, for each incubation treatment, hatch counts were recorded within a specific hatch window (12h). On d0, two birds per treatment were euthanized and intestinal contents and tissue collected. All remaining poults in all treatments were housed in one of 40 pens in a brooder and placed on a starter diet for 1wk. Intestinal samples and excreta contents were collected from each replicate pen on d3 and d7. The hatchability and yolk-free hatch weights were not affected by probiotic injection compared to negative control. Poult BW was higher in the control poults at d0 maintaining the difference through d7 (P<0.05). Feed conversion for the probiotic poults was

improved through d3 of life over negative control birds ($P<0.05$), but this effect was lost by d7. While FCR was improved, apparent Ca and P retention was not altered at d3 and d7 by probiotic injection and there was no main effect of probiotic injection on intestinal morphology. In conclusion, *in ovo* probiotics can be safely injected into turkey eggs and improve feed efficiency through the first day three of life.

INTRODUCTION

The United States has approximately 30 million turkey eggs in incubators each month (NASS, 2014). Even with the large demand for poults, the number of commercial hatcheries within the United States is decreasing. Therefore, commercial hatcheries must ship poults farther distances, increasing the time before poults are provided feed and water post-hatch. The main issue is that birds often have no access to feed or water up to 72h after hatch when considering the hatch window, bird handling, and transport time (Belo et al., 1976; Moran, 1989; 1990; Romanini et al., 2013). Fasting at any age for a period of 48h reduces the BW of birds, crypt proliferation and villus area in the duodenum (Geyra et al., 2001a). Fasting during the first 48h post-hatch has been shown to slow intestinal growth, reduce the number of total cells and percentage of proliferating cells in the crypt while increasing numbers of apoptotic enterocytes up to d5 post-feeding (Geyra et al., 2001a; Gaffga et al., 2012). Pchavov and Noy (1993) reported post-hatch poults experiencing a 48h fast had greater than a 10% reduction of BW. Also, poults with a similar delay in access to feed had fewer bacteria in the digesta (Geyra et al., 2001b; Potturi et al., 2005), intestine weight was reduced by 30%, and villus surface area decreased by over 25% after a 72h fast (Smirnov et al., 2004).

Body weight reduction and the regression of the intestine occur simultaneously with other challenges to homeostasis. Soon after the poult arrives on the farm, the poult must transition to a carbohydrate dense diet from the yolk that provided a lipid dense nutrient source. In preparation for the dietary switch the intestine begins to mature. The absorptive surface area increases during this maturation as the villi start to elongate at the end of incubation, and continue to grow after hatch (Uni et al., 2003). Enterocytes in the villi, begin to produce nutrient transporters and digestive enzymes, which facilitates increased nutrient absorption in the intestine (Uni et al., 2003). There are several detectable nutrient transporters seen throughout intestinal development, but the class that is most essential to glucose is the GLUT or solute carrier family. Glucose transport is done through several mechanisms but is mostly facilitated by glucose transporter (GLUT) and sodium glucose linked transport (SGLT) transporters. There are several GLUT isoforms (GLUT1, 2, 3, and 8) in poultry (Kono et al., 2005). The expression of GLUT2 in broiler intestines increased linearly from E20 to d14 (Gilbert et al., 2007). Nutrient transporters help facilitate the absorption of peptides as well as glucose. Age is important in the expression of the peptide transporter, PepT1, reported to be seen as early as E23 in turkeys with an approximate threefold increase in expression observed five days prior to hatch (Van et al., 2005). Based on PepT1 expression patterns in turkeys, small peptides can be transported across the lumen at hatch (de Oliveira et al., 2009).

Commensal bacteria can assist in the development of the intestine. Bacteria in the digestive tract of poultry often adhere to the epithelium helping to prevent

them from being removed from the intestine. In chickens, the mucosal secretions are a nutrient source for commensal bacteria (Smirnov et al., 2005). If bacteria persist for an adequate period of time, they can develop a biofilm on the intestine, thus helping maintain immune homeostasis (Maol and Servin, 2006). Dietary supplementation with commensal or probiotic bacteria benefits the host as well. When both *Lactobacillus* spp. and *Bifidobacteria* spp. were supplemented in the feed, an increase in intestinal villi length and villus height:crypt depth ratio was observed (Altekruse et al., 2006; Chichlowski et al., 2007; Rahimi et al., 2009). Most studies have examined these benefits post hatch. Probiotic bacteria administered prior to hatch may benefit the bird in the same way. Therefore, the objectives of this study were to determine the effect of *in ovo* probiotics on poult hatchability and to investigate the ability of a probiotic injection to mitigate the impact of delayed feed access on poult performance.

MATERIALS AND METHODS

This research was approved by the Animal Care and Use Committee at Michigan State University. The approval number was AUF #05/13-103-00.

Probiotic preparation

The microencapsulated probiotic contained two strains of *Lactobacillus reuteri*, a single strain of *Enterococcus faecium*, *Bifidobacteria animalis* and *Pediococcus acidilacti* (Biomin, Herzogenburg, Austria). To determine the concentration of organisms, the probiotic mixture was suspended in sterile PBS and plated. The four medias used were: Luria-Bertani agar (Acumedia, Lansing, MI) for general gram-negative bacteria, Bifidiobacteria agar (HiMedia Laboratories Ltd.

Mumbai, India) for *Bifidobacteria* spp, de Man, Rogosa and Sharpe agar (HiMedia Laboratories Ltd. Mumbai, India) for *Lactobacillus* spp. plus *Pediococcus* spp. and Enterococcsel agar (a modified esculin bile agar; Becton Dickinson and Company, Franklin Lakes, NJ) for *Enterococcus* spp. To create solutions of 10^6cfu/ml, the probiotic mixture was weighed (0.423g) and suspended in PBS to create 10^8cfu/ml solutions. The solutions were then diluted to 10^6cfu/ml in sterile PBS (45ml) on the day of the sample injection (E25). Solutions were stored on ice (~4h) and vortexed for 15s immediately prior to egg injection.

Birds and Husbandry

Three groups of 330 turkey eggs (990 eggs total) were individually weighed, set in an incubator (Petersime Model 5; Petersime Incubator Co., Gettysburg, PA), and incubated for 24d. The placement of each group of eggs in the incubator was offset by 24h, so that the last group of 330 eggs went in the incubator 48h after the first. All groups were obtained from the same breeder farm and placed in an egg cooler (15°C) for storage prior to incubation; no group was stored longer than 4d. The first group placed in the incubator was labeled 48h, the second group 24h and the last group 0h. Incubator conditions (37.5°C dry bulb and 29.2°C wet bulb) were monitored throughout the trial using General Tools H10 data loggers (General Tools, New York, NY). All eggs were candled on E24, and infertile eggs were removed from the study. The remaining eggs had the lowest point of the air cell marked. On the day of injection (E25 for each group: 25d, 26d, 27d of the trial) any externally pipped eggs were removed from the study. The remaining eggs (n ~ 300/group) were returned to the incubator and randomly assigned, using the experimental animal

allotment program, to three treatments based on initial egg weight (EAAP, 2009). The treatments were: Negative Control (NC; non-injected), Control (CON; 1 mL injection of sterile saline), Probiotic (PRO; 1 mL of probiotic solution at 10^6 cfu/mL). There were five replicates per treatment and 19 eggs per replicate. After randomization, eggs were removed from the incubator one replicate at a time. The large end of the egg that was to be injected was wiped with an alcohol disinfectant wipe. Negative control treatment eggs were left out of the hatcher for the same period of time as eggs in the other two treatments. A Dremel stylus rotary tool (Model # 1100-01: Dremel, Racine, WI), was used to drill a hole into the lowest point of the egg's air cell. An Allflex MR2 repeater syringe (Allflex USA INC) equipped with a 22 ga 1" needle was used to inject each egg with 1 ml of probiotic solution (10^6 cfu/ml). Needles were changed and the repeater syringe was rinsed with sterile saline between each replicate. Following injection, the drilled holes were sealed with paraffin wax. The PRO and NC eggs were placed in pedigree baskets and randomly placed in one of two Surepip hatchers (Agro Environmental Systems Inc., Dallas, Georgia, USA). The CON eggs were placed in a hatcher portion of the Petersime incubator. Hatcher conditions were monitored with data loggers to ensure consistent conditions. Replicates were randomized within the respective hatchers.

Beginning on d26 of the trial, hatch counts were recorded every 12h. The following definitions were used: a hatched poult had completely cleared the shell; a pipped poult had any portion of the shell cracked but failed to clear the shell; an unhatched poult had a completely intact shell. Hatched birds were divided in

separate portions of the pedigree baskets to prevent crossover of the hatch window. Only the birds from the hatch window with the most birds (E27 to E27.5) from all treatments were selected for the trial, all other hatch birds were removed from the study. Therefore, 60 birds per injection treatment/placement combination and 12 birds per individual replicate were used during the trial. At E28 for each placement group (d28, d29, d30 of the trial), final hatch counts were recorded. The birds from the three placement groups were not the same age and hatched on different days. Due to offset placement of the eggs, birds from the 48h and 24h were left in the hatcher for 48h and 24h without food or water to mimic hatch window and shipping conditions. Once placed in brooder battery cages, all birds were given *ad libitum* access to feed and water for seven days. Poults were provided a standard industry starter diet that met or exceeded NRC nutrient requirements (NRC, 1994).

Sample Collection

Hatch day for the 0h eggs, (at d0, d3 and d7 on feed), four poults per replicate were euthanized by cervical dislocation for intestinal sample collection. For cecal content and yolk sac collection, the euthanized bird's whole body was dipped in an antiseptic solution one bird at a time. The bird was removed from the antiseptic solution and placed on a clean disinfected cutting board. The abdominal cavity was opened with sterile scissors, dipped in alcohol and flame dried between each poult. Gloves were changed after the abdominal cavity was opened to prevent contamination from the poult exterior. Using two sets of forceps, the yolk sac and both cecal horns were extracted from the bird. The yolk sac was placed in a weigh boat and yolk-free BW was calculated based on difference between total BW and

yolk weight. Cecal contents, from two of the four poults, were pooled by gently squeezing the contents into a 1.5 mL sterile microcentrifuge tube (Denville Scientific) using the forceps to prevent glove contamination. Sterile forceps and gloves were used for each bird to prevent cross contamination. Samples were immediately chilled on ice until 25% of the poults were collected. Samples were taken to the laboratory to prevent prolonged exposure to the ice and ensure viability of samples for the dilution series.

Intestinal sections approximately 2.5 cm in length were collected from the ascending portion of the duodenal loop and the midway point between Meckel's diverticulum and the ileo-cecal junction of the ileum. Intestinal contents were washed with chilled sterile PBS then placed in 10% neutral buffered formalin and stored until processed. Samples (30 mg) were collected from each intestinal section (duodenum and ileum) proximal to the previous sampling point and placed in RNAlater (Qiagen, Valencia CA) filled microcentrifuge tubes. The microcentrifuge tubes were placed on ice for 4h and afterwards transferred to a -80°C freezer until processed. Two sets of whole pen excreta samples were collected for a 24h period starting on d2 and d6 and ending on d3 and d7, respectively. Excreta contents were immediately placed on ice, and then transported to the laboratory for storage at 0°C.

Cecal content plating

Each cecal sample was a pool from two birds. For the six serial dilution series, 30 µl of each cecal sample was pipetted into 270 µl of sterile PBS and the mixture vortexed for 15s. 30 µl of the resulting mixture was pipetted into 270 µl of sterile PBS and the mixture vortexed for 15s. The pipetting procedure repeated until

the dilution series was complete. After the dilution series was made 50 µl of each serial dilution and the pure sample were pipetted onto the center of each of four different media plates and spread using a L-shaped glass spreader rod. Rods were dipped in alcohol and passed through the flame between each use. The four medias used were: Luria-Bertani agar (Acumedia, Lansing MI) for general gram-negative bacteria, Bifidiobacteria agar (HiMedia Laboratories Ltd. Mumbai, India) for *Bifidobacteria* spp, de Man, Rogosa and Sharpe agar (HiMedia Laboratories Ltd. Mumbai, India) for *Lactobacillus* spp. plus *Pediococcus* spp. and Enterococcsel agar (a modified esculin bile agar; Becton Dickinson and Company, Franklin Lakes, NJ) for *Enterococcus* spp. All medias were made according to manufacturer instructions and stored at 15°C prior to being used. Plates were incubated for 24h at 37°C in an incubator (Precision Industries model# 30M,Chicago, IL), removed, and colony counts were made. Only counts from a single plate from the dilution series with approximately 25 to 300 total colonies was selected for counting. The plate was placed on a lightbox and colonies were marked and a lab cell counter was used to record counts.

Nutrient Transporters

Intestinal samples from each poult were thawed on ice, lysed, and homogenized using the Qiashredder homogenization kit (Qiagen, Valencia, CA). The RNA was isolated using the Qiagen RNeasy kit (Qiagen, Valencia, CA) following manufacturer's instructions. The RNA was eluted in 50 µl of nuclease-free water, and a quality check representative sample (12%) of all the RNA was loaded onto nanochips and run on an Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto,

Calif) to determine purity. Samples had a RIN number above 8. The cDNA was synthesized from 500 ng of RNA using the high-capacity cDNA reverse transcriptase kit (Applied Biosystems, Foster City, CA). Real-time PCR reactions were performed with SYBR Green Master mix and an Applied Biosystems 7300 system at 50°C for 2min, 95°C for 10min, 40 cycles of 95°C for 15s, and 60°C for 1min. The RNA test samples were run with each primer set (Table 1) to test for genomic DNA contaminations. Samples were run in triplicate and a Ct value of seven or more cycles fewer than the cDNA sample were accepted. Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method with β-actin and TATA binding protein as an endogenous control (Livak and Schmittgen, 2001). The average ΔCt of the control samples was used to calculate ΔΔCt values. ΔΔCT was calculated as follows: $\Delta\Delta CT=(CT(target,control)-CT(ref,control)-(CT(target,treatment\ or\ placement)-CT(ref,\ treatment\ or\ placement))$. Samples were only compared to samples from birds of the same age.

Morphology samples

Each fixed intestinal tissue was cut into 10 mm sections and placed into a tissue cassette. The tissues were processed by dehydration through a series of graded alcohols, cleared with xylene, and embedded in paraffin. A paraffin section from each bird (5 μm thickness) was mounted onto slides. Slides were stained with hematoxylin and eosin (HE) stain. Measurements of villi height (VH: villus tip to crypt opening) and crypt depth (CD: crypt opening to the base of the crypt) were made using Nikon NIS Elements software. The intestinal section from each bird was measured, with three villi and three crypt measurements taken on each of the

sections. The ratio of VH to CD (VCR) was calculated from these measurements. The pen average of the group of four birds was used for statistical analysis.

Excreta samples

On d2 and d6, feed and excreta pans from each pen were removed, cleaned, lined with paper, and replaced. Twenty-four hours later, excreta was collected, and placed in plastic bags. The total collection during this time period was used for determination of apparent nutrient and energy retention. All samples were freeze-dried and ground using the Cyclotec Sample Mill 1093 (FOSS North America, Eden Prairie, MN) with a 1 mm screen to achieve a uniform grind. Approximately 0.4 g of dried ground sample was weighed into a digestion vessel to which 10 mL of 70% nitric acid (Omni Trace, EMD Chemicals, Inc., Gibbstown, NJ) was added. The sample and acid solution was allowed to sit overnight (~16h) at room temperature to predigest the sample. The following day, vessels were placed in the microwave digester, with the digestion program: 1200 watts with a 30min ramp to 160°C under pressure of 190 PSI, hold samples for 10min, and cool down for 5min. After additional cooling in a fume hood for 10min, vessels were vented, and 2 ml of 30% hydrogen peroxide (Sigma-Aldrich, St. Louis, MO) was added. The sample digest was then transferred to a 25 ml volumetric flask and allowed to cool completely; double deionized (ddi) H_2O was added to bring the volume to 25 ml. Samples were transferred to 50 ml polypropylene tubes and stored at room temperature until Ca and P analysis was performed. All glassware used in the digestion process was acid washed using 30% nitric acid and rinsed with ddi H_2O to remove any residual minerals.

Calcium assay

Calcium concentration was determined by atomic absorption spectroscopy. The microwave digested samples were diluted 1:100 in a 1% $LaCl_3$ (lanthanum chloride, Sigma-Aldrich, St. Louis, MO) solution. The $LaCl_3$ acts as an ionization suppressant to eliminate the phosphate interference, which occurs in an air-acetylene flame. Excreta and feed samples were analyzed for Ca using an AA7000 Series atomic absorption spectrophotometer (Shimadzu Scientific Instruments, Inc., Columbia, MD). These samples were analyzed against a five point, matrix-matched standard curve (Ca standard source: VWR International, West Chester, PA) ranging in concentration from 1 to 5 µg/ml Ca. Peach leaves, the organic standard, (NIST, Gaithersburg, MD) were simultaneously analyzed to maintain instrument accuracy. Apparent calcium retention was reported as mg of Ca in excreta per mg of Ca in feed.

Phosphorus assay

Spectrophotometery was used to determine P in the microwave digested samples. Phosphate ions react with molybdate complexes, which in the presence of a reducing agent (Elon), are converted to molybdenum-blue to be measured in a spectrophotometer (Kaplan and Pesce, 1989). The MS solution contained 2.5 g of molybdate (MS), 7 ml of sulfuric acid, and ddi H_2O to bring the final volume to 500 ml. The Elon solution contained 1.5 g of sodium bisulfate, 0.5 g of Elon, and ddi H2O to make the final volume 50 ml (Gomori, 1942). To analyze the microwave digested sample, two mililiters of sample were diluted 1:10 with ddi H_2O. Standards were made by diluting 15 mg/dL stock solution of P (Phosphate (P) Standard, 1000 ppm, LabChem, Inc., Pittsburgh, PA) with ddi H_2O to make 5, 4, 3, 2, 1.5, 1, and 0 mg/dL

(Gomori, 1942). Into a 96-well microplate, 50µl of each standard and sample were pipetted, in duplicate. Two hundred and fifty microliters of MS solution and 25 µL of Elon were pipetted into each well. The plate was incubated for 45 min at room temperature on a plate shaker (Brinkmann TiterMix 100) at 600 rpm and read at 700 nm on the plate reader (SpectraMax 384, Molecular Devices). The SpectraMax determined the P concentration of all samples. The measurement was then adjusted by DM and amount of sample used in the microwave digestion to yield the final concentration. Apparent phosphorus retention was reported as mg of P in excreta per mg of P in feed.

Energy

Ground feed and excreta samples were formed into 1.0 g pellets using a Parr pellet press modified with a hydraulic jack, and the amount of energy in each sample was determined via bomb calorimetry following manufacturer instructions (Parr Instrument Co). The sample pellets were placed into a capsule that was placed in the loop of the bomb head. A Parr 45C10 Fuse Wire (34 B&S gage, nickel-chromium resistance wire) approximately 10 cm in length was attached to the two fuses of the bomb head and bent to touch the sample pellet. The bomb head was secured into the bomb cylinder. The bomb was filled with oxygen to bring the pressure to 32 atm. The bomb was placed into a calorimeter bucket filled with 2000 ml of diH_2O and placed into the calorimeter. The two ignition wires were pushed into the terminal sockets on the bomb head. The water temperature of the calorimeter was allowed to reach equilibrium and the temperature recorded. An electrical surge was sent down the terminals into the bomb igniting the oxygen and combusting the sample. A

second temperature reading was taken approximately six min later, after a stabilized temperature had been reached. The remaining portion of the fuze wire was measured and a calorie correction determined from the length. A benzoic acid pellet was used to confirm the standardized values of each bomb. Therefore, the energy was calculated based on the formula below:

$$\text{Heat of Combustion (cal/g)} = \frac{(\text{Ft} - \text{It}) \times \text{energy standard of bomb} - (\text{calories of wire burned})}{\text{sample dry wt. (g)}}$$

Ft= Final temperature

It= Initial temperature

Standard= benzoic acid

Dry Matter

Empty crucibles were placed in a constant temperature oven (Yamato DNF 600) at 105°C at least 2h prior to hot weighing. Each crucible was hot weighed and 0.5g of sample was added and weighed, in duplicate, and placed into the crucible. The dry matter content of the feed and excreta was measured by drying the samples at 105°C for 24h.

Statistical Analysis

All parameters were analyzed using the PROC MIXED analysis of SAS (v 9.3) with the LSmeans procedure PROC MIXED in SAS PC Windows Version 9.2 software, (SAS Institute Inc., Cary, NC). The model ($y = \mu + \tau_i + \beta_j + \tau\beta_{ij} + \epsilon_{ijk}$) included the main effects of treatment (τ) and delayed access to feed (β) as well as the interaction

between treatment and delay ($\tau\beta$). Interactions are presented as a comparison of single treatments across various placements. Comparisons across time were not used in the model. Pen or pedigree basket was used as the experimental unit for all measurements, except nutrient transporters which were analyzed on a per bird basis. Differences between means were tested using the Pdiff option of the LSmeans statement with significance accepted at $P<0.05$.

RESULTS

There were no differences in hatchability except in the 48h delayed treatment, where the control treatment had a higher hatchability than negative control and probiotic birds (Table2; $P<0.05$). There were no differences in hatchability between negative control or probiotic poults at any time point. As expected, the percent of unhatched and pipped eggs mirrored the hatchability, with the control treatment having a lower percent of unhatched eggs than probiotic and negative control treatments at 48h. Though there was no difference in the percentage of hatched or unhatched, the 0h control injected birds had a higher pip count than the negative control and probiotic injected birds ($P<0.05$). There was also no difference in mortality (data not shown).

The counts for all bacteria were one to two logs higher in the probiotic treated eggs compared to the other treatments (Table 3; $P<0.001$). The 48h-delayed poults had higher *Bifidobacteria and Lactobacillus* counts than the 0h birds ($P<0.05$). The 48h-Control birds had higher *Bifidobacteria and Lactobacillus* counts than the 0h- and 24h-Control birds. The 48h-Probiotic treatment combination had higher

number of *Enterococcus* colonies than the 0h- and 24h- times with probiotic injections.

The yolk weight of the 48h-negative control poults were not significantly lower than the 24h-negative control poults, whereas the 48h-control and probiotic birds were compared to their respective injections (data not shown). The 48h-control and 48-probiotic birds had a larger increase in BW from d0 to d3 than the 48h-negative control birds (Figure 1; $P<0.05$). There was a main effect of delay on BW as the highest BW was measured in the 0h delayed treatment on d0 (Table 4; $P<0.05$). The BW was influenced by yolk disappearance, as the yolk was reduced by approximately 3g/d across the delayed placement treatments. The control injected birds had a higher BW through d7 posthatch ($P<0.05$). There was no difference in BW between negative control and probiotic injected birds throughout the trial. The percent yolk and the overall residual yolk or yolk free BW was not ($P>0.05$) different amongst any of the injection treatments. The yolk disappearance was higher in probiotic (6.8g) and control injected birds (7.2g) over the 48h in the hatcher compared to negative control (4.8g).

Feed conversion from d0 to 3 was highest in birds from the non-injected negative control eggs compared to birds from eggs given a probiotic injection (Figure 2; $P<0.05$), but there were no difference from d3 to7. The overall FCR (d0 to 7) was improved in the 24h- and 48h-negative control poults compared to the 0h-negative control poults. This was the same in the control, but there were no differences amongst the various placement groups for the probiotic poults. The 24h

and 48h delayed placement poults had better FCR than the non-delayed birds (Table 5; $P<0.001$) at d3 and d7.

There was no interaction between injection and placement on apparent Ca and P retention in young poults (Figure 3; $P=0.81$), and there were no main effects of injection ($P=0.77$). The delayed placement on feed by 48h increased P digestibility over 0h poults at 3d of age ($P=0.03$), but was lost by d7. The results were not compared overtime but the numerical mean average retention for both minerals were higher in the d7 birds. There were no differences in energy at either d3 or d7 (Table 6).

There were differences in duodenal and ileal morphology (Table 7, 8). There were no differences in villus height, crypt depth or ratio due to *in ovo* injection in the duodenum or ileum at d0 and d7. However, birds placed on feed immediately after hatch had shorter villi and as a result lower villus height crypt depth ratio (VCR) in the duodenum and ileum than those offered feed at 24h or 48h ($P<0.02$). The increased VCR, but not height, was maintained in the duodenum of the 48h and 24h birds on d7 ($P=0.006$). These differences were not seen on d7 in the ileum. The ileal VCR was higher in the 48h-probiotic birds compared to the 0h- or 24h-probiotic birds on d0 and 0h-probiotic birds on d7 (Figure 4; $P<0.05$). The 0h-negative control VCR was the lowest at d7 in the duodenum (Figure 5; $P<0.05$). The 24h-negative control was highest whereas the 24h-probiotic and 24h-control groups were intermediary to the delay treatments treated with the same injection.

Nutrient transporter levels were highly variable through the study. In the duodenum, birds in the 24h placement treatment that were not injected (negative

control) had a 16-fold increase in PepT1 on d3 compared to 0h-negative control birds (Table 9; $P<0.001$). This increase was not seen at d0 or d7. GLUT2 was increased in both the 0h-control and 0h-probiotic birds over 0h-negative controls in the d0 birds($P<0.001$). There were no differences in duodenal GLUT2 expression on d3 or d7. Both the 0h-control birds had increased ieal PepT1 expression compared to 0h-negative control birds regardless of placement at d0 (Table 10; $P<0.05$). The 24h-probiotic birds had higher ileal PepT1 expression than the 24h-negative control birds. The 0h-probiotic birds had a higher ileal GLUT2 expression than the other treatments on d0 ($P<0.05$), but there were no difference on 3 or d7.

DISCUSSION

There was a varied response to the probiotic injections in the parameters measured. The injection improved performance of the host in some parameters, but did not affect overall performance of the poult. Though the overall hatchability in the current study was lower than expected, the hatchability was still within average for commercial turkey eggs (Schaal and Cherian, 2007). The opening and closing of the hatcher could have contributed to these results, as incubating eggs are sensitive to temperature and humidity fluctuations, which could have contributed to a reduction in overall hatchability. Treatment means were lower than expected but the lack of difference among treatments supports previous research (Chapter 1) demonstrating that probiotics can be safely injected into the egg.

The increased bacterial load in the intestine of the probiotic treated poults indicates that the birds were able to ingest the bacteria and the bacteria remained

viable until DOH. These results were similar to the first study (Chapter 1), where each of the four bacteria types had a 1 to 2 log increase. The performance parameters provide some insight to the overall effect the increased bacteria had on the body. Poults in the control treatment had heavier BWs throughout the trial, yet the reason for this is unknown. One possible cause that can be ruled out is egg weight. Egg weight has been reported to be related to hatch weight and performance (Moran, 1990). Eggs were initially weighed, and egg weights were evenly distributed across all treatments and replicates, so that egg weight was uniform (within 0.5 g) across treatments. The impact of saline on BW is still unclear. With no difference in yolk or percent yolk, and yolk absorption does not appear to be a contributing factor to the differences in BW observed here. The reduction in BW among the delayed placement poults irrespective of injection are in agreement with previous studies (Belo et al., 1976; Moran, 1989; 1990) and validates the 24h difference in age and time off feed. In Chapter 1, there were no differences in yolk weight or yolk free BW. Bacteria were injected into the amnion so that the bacteria could not enter the yolk, thus bacteria should not be able to utilize the yolk as a nutritive source. Turkey poults begin swallowing the amnion late in incubation just prior to internal pipping and should swallow the bacteria (Uni and Ferket, 2004). The injection site and swallowing by the embryo are factors that could contribute to observations that the presence of bacteria do not alter yolk weight or yolk metabolism.

Control poults may have had higher BW because these birds had increased fluid in the egg. The increase in fluid would reduce moisture loss resulting in

increased BW. As previously mentioned, the multiple door openings may have contributed to increased moisture loss of all the eggs. Injection of saline potentially reduced dehydration, however, the probiotic was suspended in sterile PBS and this did not alter d0 BW. Poults injected with 1.5 ml of saline and various levels of protein and carbohydrates had increased BW at DOH in three different studies, yet these BW differences were no longer apparent by d3 or d7 (Geyra et al., 2001a; Foye et al., 2006; Gaffga et al., 2012). These studies contrast the current study where the saline birds maintained BW differences through d7. The injection of nutrients or probiotics could alter metabolism and thus account for these differences, whereas saline may only alter hydration status.

Birds can spend 24 to 48h without access to feed from the time of hatch, but usually this results in dehydrated birds upon arrival at the farm. One concern in regards to long shipment are the decreases in both metabolism and yolk reserves. This could lead to decreased BW and higher mortality. The BW and the yolk reserves were lower in the 48h delay treatment. The decrease in BW should correlate with a decreased VCR as the villus should regress in response to the fast, but in the current study the VCR response was just the reverse. In laying hens, a fast of 24h reduced villus length (Yamauchi et al., 1996; Geyra et al., 2001b; Potturi et al., 2005). Contrastingly in broilers, ileal villus height was unaffected by a 24h fast (Thompson and Applegate, 2006; Kellett et al., 2008). The 48h delayed access to feed reduced BW, but this difference was lost by d7. The increase in VCR for 48h birds in both the duodenum and ileum on 0d may be the reason the poults were able to have a higher BW by d3.

The probiotic egg injection in this study was only able to influence performance through the first three days of life. In the few studies that examined the effect of a single dose of probiotics, no intestinal development post hatch was investigated. The transit time of the bacteria through the intestine could be the reason that bacterial injections had a short duration of effectiveness. In rats, single doses of *Lactobacillus casei*, showed that bacteria have a short adhesion time to the intestine (de Oliveira et al., 2009; Saxami et al., 2012). The bacterial population in the intestine is influenced by diet as well as environment. Though the bacterial counts at d3 or d7 were not examined, the diet could have shifted bacterial populations after the injection.

Poults rely on dietary sources for Ca once the poult hatches. After hatch, there is a tremendous need for Ca to maintain skeletal growth. The Ca and P digestibility was expected to increase in the probiotic injected birds. The poults may have absorbed adequate amount of Ca from the egg and coupled with adequate dietary amounts, intake of Ca may have been at near optimal levels. The amount of Ca in the diet may have been adequate for bird development. Little research has been done on probiotic and Ca in optimally fed animals. Calcium absorption from rats was not altered by probiotics when fed adequate Ca levels (Moran, 1990; Scholz-Ahrens et al., 2007). Body stores of Ca and P were not factored into the digestion calculations, and adequate reserves may have limited Ca absorption. Calcium and P are influenced by one another and the ratio between the two is important. A ratio that is too high or too low can reduce Ca absorption. Our ratio (1.87) as prescribed by the NRC (1994) and the genetic company should be optimal

for the poults at that age. A shift in the ratio may influence the effect of probiotics on P and Ca absorption.

The probiotic treated birds had greater villus height to crypt depth ratio after a 48h delay, therefore, duration of bacterial exposure may impact probiotic efficacy. The extra two day exposure to the bacteria may have been enough to significantly impact the intestine. Four week old female broilers given an intragastric injection of 10^7 cfu of *Lactobacillus spp.* had increased weight d8 later and improved feed conversion over a d29 period (Gilbert et al., 2007; Khan et al., 2007). Chickens and ducks given a single dose of 4 x 10^{10} cfu of *Lactobacillus spp.* at d4 of age had increased BW d56 later (Van et al., 2005; Angelakis and Raoult, 2010). The amount of *Lactobacillus spp.* in the intestine was higher at d60 of age as well. The increase in villi length would result in an increase in absorptive surface area for the intestine. This could explain why a single dose of probiotics can positively influence the performance of poultry.

Nutrient transporters were highly variable amongst all of the sample replicates, but there was an increase in the GLUT2 transporter in the duodenum and ileum of the 0h-probiotic poults over the 0h-negative control birds. An increase in GLUT2 could have led to an increase in glucose from the diet. Increases in GLUT2 expression lead to an increase in energy absorption from the diet in weanling pigs (Gabler et al., 2007; Schaal and Cherian, 2007). Gilbert (2007) demonstrated that GLUT2 increases linearly with age in chickens. The probiotic poults had an improved efficiency through d3 of life. These factors examined together indicate a more mature and more functional intestine in the turkey poult administered

probiotic bacteria *in ovo*. These increases in development could ease the transition to feed and reduce overall feed costs in turkey production.

The influence of probiotic bacteria on the intestine is beneficial to performance. Probiotics improve intestinal morphology after a delayed access to feed; and a single injection into the egg appears to improve FCR for up to three day post injection. Future studies looking at the combined effect of probiotics *in ovo* and probiotics in the feed could produce an even greater benefit. The results indicate that probiotics can safely be injected into the egg and consequently, produce a better performing poult after hatch. *In ovo* probiotics may be a beneficial management practice to adopt since the supplementation of probiotics pre-hatch appears to improve performance early in life.

APPENDIX

Table 2.1: Primers used for real-time PCR on turkey intestinal samples[1]

Gene	Sequence	Tm (°C)	Amplicon length (bp)
Pept1 F	5'-GTTTCTAGCTTGCGGTCGGA-3'	60.4	120
Pept1 R	5'-TCCAAAATCCGTGTCACCCA-3'	59.5	
GLUT2 F	5'-AAAGAGAGTGTCCATCGGGC-3'	59.8	104
GLUT2 R	5'-CGTTGATGCCTGAGAACTGC-3'	59.6	
Beta-actin F	5'-CTGGCCGTGACCTGACGGAC-3'	65.2	238
Beta-actin R	5'-GCCTCGGGGCACCTGAACCT-3'	66.8	
TATA Binding protein F	5'-CACGTGTGTGGCCATTCTTG-3'	60.0	107
TATA Binding protein R	5'-CCACGTTGAAGAGCTCTGGT-3'	60.0	

[1]Primers designed by the NCBI Primer Blast software program (http://www.ncbi.nlm.nih.gov/tools/primer-blast/).

Table 2.2: Hatchability of turkey poults administered *in ovo* probiotics administered on d25 of incubation[1]

	Hatched[2]			Pipped[3]			Unhatched[4]		
	Negative Control[5]	Control	Probiotic	Negative Control	Control	Probiotic	Negative Control	Control	Probiotic
0h[6]	62.1	56.8	62.1	11.6[b]	19.0[a]	12.6[b]	26.3	24.2	25.3
24h	70.3	70.5	64.2	8.4	5.3	10.5	21.2	24.0	25.3
48h	74.7[b]	86.1[a]	68.4[b]	2.1[b]	9.5[a]	9.5[a]	23.2[a]	4.2[b]	22.1[a]
SEM	4.9	4.9	4.9	2.3	2.3	2.3	4.9	4.9	4.9

[a,b] Means within row for each treatment with no common superscript are different ($P<0.05$)

[1] A probiotic mixture of *L. reuteri, B. animalis, E. faecium, and P. acidilacti.*

[2]Percentage of poults that had hatched - completely cleared the shell at 27.5d of incubation.

[3] Percentage of poults that had started to hatch - cracked the shell at 27.5d of incubation.

[4] Percentage of poults that had not started to hatch - shell completely intact at time of count.

[5] Mean of 5 replicates of 19 eggs each. Negative Control - non-injected eggs; control - sterile saline injected eggs; probiotic - eggs injected with 1 ml of probiotic inoculations at 10^6cfu/ml. All treatment groups were in their own separate hatcher.

[6] 0h-poults not held in the hatcher following hatch; 24h-poults held in the hatcher for 24h prior to feed and water access; 48h-poults held in the hatcher for 48h prior to feed and water access. Turkey eggs were offset to ensure all poults were placed on feed and water at the same time.

Table 2.3: Total cecal bacterial populations (log_{10}cfu/ml) from poults administered *in ovo* injections on d25 of incubation as determined by cultivable plate count on four different medias[1]

	Bacteria			
Placement[2]	BIF[4]	ENT[5]	LP[6]	GN[7]
0h	8.24[b]±0.29	7.56±0.29	7.28[b]±0.3	7.96±0.27
24h	8.40[ab]±0.27	7.88±0.27	7.77[ab]±0.3	8.62±0.26
48h	8.81[a]±0.29	8.54±0.29	8.48[a]±0.3	8.91±0.26
P-value	0.05	0.21	0.05	0.23
Injection[3]				
Negative Control	7.69[b]±0.27	7.69[b]±0.27	7.65[b]±0.27	6.99[b]±0.3
Control	8.24[b]±0.29	7.14[b]±0.29	8.32[b]±0.26	7.16[b]±0.3
Probiotic	9.52[a]±0.29	9.15[a]±0.29	9.52[a]±0.26	9.39[a]±0.3
P-value	<0.001	<0.001	<0.001	<0.001

[a,b] Means and SEM within column for each item with no common superscript are different (P<0.05)

[1] A probiotic mixture of *L. reuteri, B. animalis, E. faecium, and P. acidilacti.*

[2]Five replicate groups with a two-bird pool of cecal contents. 0h-poults not held in the hatcher following hatch; 24h-poults held in the hatcher for 24h prior to feed and water access; 48h-poults held in the hatcher for 48h prior to feed and water access. Turkey eggs were offset to ensure all poults were placed on feed and water at the same time.

[3]Negative Control - non-injected eggs; control - sterile saline injected eggs; probiotic - eggs injected with 1 ml of probiotic inoculations at 10^6cfu/ml. All treatment groups were in their own separate hatcher.

[4]BIF- *Bifidobacteria* count

[5]ENT-*Enterococcosel* count

[6]LP-*Lactobacillus* and *Pediococcus* count

[7]GN- Gram-negative bacteria count

Table 2.4: Body weight of turkey poults with either a delayed placement on feed and/or an *in ovo* probiotic injection administered at 25d of incubation[1]

	d0			Total BW (g)		
	Yolk Free BW (g)	Yolk Wt (g)	Yolk[4] (%)	d0[5]	d3	d7
Placement[2]						
0h	53.40±1.70	16.47^{a}±0.57	20.62^{a}±1.08	69.95^{a}±0.43	77.01^{c}±1.82	137.6^{b}±6.91
24h	55.37±1.70	13.61^{b}±0.57	16.45^{b}±1.08	65.15^{b}±0.43	82.38^{b}±1.74	149.77^{ab}±7.17
48h	56.71±1.70	10.53^{c}±0.57	12.12^{c}±1.08	59.49^{c}±0.43	88.09^{a}±1.93	160.15^{a}±7.34
P-value	0.23	<0.001	<0.001	0.003	0.011	0.78
Treatment[3]						
Negative Control	57.30±1.70	12.98±0.57	15.24±1.08	64.24^{b}±0.43	78.74^{b}±1.93	146.71^{b}±7.58
Control	53.33±1.70	13.54±0.57	16.74±1.08	66.44^{a}±0.43	87.09^{a}±1.82	153.28^{a}±6.91
Probiotic	54.85±1.70	14.09±0.57	17.22±1.08	63.91^{b}±0.43	81.64^{b}±1.74	147.54^{ab}±6.91
P-value	0.21	0.45	0.38	<0.001	<0.001	0.05

^{a,b} Means and SEM within column for each item with no common superscript are different (P<0.05)

[1] A probiotic mixture of *L. reuteri, B. animalis, E. faecium, and P. acidilacti.*

[2]Mean of 5 replicate groups of a two bird pool of cecal contents. 0h-poults not held in the hatcher following hatch; 24h-poults held in the hatcher for 24h prior to feed and water access; 48h-poults held in the hatcher for 48h prior to feed and water access. Turkey eggs were offset to ensure all poults were placed on feed and water at the same time.

[3]Negative - non-injected eggs; control - sterile saline injected eggs; probiotic - eggs injected with 1ml of probiotic inoculations at 10^{6}cfu/ml. All treatment groups were in their own separate hatcher.

[4]Yolk weight as a percentage of body weight

[5] Days on feed

Table 2.5: Body weight of turkey poults with either a delayed placement on feed and/or an *in ovo* probiotic injection administered at 25d of incubation[1]

	d0			Total BW (g)		
	Yolk Free BW (g)	Yolk Wt (g)	Yolk[3] (%)	d0[4]	d3	d7
Interaction						
0h-Negative	55.98±2.94	15.90[a]±0.98	19.28[a]±1.91	69.58[a]±0.75	73.66±3.02	136.53±11.49
24h-Negative	58.26±2.94	11.92[b]±0.98	13.71[b]±1.91	64.48[b]±0.75	80.94±3.02	148.00±12.85
48h-Negative	57.65±2.94	11.13[b]±0.98	12.72[c]±1.91	58.66[c]±0.75	81.63±3.90	155.59±14.84
P-value	0.33	0.05	0.001	0.001	0.09	0.31
0h-Sham	52.88±2.94	17.66[a]±0.98	20.37[a]±1.91	70.25[a]±0.75	78.80[b]±3.38	132.56[b]±12.85
24h-Sham	54.01±2.94	14.2[b]±0.98	18.21[a]±1.91	67.12[b]±0.75	87.21[b]±3.02	156.29[a]±11.49
48h-Sham	57.66±2.94	10.43[c]±0.98	11.63[b]±1.91	61.94[c]±0.75	95.04[a]±3.02	170.98[a]±11.49
P-value	0.38	0.05	0.001	0.001	0.001	0.05
0h-Pro	51.35±2.94	15.86[a]±0.98	22.22[a]±1.91	70.03[a]±0.75	78.56[b]±3.02	143.72±11.49
24h-Pro	53.85±2.94	14.73[b]±0.98	17.43±1.91	63.85[b]±0.75	78.75[b]±3.02	145.02±12.85
48h-Pro	54.81±2.94	10.04[c]±0.98	12.01±1.91	57.86[c]±0.75	87.60[a]±3.02	153.86±11.49
P-value	0.47	0.05	0.001	0.001	0.05	0.49

[a,b] Means and SEM within column for each item with no common superscript are different ($P<0.05$)

[1] A probiotic mixture of *L. reuteri, B. animalis, E. faecium, and P. acidilacti.*

[2]Mean of 5 replicate groups of a two bird pool of cecal contents. 0h-poults not held in the hatcher following hatch; 24h-poults held in the hatcher for 24h prior to feed and water access; 48h-poults held in the hatcher for 48h prior to feed and water access. Turkey eggs were offset to ensure all poults were placed on feed and water at the same time. Negative - non-injected eggs; control - sterile saline injected eggs; probiotic - eggs injected with 1ml of probiotic inoculations at 10^6cfu/ml. All treatment groups were in their own separate hatcher.

[3]Yolk weight as a percentage of body weight

[4] Days on feed

Table 2.6: Apparent energy retention of poults who experienced either delayed placement on feed and/or an *in ovo* probiotic injection administered at 25d of incubation[1]

	Apparent energy retention (%)							
	Placement[2]				Injection[3]			
	0h	24h	48h	P-value	Negative	Control	Pro	P-value
d3	4.3±1.0	5.9±1.0	3.7±1.0	0.17	4.2±0.4	5.0±0.4	4.9±0.4	0.77
d7	5.9±1.5	8.3±1.5	8.2±1.5	0.21	6.1±1.0	7.6±1.0	7.1±1.0	0.45

[1] A probiotic mixture of *L. reuteri, B. animalis, E. faecium, and P. acidilacti.*
[2]Mean of 5 replicate groups of a 12 birds per pen each. 0h-poults not held in the hatcher following hatch; 24h-poults held in the hatcher for 24h prior to feed and water access; 48h – poults held in the hatcher for 48h prior to feed and water access. Turkey eggs were offset to ensure all poults were placed on feed and water at the same time
[3]Negative - non-injected eggs; control - sterile saline injected eggs; probiotic - eggs injected with 1ml of probiotic inoculations at 10^6cfu/ml. All treatment groups were in their own separate hatcher

Table 2.7: The main effect of *in ovo* probiotic injection administered at 25d of incubation or time delay to feed on duodenal morphology of young poults[1]

	Injection[2]				Placement			
	Negative Control[2]	Control	Probiotic	P-value	0[2]	24	48	P-value
Duodenum[4] d0								
Villus Height	661.4±29.6	682.8±29.6	661.2±29.6	0.84	597.9[b]±32.8	723.3[a]±32.8	684.1[a]±32.8	0.02
Crypt Depth	64.0±3.3	71.4±3.3	73.6±3.3	0.08	70.7±3.3	68.8±3.3	69.5±3.3	0.91
Ratio	11.1±0.5	10.0±0.5	9.4±0.5	0.08	8.9[b]±0.6	11.2[a]±0.6	10.3[a]±0.6	0.01
Duodenum d7								
Villus Height	1310.2±64.1	1253.7±64.1	1203.0±64.1	0.46	1139.5±61.6	1336.2±61.6	1291.2±61.6	0.06
Crypt Depth	72.4±3.6	78.0±3.6	74.3±3.6	0.51	80.9±3.5	74.9±3.5	68.9±3.5	0.05
Ratio	20.3±1.2	17.2±1.2	17.7±1.2	0.16	15.2[b]±1.2	19.2[a]±1.2	20.8[a]±1.2	0.006

[a,b] Means within column within same group for each item with no common superscript are different ($P<0.05$)
[1] A probiotic mixture of *L. reuteri, B. animalis, E. faecium, and P. acidilacti.*
[2] Mean of 5 replicate groups of 12 birds per pen each. Negative Control - non-injected eggs; control - sterile saline injected eggs; probiotic - eggs injected with 1 ml of probiotic inoculations at 10^6cfu/ml. All treatment groups were in their own separate hatcher.
[3] 0h-poults not held in the hatcher following hatch; 24h-poults held in the hatcher for 24h prior to feed and water access; 48h-poults held in the hatcher for 48h prior to feed and water access. Turkey eggs were offset to ensure all poults were placed on feed and water at the same time.
[4] All measurement in µm
[5] Ratio of villus height to crypt depth

Table 2.8: The main effect of *in ovo* probiotic injection administered at 25d of incubation or time delay to feed on ileal morphology of young poults[1]

	Injection[2]				Placement			
	Negative Control[3]	Control	Probiotic	P-value	0[3]	24	48	P-value
Ileum[4] d0								
Villus Height	278.1±26.2	329.9±26.2	323.7±26.2	0.20	254.4[b]±26.2	316.5[a]±26.2	360.8[a]±26.2	0.01
Crypt Depth	56.9±3.6	58.9±3.6	55.0±3.6	0.70	57.2[ab]±3.6	62.6[a]±3.6	51.1[b]±3.6	0.05
Ratio[5]	5.2±0.6	6.1±0.6	6.5±0.6	0.22	4.5[b]±0.6	5.4[b]±0.6	7.9[a]±0.6	0.001
Ileum d7								
Villus Height	484.2±19.5	503.9±19.5	471.7±19.5	0.46	480.5±20.4	488.2±20.4	491.1±20.4	0.92
Crypt Depth	69.6±2.7	69.8±2.7	74.1±2.7	0.41	74.5±2.9	67.8±2.9	71.1±2.9	0.21
Ratio	7.4±0.5	7.6±0.5	6.8±0.5	0.41	6.9±0.5	7.5±0.5	7.3±0.5	0.21

[a,b] Means within column within same group for each item with no common superscript are different ($P<0.05$)

[1] A probiotic mixture of *L. reuteri, B. animalis, E. faecium, and P. acidilacti.*

[2] Mean of 5 replicate groups of 12 birds per pen each. Negative Control - non-injected eggs; control - sterile saline injected eggs; probiotic - eggs injected with 1 ml of probiotic inoculations at 10^6cfu/ml. All treatment groups were in their own separate hatcher.

[3] 0h-poults not held in the hatcher following hatch; 24h-poults held in the hatcher for 24h prior to feed and water access; 48h-poults held in the hatcher for 48h prior to feed and water access. Turkey eggs were offset to ensure all poults were placed on feed and water at the same time.

[4] All measurement in µm

[5] Ratio of villus height to crypt depth

Table 2.9: The interaction between placement on feed and *in ovo* injection on the relative gene expression of PepT1 and GLUT2 in the duodenum of young turkey poults[1]

	PepT1			GLUT2		
	d0	d3	d7	d0	d3	d7
Interaction[2]	Mean[3]± St.Dev	Mean±St.Dev	Mean±St.Dev	Mean±St.Dev	Mean±St.Dev	Mean±St.Dev
0h-Negative	1.00±1.63	1.00^{b}±1.43	1.00±1.70	1.00^{c}±1.67	1.00±1.46	1.00±1.75
24h-Negative	1.10±1.57	16.26^{a}±1.7	0.15±2.32	0.53^{c}±1.61	1.00±1.75	1.00±2.43
48h-Negative	0.90±1.49	0.56^{b}±2.32	0.32±1.99	0.47^{c}±1.52	1.00±2.43	1.00±2.06
0h-Control	2.25±1.57	1.99^{b}±1.7	0.55±1.81	19.21^{a}±1.61	1.99±1.75	1.06±1.87
24h-Control	2.22±1.52	0.75^{b}±1.49	1.02±1.70	0.50^{c}±1.56	0.05±1.52	2.68±1.75
48h-Control	1.18±1.52	0.84^{b}±1.52	0.35±1.52	0.49^{c}±1.56	0.90±1.56	1.07±1.56
0h-Pro	2.04±1.57	1.11^{b}±1.41	0.18±1.81	8.39^{b}±1.61	1.11±1.44	0.36±1.87
24h-Pro	2.08±1.52	1.80^{b}±1.52	0.19±2.32	0.41^{c}±1.56	0.11±1.56	1.24±2.43
48h-Pro	0.66±1.57	0.60^{b}±1.49	0.67±1.57	0.16^{c}±1.61	0.44±1.52	2.08±1.61
P-value	0.35	<0.001	0.36	<0.001	0.49	0.51

[a,b] Means within column within with no common superscript are different (P<0.05)

[1] Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method

[2] Interaction between duration of delayed placement on feed and in ovo injection treatment. Negative Control - non-injected eggs; control - sterile saline injected eggs; probiotic - eggs injected with 1ml of probiotic inoculations at 10^6 cfu/ml. All treatment groups were in their own separate hatcher.

[3] 0h-poults not held in the hatcher following hatch; 24h-poults held in the hatcher for 24h prior to feed and water access; 48h-poults held in the hatcher for 48h prior to feed and water access. Turkey eggs were offset to ensure all poults were placed on feed and water at the same time.

[4] Mean of 5 replicate groups of 4 birds per pen each.

Table 2.10: The interaction between placement on feed and *in ovo* injection on the relative gene expression of PepT1 and GLUT2 in the ileum of young turkey poults[1]

	PepT1			GLUT2		
	d0	d3	d7	d0	d3	d7
Interaction[2]	Mean[3]± St.Dev	Mean±St.Dev	Mean±St.Dev	Mean±St.Dev	Mean±St.Dev	Mean±St.Dev
0h-Negative	1.00^{b}±1.49	1.00±1.52	1.00±2.21	1.00^{b}±1.64	1.00±1.58	1.00±2.34
24h-Negative	1.87^{b}±1.52	1.95±1.67	1.34±2.21	1.04^{b}±1.68	1.34±1.76	1.79±2.34
48h-Negative	1.40^{b}±1.52	0.56±1.88	6.23±2.65	1.55^{b}±1.68	0.66±1.99	1.05±2.84
0h-Control	4.78^{a}±1.39	2.92±2.07	0.63±2.65	2.36^{b}±1.51	2.40±2.22	0.28±2.84
24h-Control	4.13^{ab}±1.52	1.13±1.61	2.52±1.75	1.39^{b}±1.68	0.76±1.68	0.27±1.83
48h-Control	2.31^{b}±1.47	0.27±1.52	1.26±1.99	1.94^{b}±1.61	0.38±1.58	0.82±2.09
0h-Pro	2.95^{ab}±1.60	1.12±1.49	0.72±2.21	7.90^{a}±1.79	0.56±1.55	0.28±2.34
24h-Pro	6.12^{a}±1.43	0.43±1.67	2.52±1.99	3.06^{b}±1.55	0.60±1.76	1.93±1.93
48h-Pro	1.98^{b}±1.43	0.60±1.61	2.08±1.99	2.61^{b}±1.55	0.48±1.68	0.25±2.09
P-value	0.05	0.28	0.35	0.05	0.61	0.58

[a,b] Means within column within with no common superscript are different (P<0.05)

[1] Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method

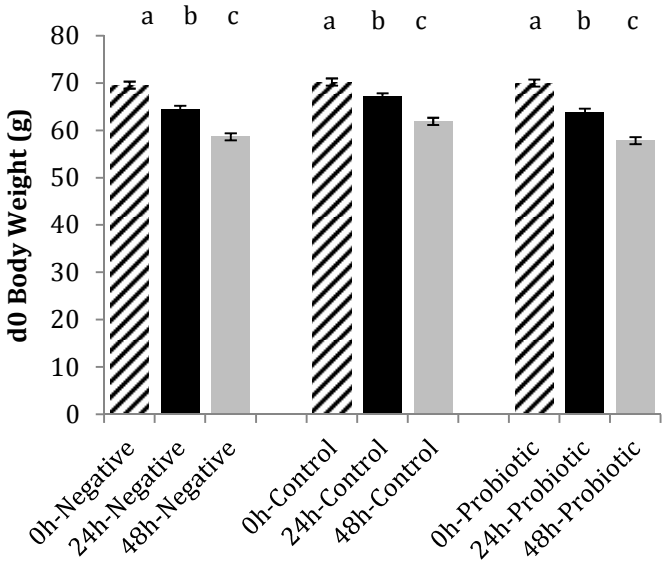
[2] Interaction between duration of delayed placement on feed and in ovo injection treatment. Negative Control - non-injected eggs; control - sterile saline injected eggs; probiotic - eggs injected with 1ml of probiotic inoculations at 10^{6}cfu/ml. All treatment groups were in their own separate hatcher.

[3] 0h-poults not held in the hatcher following hatch; 24h-poults held in the hatcher for 24h prior to feed and water access; 48h-poults held in the hatcher for 48h prior to feed and water access. Turkey eggs were offset to ensure all poults were placed on feed and water at the same time.

[4] Mean of 5 replicate groups of 4 birds per pen each

Figure 2.1: The BW of poults who experienced a delayed placement on feed and with or without an *in ovo* probiotic injection A.) Poults at d0 from either negative control (non-injected eggs), control (sterile saline injected eggs at d25 of incubation) or probiotic (eggs injected with 1 ml of probiotic inoculations at 10^6 cfu/ml at d25 of incubation) eggs than subjected to a 48h, 24h or 0h delay placement at hatch. B.) Poults at d3 from either negative control (non-injected eggs), control (sterile saline injected eggs at d25 of incubation) or probiotic (eggs injected with 1 ml of probiotic inoculations at 10^6 cfu/ml at d25 of incubation) eggs than subjected to a 48h, 24h or 0h delay placement at hatch. C.) Poults at d7 from either negative control (non-injected eggs), control (sterile saline injected eggs at d25 of incubation) or probiotic (eggs injected with 1 ml of probiotic inoculations at 10^6 cfu/ml at d25 of incubation) eggs than subjected to a 48h, 24h or 0h delay placement at hatch. All treatment groups were in their own separate hatcher. 0h-poults not held in the hatcher following hatch; 24h-poults held in the hatcher for 24h prior to feed and water access; 48h-poults held in the hatcher for 48h prior to feed and water access. Turkey eggs were offset to ensure all poults were placed on feed and water at the same time. [a,b] Bars within same group with no common superscript differ significantly ($P<0.05$)

A



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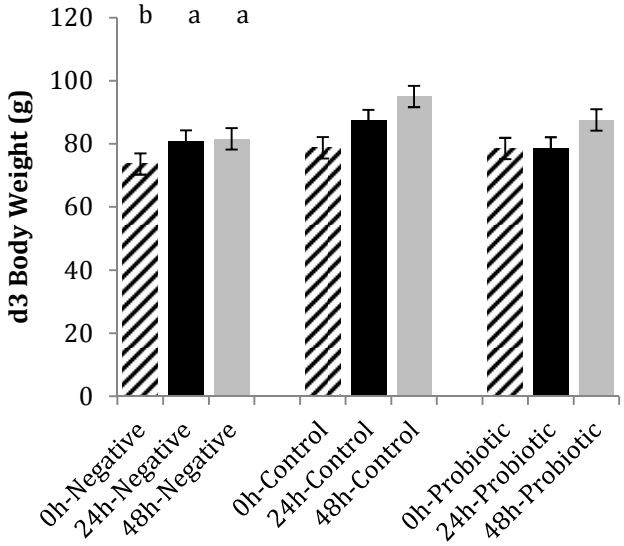

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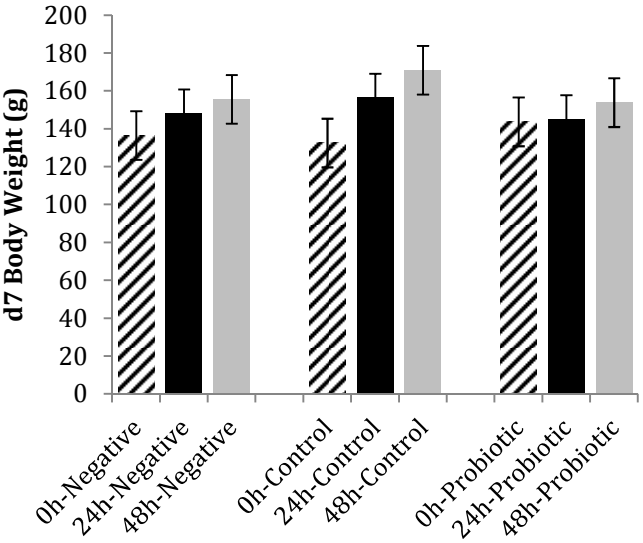
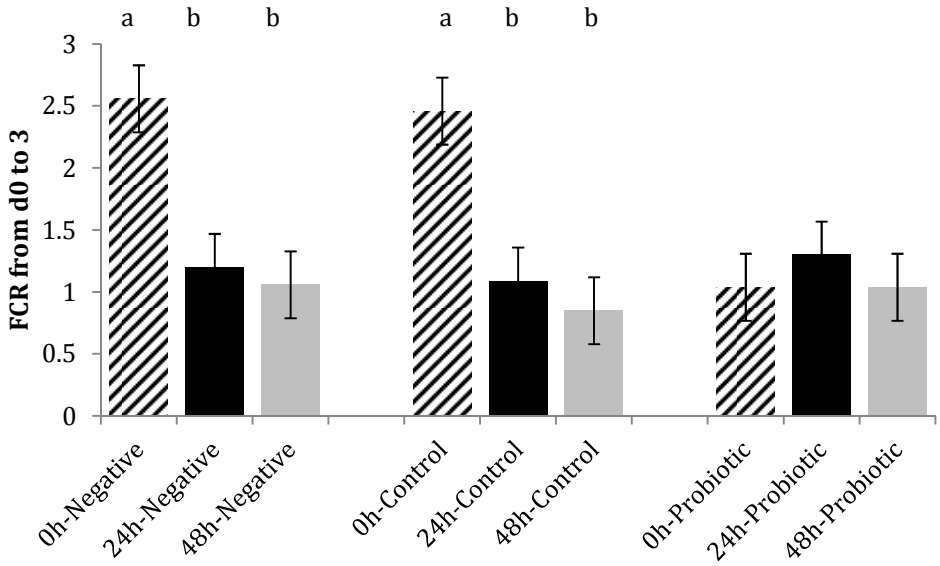


Figure 2.2: The FCR of poults who experienced a delayed placement on feed and either an *in ovo* probiotic injection A.) FCR from d0 to 3 of poults from either negative control (non-injected eggs), control (sterile saline injected eggs at d25 of incubation) or probiotic (eggs injected with 1 ml of probiotic inoculations at 10^6 cfu/ml at d25 of incubation) eggs than subjected to a 48h, 24h or 0h delay placement at hatch. B.) FCR from d3 to 7 of poults from either negative control (non-injected eggs), control (sterile saline injected eggs at d25 of incubation) or probiotic (eggs injected with 1 ml of probiotic inoculations at 10^6 cfu/ml at d25 of incubation) eggs than subjected to a 48h, 24h or 0h delay placement at hatch. C.) FCR from d0 to 7 of poults from either negative control (non-injected eggs), control (sterile saline injected eggs at d25 of incubation) or probiotic (eggs injected with 1 ml of probiotic inoculations at 10^6 cfu/ml at d25 of incubation) eggs than subjected to a 48h, 24h or 0h delay placement at hatch. All treatment groups were in their own separate hatcher. 0h-poults not held in the hatcher following hatch; 24h-poults held in the hatcher for 24h prior to feed and water access; 48h-poults held in the hatcher for 48h prior to feed and water access. Turkey eggs were offset to ensure all poults were placed on feed and water at the same time. [a,b] Bars within same group with no common superscript differ significantly ($P<0.05$)

A



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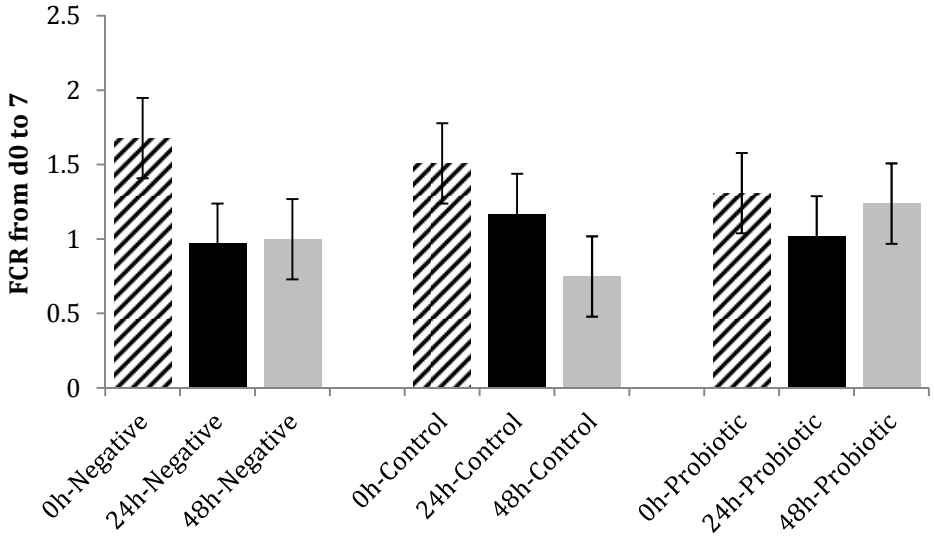

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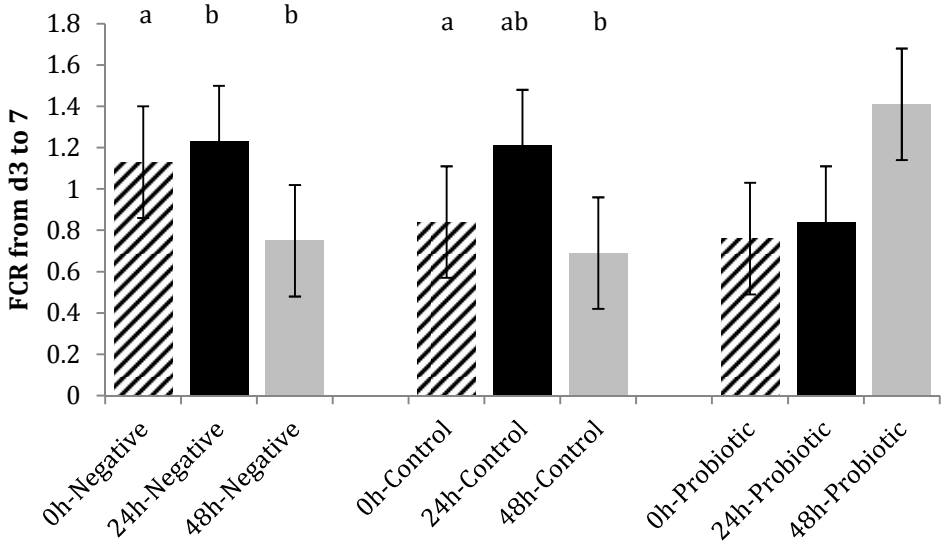
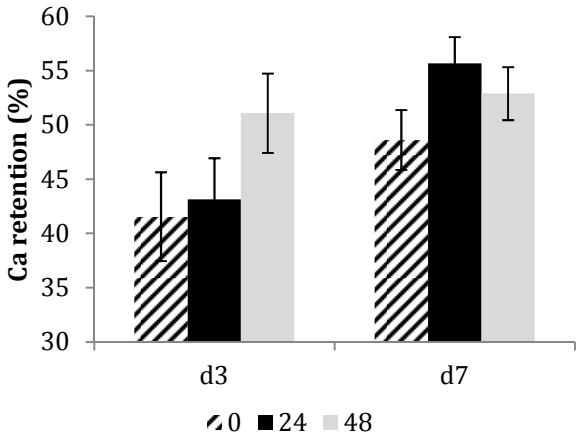


Figure 2.3: Apparent calcium and phosphorus retention in 3 or 7d old poults who experienced a delayed placement on feed with or without an *in ovo* probiotic injection A. Calcium digestibility of *in ovo* injected poults (P=0.679). B. Phosphorus digestibility of *in ovo* injected poults (P=0.179). C. Calcium digestibility of poults with delayed placement of feed (P=0.679). D. Phosphorus digestibility poults with delayed placement of feed (P=0.679). 0h-poults not held in the hatcher following hatch; 24h-poults held in the hatcher for 24h prior to feed and water access; 48h-poults held in the hatcher for 48h prior to feed and water access. Turkey eggs were offset to ensure all poults were placed on feed and water at the same time. Negative Control - non-injected eggs; control - sterile saline injected eggs; probiotic - eggs injected with 1ml of probiotic inoculations at 10^6cfu/ml. All treatment groups were in their own separate hatcher. [a,b] Bars within same group for each item with no common superscript differ significantly (P<0.05)

A.



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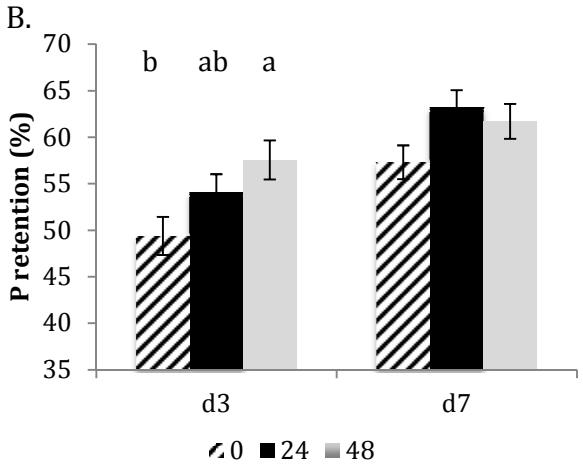
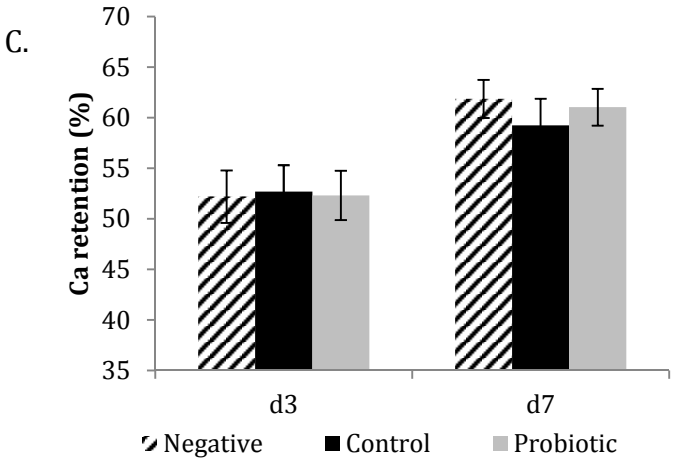




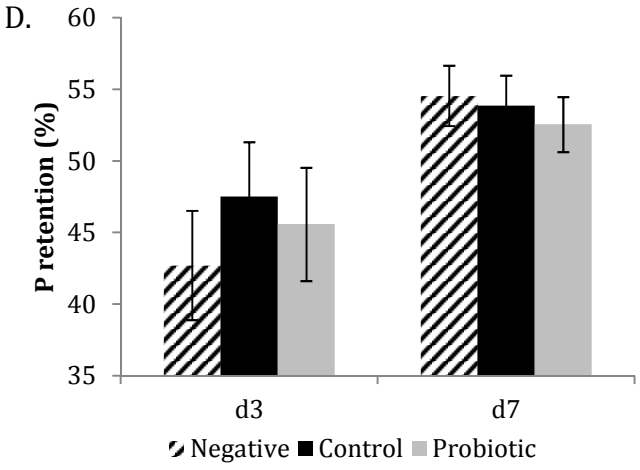
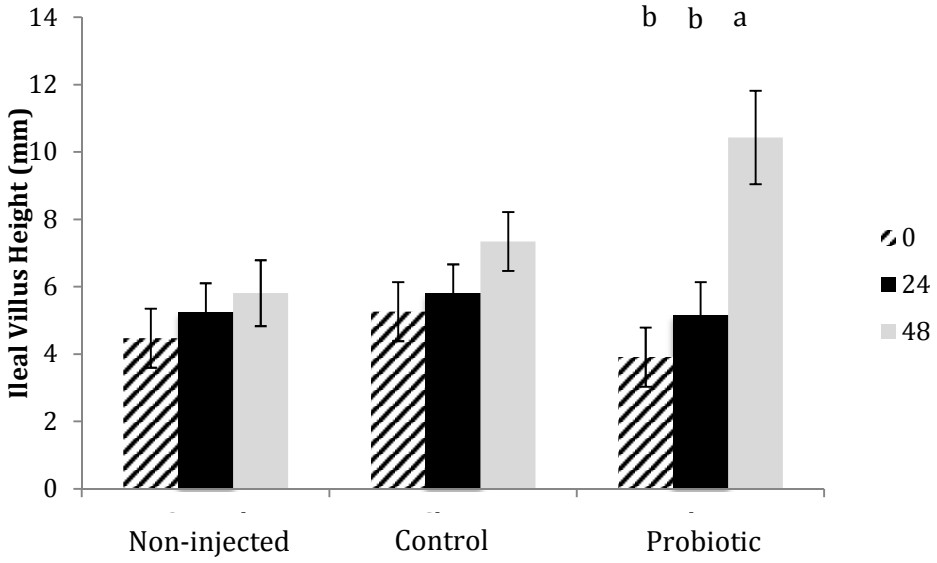


Figure 2.4: Ileal villus height to crypt depth ratio from A.) d0 turkeys whose eggs were injected with probiotics on d25 of incubation and with or without a delayed placement on feed. B.) d7 turkeys whose eggs were injected with probiotics on d25 of incubation and with or without a delayed placement on feed. Negative Control - non-injected eggs; control - sterile saline injected eggs; probiotic - eggs injected with 1ml of probiotic inoculations at 10^6 cfu/ml. All treatment groups were in their own separate hatcher. 0h-poults not held in the hatcher following hatch; 24-poults held in the hatcher for 24h prior to feed and water access; 48h-poults held in the hatcher for 48h prior to feed and water access. Turkey eggs were offset to ensure all poults were placed on feed and water at the same time. [a,b] Bars within same group with no common superscript differ significantly (P<0.05)

A.



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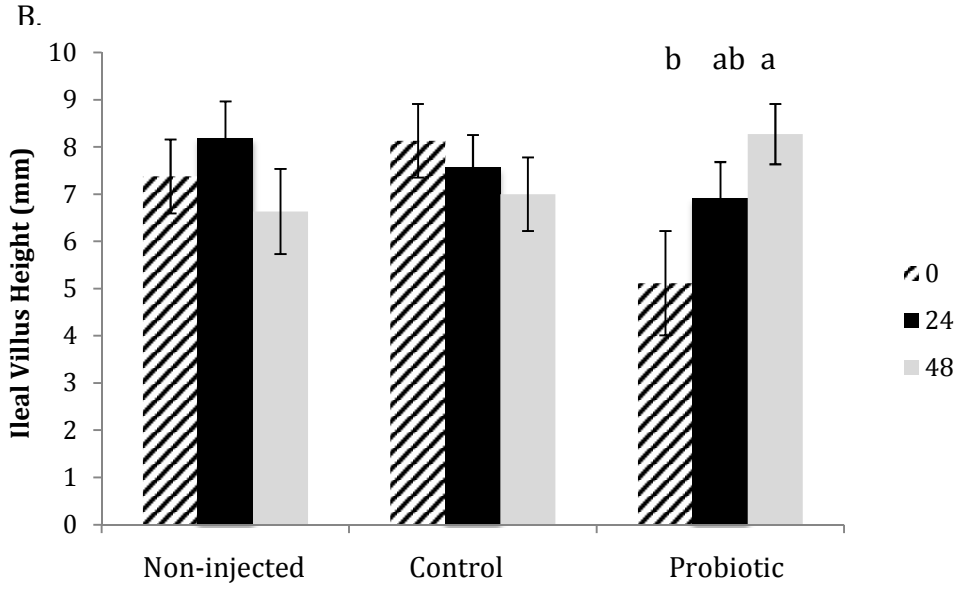
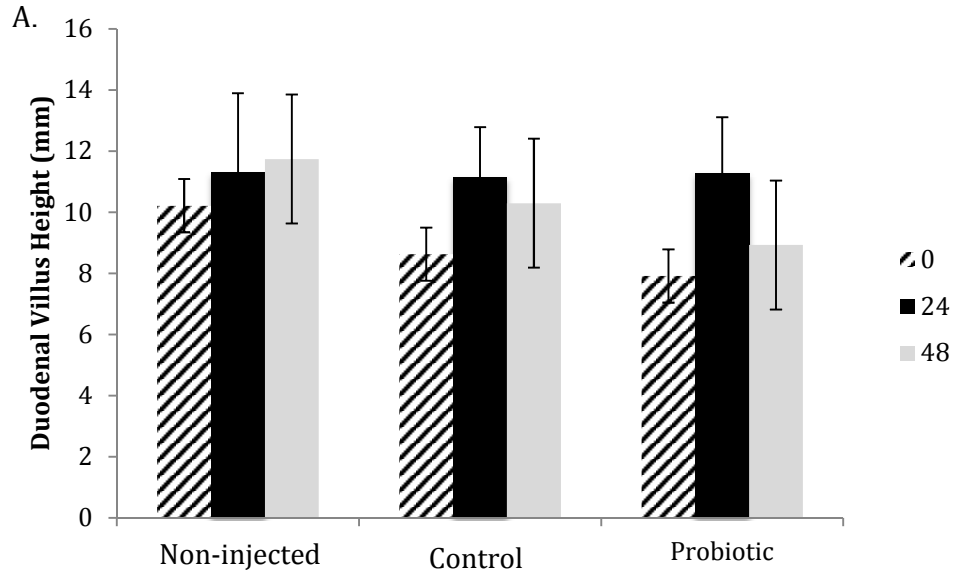
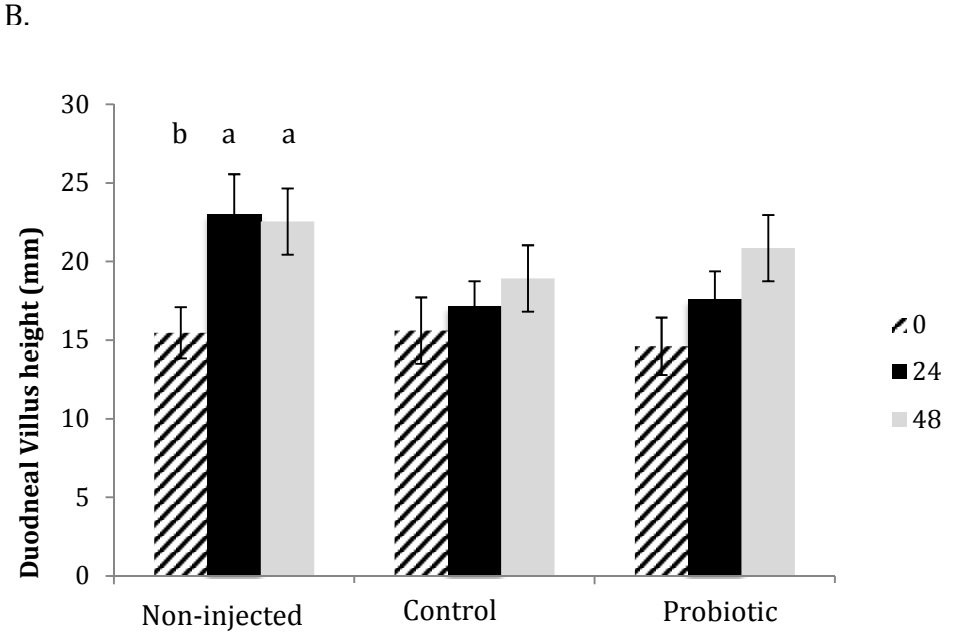


Figure 2.5: Duodenal villus height to crypt depth ratio from A.) d0 turkeys whose eggs were injected with probiotics on d25 of incubation and with or without a delayed placement on feed. B.) d7 turkeys whose eggs were injected with probiotics on d25 of incubation and with or without a delayed placement on feed.. Non-injected control - non-injected eggs; control - sterile saline injected eggs; probiotic - eggs injected with 1ml of probiotic inoculations at 10^6 cfu/ml. All treatment groups were in their own separate hatcher. 0h-poults not held in the hatcher following hatch; 24h-poults held in the hatcher for 24h prior to feed and water access; 48h-poults held in the hatcher for 48h prior to feed and water access. Turkey eggs were offset to ensure all poults were placed on feed and water at the same time. [a,b] Bars within same group for each item with no common superscript differ significantly (P<0.05)





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CHAPTER 3: BONE CHARACTERISTICS AND MINERAL DIGESTION IN YOUNG TURKEY POULTS: THE COMBINED EFFECT OF *IN OVO* PROBIOTICS AND LOW CALCIUM AND PHOSPHORUS DIETS

ABSTRACT

The use of probiotics can increase intestinal maturity, increase BW, improve FCR, and mineral absorption. *In ovo* probiotics may increase the performance of the host in the same way as those presented in the diet. Therefore, the objective of this study was to determine the impact of *in ovo* probiotics on performance and bone mineralization in turkeys fed a diet with or without a 20% dietary reduction in Ca and P. Fertile turkey eggs were candled on 25d and allocated to one of two treatments: non-injected eggs (Control) or eggs injected with one ml of probiotic solution (concentration of 10^6 cfu/ml). Each treatment had 20 replicates that contained 23 eggs per replicate. Eggs were transferred into two separate hatchers, and hatch counts were recorded on 28d. Four poults per replicate were sampled for bone measurements, and the ceca contents from two birds were pooled to assess bacterial counts. The remaining poults were allocated to brooder battery pens and assigned to a control diet (100% NRC, 1994) or a Ca and P deficient diet (80% NRC, 1994) for 21d. Four poults per pen were euthanized for bone and intestinal samples on 7d and 21d after hatch. The probiotic injection reduced the hatchability of birds, (87% vs. 61%), however, the bacterial load in the intestine was increased by over 3 $\log_{10}$ cfu/ml at DOH by probiotic injection. Probiotic injected birds had lower BW and shorter, narrower tibias and femurs at 7 and 21d post hatch. Poults that received the *in ovo* injection had a two-fold increase in mortality vs. non-injected poults ($P<0.001$). Poults fed the mineral deficient diets had a 12% higher rate of mortality compared to control birds ($P<0.05$). There were no differences in bone mineralization due to dietary treatment. These results

indicate that *in ovo* probiotics (10^6 cfu/ml) have negative effects on poult growth and performance in the first 21d post hatch especially with a nutritional challenge.

INTRODUCTION

Calcium is the most abundant mineral in the turkey's body, and an adequate intake is important to build maximal bone strength. Mixing errors or variation in feedstuff nutrient concentrations can lead to skeletal issues resulting in higher mortality or underperforming birds (Oviedo-Rondón et al., 2006). Diets deficient in Ca cost producers millions of dollars each year (Cook, 2000). Dietary feed additives, such as phytase, are often incorporated in the diet to help offset or reduce nutrients that must be added to the diet (Atia et al., 2000; Applegate et al., 2003). Many feed additives are derived from bacteria. This process normally involves *A. niger*, *Peniophora lycii* and *E. coli* bacteria that are selected and grown, and the additive is isolated from the bacteria (Selle and Ravindran, 2007). Feeding live bacteria (probiotics) could potentially provide the same benefits as the purified metabolites derived from them.

Probiotic bacteria have been studied over the years and have been found to improve the intestinal health of the host (Haghighi et al., 2006). The bacteria are often selected from naturally present commensal strains that originate from the host species (Walker, 2008). Patterson (2003) demonstrated that probiotic bacteria could help offset the effects of several disease challenges because the bacteria can alter the gut microbiota and immune system of the host. Probiotics are most effective given prior to the onset of a disease challenge to allow the bacteria time to reach the intestine and alter microbiota before the pathogenic bacteria reach the gut from the environment (Audisio et al., 2000). The earliest exposure to pathogenic bacteria is in the hatchery, and in order to deliver commensal

bacteria prior to hatch, the probiotics need to be presented *in ovo.* Previous research trials (Chapter 2) demonstrated that *in ovo* probiotics can benefit poults early in life in a non-challenge setting, but the impact during a nutritional challenge is unclear. Therefore, the objective of the study was to determine the impact of *in ovo* probiotics on performance and bone mineralization in turkeys fed a diet with a nutritional challenge of a 20% dietary reduction in Ca and P.

MATERIALS AND METHODS

The Michigan State University Institutional Animal Care and Use Committee approved all procedures. The approval number was AUF #05/13-103-00.

Probiotic preparation

The microencapsulated probiotic contained two strains of *Lactobacillus reuteri*, a single strain of *Enterococcus faecium*, *Bifidobacteria animalis* and *Pediococcus acidilacti* (Biomin, Herzogenburg, Austria). To determine the concentration of organisms, the probiotic mixture was suspended in sterile PBS and plated. To create solutions of 10^6 cfu/ml, the probiotic mixture was weighed (0.423 g) and suspended in PBS to create 10^8 cfu/ml solutions. The solutions were then diluted to 10^6 cfu/ml in sterile PBS (25 ml) the day of the sample injection (E25). Solutions were stored on ice (~4h) and vortexed for 15s immediately prior to egg injection.

Bird incubation and treatments

One thousand turkey eggs were individually weighed, set in an incubator (Petersime Model 5; Petersime Incubator Co., Gettysburg, PA), and incubated for 24d. Incubator

conditions (37.5°C dry bulb and 29.2°C wet bulb) were monitored throughout the trial using General Tools H10 data loggers (General tools, New York, NY). All eggs were candled on E24, infertile and externally pipped eggs removed, and the lowest point of the air cell marked. The remaining eggs were returned to the incubator and randomly assigned, using the experimental animal allotment program to one of two treatments based on initial egg weight (EAAP, 2009). The average difference in egg weight in each replicate was less than 0.5 g. There were 20 replicates per treatment with 23 eggs per replicate. The large end of the egg was wiped with an alcohol disinfectant wipe. A Dremel stylus rotary tool (Model # 1100-01: Dremel, Racine, WI) was used to drill a hole into the lowest point of the egg's air cell. An Allflex MR2 repeater syringe (Allflex USA INC) equipped with a 22ga 1" needle was used to inject each egg with 1 ml of probiotic solution (10^6 cfu/ml). Needles were changed, and the repeater syringe was rinsed with sterile saline between each replicate. Following injection, the drilled holes were sealed with paraffin wax. The control eggs were not drilled or injected, but were removed from the incubator for the same amount of time as the probiotic treatment. Each replicate was placed in a pedigree basket and assigned to one of two Surepip hatchers (Agro Environmental Systems Inc., Dallas, Georgia, USA) resulting in separate hatchers for each egg treatment. The pedigree baskets were randomly placed throughout the assigned hatcher.

Sampling

Hatch counts were recorded on E28 using the following definitions: a hatched poult had completely cleared the shell; a pipped poult had any portion of the shell cracked but failed to clear the shell; an unhatched poult had a completely intact shell. All poults were removed from the hatcher one replicate at a time, and total BW recorded. For cecal content

and yolk sac collection, four birds per replicate were euthanized by cervical dislocation and dipped in an antiseptic solution. Each bird was removed from the antiseptic solution and placed on a clean disinfected cutting board. The abdominal cavity was opened with sterile scissors, which were dipped in alcohol, and flame dried between each poult. Gloves were changed after the abdominal cavity was opened to prevent contamination from the poult exterior. Using two sets of forceps, the yolk sac and both cecal horns were extracted from the bird. The yolk sac was placed in a weigh boat and yolk-free BW was calculated based on difference between total BW and yolk weight. Cecal contents, from two of the four poults, were pooled by gently squeezing the contents into a sterile 1.5 mL microcentrifuge tube (Denville Scientific) using the forceps to prevent glove contamination. Sterile forceps and gloves were used for each bird to prevent cross contamination. Samples were immediately chilled on ice until 25% of the poults had been collected, at which point, samples were taken to the laboratory to prevent prolonged exposure to the ice and ensure viability of samples for the dilution series.

The remaining birds (n = 8/replicate) were placed in brooder batteries by treatment and assigned to one of two diets. The control diet was formulated to meet or exceed all nutrient NRC requirements (NRC, 1994). The Ca and P deficient diet was formulated with a 20% reduction in Ca and P concentrations (Table 1). One kilogram feed samples were collected from each diet for determination of Ca and P. Four poults per pen representing the average weight were selected at 7d and 21d for a total of 40 birds per diet and injection combination. Femurs and tibias were collected from each bird for the determination of bone morphology and ash content.

Bone Ash

Muscle tissue was removed from the bone using a #10 scalpel blade, and the length and width (measured at 50% of length) of each bone were measured. The bones were divided with a scapel blade into epiphyseal and diaphyseal sections with the epiphyseal section defined as the proximal and distal 25% of the bone, and the diaphyseal was defined as the middle 50% of the bone. Bones were placed in a soxhlet and the fat removed via ethyl ether (Avantor Performance Materials) extraction for 48h (AOAC International method 920.39. 2006). Following fat extraction, bones were placed in crucibles and put into a drying oven (American Scientific Products Model DN-81) for a minimum of 24h at 105°C. To determine percent ash, dried bones were removed, weighed while hot in a crucible, and placed in an ashing oven (Thermolyne Furnace Type 30400) for approximately 36h at 600°C. Crucibles were removed from the ashing oven and hot weighed. To determine percent ash of the bone, the ash weight was divided by the ether extracted dry weight.

Cecal content plating

Each cecal sample was a pool from two birds. For the six serial dilution series, 30 μl of each cecal sample was pipetted into 270 μl of sterile PBS. The mixture was vortexed for 15s, and the pipetting procedure repeated to create the next dilution. Next, 50 μl of each serial dilution and the pure sample were pipetted onto the center of each of four different media plates and spread using a L-shaped glass spreader rod. Rods were flamed in alcohol between each use. The four medias used were: Luria-Bertani agar (Acumedia, Lansing MI) for general gram-negative bacteria, Bifidiobacteria agar (HiMedia Laboratories Ltd. Mumbai, India) for *Bifidobacteria* spp, de Man, Rogosa and Sharpe agar (HiMedia

Laboratories Ltd. Mumbai, India) for *Lactobacillus* spp. plus *Pediococcus* spp. and Enterococcsel agar (a modified esculin bile agar; Becton Dickinson and Company, Franklin Lakes, NJ) for *Enterococcus* spp. All medias were made according to manufacturer instructions and stored at 15°C prior to being used. Plates were incubated for 24h at 37°C in an incubator (Precision Industries model# 30M, Chicago, IL), removed, and colony counts were made. Only counts from a single plate from the dilution series with approximately 25 to 300 total colonies was selected for counting. The plate was placed on a lightbox and a marker was used to mark counted colonies, and a lab cell counter was used to keep track of the counts.

Excreta samples

On 6d and 20d of the trial, excreta pans were removed, cleaned, lined with paper, and replaced. Twenty-four hours later, all excreta was collected and placed in plastic bags for apparent Ca and P retention. All samples were freeze-dried (FTS Systems, Inc, Warminster, PA USA. Model: TRI9C0T002) and ground using the Cyclotec Sample Mill 1093 (FOSS North America, Eden Prairie, MN) with a 1 mm screen to achieve a uniform grind. Ground samples were digested in nitric acid (OmniTrace, 67-70%, EMD Millipore, Billerica, MA.) using a MARS 5 microwave digestion system (CEM Corporation, Matthews NC) in accordance with procedures of Spears and Lloyd (2001). All glassware used in the digestion process was acid washed using 30% nitric acid and rinsed in distilled de-ionized (ddi) H_2O in order to remove any residual minerals. Approximately 0.4 g of sample was weighed into a digestion vessel and 10 mL of 70% nitric acid (Omni Trace, EMD Chemicals, Inc., Gibbstown, NJ) was added. The sample and acid solution was allowed to sit overnight (~16h) at room temperature to predigest the sample. The following day, vessels were

placed in the microwave digester, with the digestion program: 1200 watts with a 30min ramp to 160°C under pressure of 190 PSI, hold samples for 10min, and cool down for 5min. After additional cooling in a fume hood for 10min, vessels were vented, and two milliliters of 30% hydrogen peroxide (Sigma-Aldrich, St. Louis, MO) was added. The sample digest was then transferred to a 25 ml volumetric flask and allowed to cool completely; ddi H_2O was added to bring the volume to 25 ml. Samples were transferred to 50 ml polypropylene tubes and stored at room temperature until Ca and P were determined.

Calcium assay

Calcium concentration was determined by atomic absorption spectroscopy. The microwave digested samples were diluted 1:100 in a 1% $LaCl_3$ (lanthanum chloride, Sigma-Aldrich, St. Louis, MO) solution. The $LaCl_3$ acts as an ionization suppressant to eliminate the phosphate interference, which occurs in an air-acetylene flame. Excreta and feed samples were analyzed for Ca using an AA7000 Series atomic absorption spectrophotometer (Shimadzu Scientific Instruments, Inc., Columbia, MD). These samples were analyzed against a five point, matrix-matched standard curve (Ca standard source: VWR International, West Chester, PA) ranging in concentration from 1 to 5 µg/ml Ca. Peach leaves, the organic standard, (NIST, Gaithersburg, MD) were simultaneously analyzed to maintain instrument accuracy. Apparent Ca retention reported as mg of Ca in excreta per mg of Ca in feed.

Phosphorus assay

Spectrophotometery was used to determine P in the microwave digested samples. Phosphate ions react with molybdate complexes, which in the presence of a reducing agent

(Elon), are converted to molybdenum-blue to be measured in a spectrophotometer (Kaplan and Pesce, 1989). The MS solution contained 2.5 g of molybdate (MS), 7 ml of sulfuric acid, and ddi H_2O to bring the final volume to 500 ml. The Elon solution contained 1.5 g of sodium bisulfate, 0.5 g of Elon, and ddi H_2O to make the final volume 50 ml (Gomori, 1942). To analyze the microwave digested sample, two ml of sample was diluted 1:10 with ddiH_20. Standards were made by diluting 15 mg/dL stock solution of P (Phosphate Standard, 1000 ppm, LabChem, Inc., Pittsburgh, PA) with ddi H_2O to make 5, 4, 3, 2, 1.5, 1, and 0 mg/dL (Gomori, 1942). Into a 96-well microplate, 50 µl of each standard and sample were pipetted, in duplicate. Two hundred and fifty microliters of MS solution and 25 µL of Elon were pipetted into each well. The plate was incubated for 45min at room temperature on a plate shaker (Brinkmann TiterMix 100) at 600 rpm and read at 700 nm on the plate reader (SpectraMax 384, Molecular Devices). The SpectraMax determined the P concentration of all samples. The measurement was then adjusted by DM and amount of sample used in the microwave digestion to yield the final concentration. Apparent phosphorus retention was reported as mg of P in excreta per mg of P in feed.

Energy

Ground feed and excreta samples were formed into 1.0 g pellets using a combination Parr pellet press modified with a hydraulic jack, and the amount of energy in each sample was determined via bomb calorimetry following manufacturer instructions (Parr Instrument Co). Briefly, sample pellets were placed into a capsule that was placed in the loop of the bomb head. A Parr 45C10 Fuse Wire (34 B&S gage, nickel-chromium resistance wire) approximately 10 cm in length was attached to the two fuses of the bomb head and

bent to touch the sample pellet. The bomb head was secured into the bomb cylinder. The bomb was filled with oxygen to bring the pressure to 32 atm. The bomb was placed into a calorimeter bucket filled with 2000 ml of diH_2O and placed into the calorimeter. The two ignition wires were pushed into the terminal sockets on the bomb head. The water temperature of the calorimeter was allowed to reach equilibrium and the temperature recorded. An electrical surge was sent down the terminals into the bomb igniting the oxygen and combusting the sample. A second reading was taken approximately six min later, after a stabilized temperature had been reached. The bomb was disassembled and the remaining portion of the wire was measured and a calorie correction determined from the length. A benzoic acid pellet was used to confirm the standardized values of each bomb. Therefore, the energy was calculated based on the formula below:

$$\text{Heat of Combustion (cal/g)} = \frac{(\text{Ft} - \text{It}) \times \text{energy standard of bomb} - (\text{calories of wire burned})}{\text{sample dry wt. (g)}}$$

Ft= Final temperature

It= Initial temperature

Standard= benzoic acid

Dry Matter

Empty crucibles were placed into a constant temperature oven (Yamato DNF 600) at 105°C at least 2h prior to hot weighing. Each crucible was hot weighed and 0.5 g of sample were weighed, in duplicate, and placed into the crucible. The dry matter content of the feed and excreta was measured by drying the samples at 105°C for 24h.

Statistical Analysis

All parameters were analyzed using the PROC MIXED analysis of SAS (v 9.3) with the LSmeans procedure PROC MIXED in SAS PC Windows Version 9.2 software, (SAS Institute Inc., Cary, NC). The model ($y = \mu + \tau_i + \beta_j + \tau\beta_{ij} + \epsilon_{ijk}$) included the main effects of treatment (τ) and diet (β) as well as the interaction between treatment and diet (τβ). Interactions are presented as a comparison of single treatments across various placements. Comparisons across time were not used in the model. Pen or pedigree basket was used as the experimental unit for all measurements. Differences between means were tested using the Pdiff option of the LSmeans statement with significance accepted at $P<0.05$.

RESULTS

Performance

In ovo probiotic administration reduced hatchability of turkeys ($P<0.05$), but not hatch day BW (Table 2). Probiotic injection increased the percent of poults pipped compared to controls ($P<0.05$), and increased the percentage of poults unhatched by 125% ($P<0.05$). Residual yolk was 4.28% higher and percent yolk was 2.97% higher in the probiotic injected treatment compared to the control ($P<0.05$).

There were no differences in BW at DOH and 21d between diets (Table 3, 4). There were interactions between injection and diet on BW, FI, and BWG. The deficient treated birds had increased BW, FI, and BWG compared to control for the probiotic birds, but were decreased in the non-injected birds. Mortality was 28% higher in the probiotic injected eggs through the first week of life ($P<0.01$, Table 3) with no effect on mortality from 8 to 21d (Table 4). The poult mortality was greatest when the Ca and P deficient diet was fed to

probiotic injected birds (P<0.05). The probiotic injection reduced feed intake until 7d (P=0.04), however, FCR was not affected by diet or injection throughout the trial (Tables 3, 4). There were no interactions between diet and injection on performance from 8d to 21d (Table 4).

Cecal Bacterial Counts

The probiotic injection increased cecal bacteria load for all types of determined bacteria (Table 5). *Bifidobacteria* and general gram-negative bacteria had a 3-log increase in the probiotic injected birds compared to control poults (P<0.001). *Lactobacillus* and *Enterococcus* were 4-logs higher in the probiotic injected birds than the controls (P<0.001).

Nutrient Digestibility

Birds fed the control diet had higher apparent retention of Ca and P than birds fed the deficient diet (Table 6). 7d, the poults fed a control diet and received the *in ovo* probiotic injection had higher apparent Ca retention (58%) than birds in any of the other treatment combinations (P<0.05), but the group was not significantly higher than the non-injected control group. However, these differences were not seen on 21d (P<0.05). The apparent retention of P was higher in the control diet fed birds than those fed the Ca and P deficient diet (P<0.05) at both 7d and 21d. There were no significant diet by injection interactions on P retention. There were no differences in apparent energy retention for the injection treatment or diet.

Bone parameters

All morphology is reported on whole bone measurements, whereas the ash content is separated into epiphyseal and diaphyseal sections. Poult femur length and the width of

tibia and femur bones did not respond to probiotic injection at DOH (Table 7). Tibial length was reduced in the birds injected with probiotics compared to the non-injected birds (P<0.05) on all sample days. There was a reduction of femur length on 7d and 21d in probiotic injected birds compared to the non-injected (P<0.05). Probiotic injection narrowed the tibia and femur width compared to birds not injected with the probiotic on 7d and 21d, but no effect was seen in DOH (P<0.05). There was no difference in tibial or femoral morphology for the poults fed the Ca and P deficient diet compared to control fed birds for any of the sample days. There were significant (P<0.05) interactions between the injection and dietary treatments with birds in both the tibia and femur (Figure 1). Femur length was longer in the probiotic deficient fed birds and the non-injected control birds on DOH, and this carried through for all sample days (Figure 1A). There were no differences in tibia length amongst the treatments at DOH. However, on 7d and 21d tibia length was reduced when the probiotic was injected regardless of dietary treatment (Figure 1B; P<0.05). The injected and deficient fed poults had numerically higher tibia length on d21, whereas the non-injected deficient birds' tibias were numerically shorter. Femur widths were not affected by any treatment or interaction on any of the sample days (Figure 1C). Tibia width was significantly lower on 21d when the probiotic was injected regardless of dietary treatment (Figure 1D; P<0.05). The greater length was observed when the diet had adequate Ca and P and the birds were not injected compared to the other treatments (P<0.05).

Tibial epiphyseal ash was not changed by injection (Figure 2A). The diaphyseal ash in the tibia was higher in the probiotic injected birds than non-injected birds on DOH, but not on 7d and 21d (Figure 2C; P<0.05). Diet did not alter the epiphyseal or diaphyseal ash

content in the tibia (Figure 2B,D). There were no differences in ash content at 7d or 21d due to dietary treatment or *in ovo* injection for either the diaphyseal or epiphyseal portion of the femur or tibia (Figure 3). There was an increase in epiphyseal ash content of the femur at DOH (Figure 3A; $P<0.05$) There were no differences in either section of bone's ash as a total ash percentage for any treatment (Figure 4, 5).

DISCUSSION

In the previous studies (Chapter 1, 2), injection of *in ovo* probiotics into turkey eggs at 25d did not alter hatchability, whereas there was a 25% reduction in the current study. The exact reason for the inconsistent results across trials is unknown, but there are multiple possibilities as to why the hatchability was different in this trial compared to other. The equipment, technique and personnel were the same for each trial and can be ruled out as reason for the change. Sample plating confirmed the probiotic product was the same concentration in each trial. Initial egg weight was measured and balanced across all treatments. The eggs came from the same commercial operation, though the eggs could not be confirmed to have come from the same hens or hens of the same age. A difference in breeder hen age could explain the difference in the number of pipped eggs. Incubation in eggs from younger hens can take longer that that of older hens (Applegate, 2002). The injection could have shifted the hatch window thus decreasing the percent of poults hatched but increasing the percent of pipped eggs. The eggs that were pipped in the probiotic injected eggs contained embryos that appeared viable. The combination of pipped and hatched probiotic poults would translate into numbers more closely aligned to control poults (92% vs 82%).

Temperature plays an important role in development. A short reduction in temperature before 10d can reduce growth rate of the avian embryo, but through compensatory gain, these eggs will hatch as soon as their peers (French, 1997). A temperature reduction late in incubation as a result of the injection could slow poult development compared to the control eggs. The single probiotic injection could have chilled the embryo enough to reduce hatchability. The probiotic solution was stored on ice to prevent growth of the bacteria. The temperature of the solution was approximately 1°C, considerably lower than hatcher temperature (~37°C). The dramatic reduction in temperature of the internal environment for a short period of time seems to be enough to delay hatch, where smaller temperature reduction from longer period of time was not.

The amount of residual yolk measured in the probiotic injected birds further points to a developmental delay. The yolk sac begins to rapidly disappear as the poult approaches hatch (Sell et al., 1991). In broilers, the yolk sac is approximately 12% of BW at hatch and disappears at a rate of approximately 2.5 g/d (Huang et al., 2008). Yolk sac disappearance was roughly 3 g over a 36h period in turkey poults held without feed for the first 48h of life (Moran and Reinhart, 1980). These studies demonstrate a relatively consistent yolk disappearance of 3 g across avian species in the first few days of life, which is consistent with the difference in yolk weight between control and probiotic birds at hatch. A developmental delay equaling one day would explain the increase in residual yolk.

The cecal bacterial load were approximately one-half to one log higher in the current trial compared to the previous trials (Chapters 1, 2). An error in the probiotic solution concentration was not an issue as plating confirmed concentrations before and after the trial. In a previous study (Chapter 1), a 10^8 cfu injection reduced hatchability. The

results from that study indicate that a high level of embryonic intestinal bacteria correlates with a low hatchability. The large amounts of commensal bacteria in the current study are analogous to a necrotic enteritis outbreak. Necrotic enteritis is caused when the naturally present commensal bacteria, *Clostridium perfringenes,* over proliferates and causes disease (Lyhs et al., 2013). The data from the current experiment suggests a large commensal bacteria load actually prevents the absorption of the yolk and reduces development by a day. The previous c results (Chapters 1, 2) coupled with the current study indicate that 10^4 to 10^6 cfu of bacterial injection resulting in ~10^7 cfu of bacteria in the intestine is the optimal amount of bacteria that the intestine can handle when administered *in ovo*.

The average hatch BW was not altered by probiotic injection. However, by 7d, BW was higher in the *in ovo* control birds. Though the BW of the birds fed the control diet was higher, FCR was not different between the treatments. Both injection treatments were less than Hybrid performance goals. The FCR results are not consistent with the previous study (Chapter 2) or published literature. Broilers and turkeys given *Lactobacillus* cultures had improved BW gain and feed conversion (Kalavathy et al., 2003; Torres-Rodriguez et al., 2007). The ability of *in ovo* probiotic bacteria to assist the performance of the host could be limited to a non-nutrient challenged setting.

Previous studies on reduced nutrient diets effect on performance are more numerous but results are variable. Turkeys fed low Ca diets did not have a change in FCR (Sanders and Rowland, 1992; Roberson, 2004). A 20% reduction in NRC recommended dietary Ca, increased the BW of turkeys but did not alter FCR (Atia et al., 2000). A 25 to 40% dietary reduction in Ca reduced broiler performance (Sebastian et al., 1997; Houshmand et al., 2011). Broilers fed low P diets had reduced feed intake through the first

three weeks of life (Sebastian et al., 1997). A 25% reduction in dietary Ca reduced feed intake and growth in broilers (Houshmand et al., 2011). In the current study, a reduction in performance was seen in the low Ca and P fed birds. The poorer performance could be a result of the diets or could be the result of an unknown condition that was exacerbated by the reduced nutrient diets.

The dietary retention of Ca and P was higher in the control diets rather than the reduced nutrient diets. In broilers, low P diet had a higher retention and retention increased when Ca decreased (Ravindran et al., 1995) Bacteria in the intestine go through a natural progression and change as the bird ages (Danzeisen et al., 2013). Altering this progression may hinder absorption of nutrients early in life. The inability to retain nutrient may have contributed to mortality. In both the second and third experiment chapter the diets did not alter mortality, but the mortality was higher than expected. Bone strength was reduced in low P diets (Ravindran et al., 1995). This could reduce mobility of turkeys and result in fewer feeding. The inability of turkeys to transition to a commercial diet is due to an underdeveloped intestine as another possible reason for early life mortality (Lilburn, 1998). The probiotic injection into the egg may have exacerbated the difficulty in transitioning to feed, causing increased mortality.

Probiotic injections into the egg increased the diaphyseal ash levels of both the femur and tibia at DOH, though not significantly higher in the femur. The effect on ash was short in duration, as there were no differences at 7d. Two strains of *Bifidobacteria* increased Ca content of the tibia and femur of rats (Pérez-Conesa et al., 2007). Laying hens fed combinations for *Lactobacillius* and *Bifidobacteria* and broilers fed a combination of *Bacillus* strains had increased bone ash as well (Panda et al., 2003; Mutus et al., 2006). The

increase of bone ash in turkey poults could potentially be a benefit. The increased ash percentage could help offset skeletal problems later in life though the potential reason for increased mineralization at hatch is unclear. Probiotic bacteria can reduce pH in the intestine through production of short chain fatty acids (Fooks and Gibson, 2007) allowing for increased Ca absorption (Bronner and Pansu, 1999). The birds did not appear to have increased Ca absorption so the exact mechanism is unclear. Dietary and *in ovo* administration of probiotics could have further increased bone mineralization and provided a longer duration of benefits.

The length of the femur and tibia increased with age within birds of the same treatment in the current study, which is in accordance with studies by Bond et al. (1991), Skinner and Waldroup (1995) and Bruno et al. (2007). The length of the femur and tibia was shorter in the probiotic injected birds compared to the control birds on 7d and d21. In rats, *Bifidobacteria Longum* in combination with a prebiotic and *Lactobacillus* improved bone length and mineral absorption (Kruger et al., 2009; Rodrigues et al., 2012). In broilers, probiotics did not alter bone length or width in birds fed diets with nutrients levels at NRC recommendation or higher (Mutus et al., 2006). Turkeys are more closely related to chickens, rather than rats, and the underlying mechanism for probiotic's effects on bone are not understood. Avian species may not respond to probiotics in the same manner as mammalian species.

The probiotic injection, while influencing bone length and ash, did not alter excretion of Ca or P in the diet. The probiotic injection was expected to decrease excreta level of both minerals. Serum Ca levels were increased in production laying hens when their diets were supplemented with 200 mg of probiotics containing two *Lactobacillus* spp.

and one *Bifidobacteria* spp. (Panda et al., 2003). Commensal and probiotic bacteria alter vitamin D receptors in the host, and have been shown to improve Ca digestibility through this mechanism (Resta, 2009). Another possible mechanism is the bacteria binding of phytic acid rather than Ca. With less bound Ca, Ca absorption would increase (Scholz-Ahrens et al., 2007). In the current study, additional diets with prolonged use of probiotics could have altered digestibility.

Calcium and P digestibility was higher in birds fed the control diets. In poultry studies, the response to deficient Ca and P diet has varied. In 45wk old laying hens, a 10% reduction in P decreased Ca digestibility (Jalal and Scheideler, 2001). While non-phytate diets increased P and N digestibility in broilers (Ravindran et al., 2000). A greater reduction in Ca and P in the diet may have affected birds and altered excreta mineral concentrations. The recommended amount of Ca and P may have been adequate for early life growth needs in turkeys. The tibia did have increased length in low Ca and P fed birds. However, femurs were shorter in birds fed deficient diets, indicating that bones respond differently to dietary deficiencies. Though, there was no interaction between the diet and injection treatments, the difference in tibia and femur length between the non-injected control and probiotic was greater in the 21d birds compared to the 7d. The differences observed may have continued through the production period and warrant further investigation.

A common practice in poultry is to use bacteria derived phytase in the diet to improve P digestibility. There are several bacterial sources of microbial phytase that have proven effective, and thus usage has increased over the last 20 years (Kiarie et al., 2013). Bacterial phytase improved P retention and bone mineralization in turkeys (Applegate et al., 2003). The addition of the bacteria strains, which phytase is isolated from, could alter P

digestion in the same way as phytase addition to the diet, but this is unclear. Laying hen diets supplemented with molasses and strains of *Lactobacillus* had increased phytase activity and P retention (Nahashon et al., 1994). While the total Ca and P in the deficient diet of the current study was less than the control diet, the ratio of Ca:P was approximately the same for both diets (1.4, 1.5). Microbial phytase effectiveness was reduced in turkeys from hatch to 21d when the ratio increased to 2.0 (Qian et al., 1996). In the current study, the Ca:P ratio might have been too high for the microbes to effect digestibility. In the current experiment, the single injection of probiotics was not enough to alter digestibility, whereas continued use might have been. In rats, supplementation with probiotics for 30d improved both Ca and P digestibility (Pérez-Conesa et al., 2006). The author is not aware of a study examining a single dose probiotic in poultry and the effects on digestibility of Ca and P. The effect of daily feeding is difficult to compare to a single dose.

The trial further demonstrates that a single injection of probiotics (10^6 cfu) has a long lasting effect on the host. A shift in bacterial population in the intestine influences skeletal morphology and body size. Further research could determine if the long lasting effects could be beneficial. The current results show that even commensal bacteria can be harmful if in too large amount. Producers must protect poults from high cecal bacterial concentrations of any kind. The trial further demonstrates the potential of a single event to alter performance early in life and for that shift in production to be maintained through the first three weeks post hatch.

APPENDIX

Table 3.1: Composition, calculated, and analyzed nutrient content of diets fed from 0-21d of age

Diet (as fed)	Control [1]	Deficient
Ingredients (%)		
Corn	41.63	41.63
Soybean meal, 48% CP	48.47	48.47
Soy oil	3.00	3.00
NaCl	0.25	0.25
Copper sulfate	0.05	0.05
Sodium bicarbonate	0.20	0.20
DL Methionine	0.28	0.28
Lysine HCl	0.34	0.34
Threonine	0.13	0.13
Limestone	2.10	1.65
Monocalcium phosphate	2.70	2.00
Vitamin/mineral premix[2]	0.35	0.35
Celite	0.00	1.15
Chromic (III) Oxide	0.50	0.50
Calculated values (DM)		
ME (kcal/kg)	2875	2875
Crude protien	27.7	27.7
Ca (g/kg)	14.0	11.3
nPP (g/kg)	7.5	6.1
Ca:P ratio (g/g)	1.87	1.85
Analyzed values (DM)		
Ca (g/kg)	15.76	11.17
Total P (g/kg)	11.17	9.89
Ca:P ratio (g/g)	1.41	1.51

[1] Control are birds fed diet containing 100% of NRC Ca and P requirements; Deficient are birds fed diet containing 80% of NRC Ca and P requirements.

[2] Supplied per kilogram of diet: vitamin A, 13,233 IU; vitamin D3, 6,636 IU; vitamin E, 44.1 IU; vitamin K, 4.5 mg; thiamine, 2.21 mg; riboflavin,
6.6 mg; pantothenic acid, 24.3 mg; niacin, 88.2 mg; pyridoxine, 3.31 mg; folic acid, 1.10 mg; biotin, 0.33 mg; vitamin B12, 24.8 mg; choline, 669.8
mg; iron from ferrous sulfate, 50.1 mg; copper from copper sulfate, 7.7 mg; manganese from manganese oxide, 125.1 mg; zinc from zinc oxide,
125.1 mg; iodine from ethylene diamine dihydroidide, 2.10 mg; selenium from sodium selenite, 0.30 mg.

Table 3.2: Hatch performance of turkey poults administered *in ovo* probiotics at 25d of incubation[1]

	In ovo control[2]	Probiotic
	Mean ± SE	Mean ± SE
Hatchability[3]	87.21[a]±1.4	61.33[b]±1.4
Pipped[4]	4.99[a]±1.57	21.01[b]±1.57
Unhatched[5]	7.80[a]±1.4	17.66[b]±1.4
Hatch weight (g)	62.95±0.88	64.12±0.88
DOH Yolk weight (g)	15.52[b]±0.80	18.49[a]±0.80
Yolk [6](%)	24.64[b]±1.25	28.92[a]±1.25

[a,b] Means and standard error within row with no common superscript are different (P<0.05)

[1] A probiotic mixture of *L. reuteri, B. animalis, E. faecium, and P. acidilacti.*

[2] Mean of 20 replicate groups of 23 eggs each. Control are non-injected eggs in a separate hatcher; probiotic are eggs injected with 1ml of probiotic inoculations at 10^6cfu/ml in a separate hatcher.

[3] Percentage of poults that had hatched - completely cleared the shell at time of count

[4] Percentage of poults that had started to hatch - cracked the shell at time of count.

[5] Percentage of poults that had not started to hatch - shell completely intact at time of count

[6] Yolk weight as a percentage of hatch weight

Table 3.3: Performance of 0 to 7d old poults fed control or Ca and P deficient diets with or without *in ovo* probiotic[1] administration at 25d of incubation

Item	BW (g)	FI (g)	BWG (g)	FCR(g/g)	Mortality(%)
	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE
Injection[2]					
In ovo control	121.73[a]±2.67	72.91[a]±4.05	59.17[a]±2.88	1.26±0.24	10.86[b]±2.71
Probiotic	109.45[b]±2.67	61.08[b]±4.05	44.95[b]±2.88	1.70±0.24	38.21[a]±2.71
Diet[3]					
Control	116.20±2.67	67.19±4.05	52.05±2.88	1.45±0.24	18.97[b]±2.71
Deficient	114.98±2.67	66.80±4.05	52.07±2.88	1.51±0.24	30.10[a]±2.71
Interaction					
In ovo control x Control	124.15[a]±3.78	74.69[a]±5.73	61.98[a]±4.07	1.23±0.34	08.91[c]±3.83
In ovo control x Deficient	119.31[a]±3.78	71.12[a]±5.73	56.35[a]±4.07	1.29±0.34	12.82[c]±3.83
Probiotic x Control	108.24[b]±3.78	59.68[b]±5.73	42.11[b]±4.07	1.68±0.34	29.02[b]±3.83
Probiotic x Deficient	110.65[b]±3.78	62.48[b]±5.73	47.78[b]±4.07	1.73±0.34	47.39[a]±3.83
P-values					
Injection	<0.001	0.04	0.01	0.20	<0.001
Diet	0.75	0.94	0.96	0.86	0.006
Interaction	0.02	0.58	<0.001	0.98	<0.001

[a,b] Means and standard error within column for each item with no common superscript are different ($P<0.05$)

[1] A probiotic mixture of *L. reuteri, B. animalis, E. faecium, and P. acidilacti.*

[2]Mean of 10 replicate pens. Non-injected eggs were in a separate hatcher; probiotic are eggs injected with 1 ml of probiotic inoculations at 10^6cfu/ml and placed in a separate hatcher.

[3]Control are birds fed diet containing 100% of NRC Ca and P requirements; deficient are birds fed diet formulated to contain 80% of NRC Ca and P requirements.

Table 3.4: The performance poults from 8 to 21d post hatch fed control or Ca and P deficient diets with or without *in ovo* probiotic administration

Item	BW (g)	FI (g)	BWG (g)	FCR (g/g)	Mortality
	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE
Injection[2]					
In ovo control	525.91[a]±31.61	545.99±13.66	396.71±22.31	1.44±0.14	0.20±0.15
Probiotic	441.95[b]±35.34	474.03±15.27	330.27±24.95	1.53±0.15	0.25±0.15
Diet[3]					
Control	488.63±33.53	508.52±14.49	346.86±23.67	1.63±0.14	0.35±0.15
Deficient	479.23±33.53	511.5±14.49	380.12±23.67	1.35±0.15	0.10±0.15
Interaction					
In ovo control x Control	539.14±44.70	550.78±19.32	387.8±31.55	1.51±0.19	0.40±0.15
In ovo control x Deficient	512.68±44.70	541.19±19.32	405.61±31.55	1.37±0.20	0.00±0.15
Probiotic x Control	438.12±49.98	466.25±21.60	305.92±35.28	1.74±0.21	0.30±0.15
Probiotic x Deficient	445.79±49.98	481.82±21.60	354.63±35.28	1.33±0.22	0.20±0.15
P-value					
Injection	0.001	0.08	0.06	0.64	0.94
Diet	0.88	0.84	0.32	0.18	0.82
Interaction	0.54	0.72	0.64	0.52	0.79

[a-b] Means and standard error within column for each item with no common superscript are different

[1] A probiotic mixture of *L. reuteri, B. animalis, E. faecium, and P. acidilacti.*

[2]Mean of 10 replicate pens (8 birds/replicate). Non-injected are non-injected eggs in a separate hatcher; probiotic are eggs injected with 1 ml of probiotic inoculations at 10^6cfu/ml in a separate hatcher.

[3] Control are birds fed diet containing 100% of NRC Ca and P requirements; deficient are birds fed diet formulated to contain 80% of NRC Ca and P requirements.

* ($P<0.05$)

Table 3.5: Total cecal bacterial populations (log_{10}cfu/ml) as determined by cultivable plate count on four different medias from poults administered various amounts of *in ovo* probiotics[1] administered at 25d of incubation

	BIF[3]	ENT[4]	GN[5]	LAC[6]
Injection[2]	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE
In ovo control	7.15[b]±0.21	5.74[b]±0.21	7.13[b]±0.21	6.43[b]±0.21
Probiotic	10.42[a]±0.21	10.37[a]±0.22	10.22[a]±0.21	10.40[a]±0.22

[a,b] Means within column for each treatment with no common superscript are different ($P<0.001$)

[1] A probiotic mixture of *L. reuteri, B. animalis, E. faecium, and P. acidilacti.*

[2]Mean of 20 replicate groups of a two-bird pool of cecal contents. Non-injected are control eggs in a separate hatcher; probiotic are eggs injected with 1ml of probiotic inoculations at 10^6cfu/ml in a separate hatcher

[3] BIF- Bifidobacteria

[4]ENT-Enterococcocus

[5]GN- Gram-negative bacteria

[6]LAC-Lactobacillus and Pediococcus

Table 3.6: Apparent Ca and P retention in 7 and 21d old poults fed either control or Ca and P deficient diets with or without *in ovo* probiotic[1] injection at 25d of incubation

Item	Calcium[4] (%)		Phosphorus (%)		Energy (%)	
	day 7	day 21	day 7	day 21	day 7	day 21
	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE
Injection[2]						
In ovo control	52.34±1.34	42.59±1.95	67.11±0.84	56.35±2.61	8.5±1.07	13.8±0.8
Probiotic	54.33±1.47	44.18±2.13	66.38±0.93	54.58±2.78	6.5±1.07	15.2±0.8
Diet[3]						
Control	56.52[a]±1.34	42.09±2.13	68.17[a]±0.84	58.61[a]±2.78	6.7±1.06	14.6±0.6
Deficient	50.14[b]±1.47	44.68±1.95	65.32[b]±0.93	52.33[b]±2.61	8.4±1.06	14.8±0.6
Interaction						
In ovo control x Control	54.45[a]±1.89	39.93±2.91	67.49±1.19	59.53±3.80	8.4±1.02	14.0±1.04
In ovo control x Deficient	50.22[b]±1.89	45.24±2.60	66.73±1.19	53.17±3.58	8.2±1.02	13.5±1.04
Probiotic x Control	58.59[a]±1.89	44.25±3.11	68.84±1.19	57.68±4.06	6.2±1.02	15.4±1.04
Probiotic x Deficient	50.06[b]±2.26	44.12±2.91	63.91±1.42	51.48±3.80	7.2±1.02	15.2±1.04
P-value						
Injection	0.32	0.58	0.56	0.65	0.20	0.23
Diet	0.003	0.37	0.03	0.11	0.17	0.87
Interaction	0.29	0.35	0.10	0.98	0.28	0.77

[a,b] Means within column for each main effect or interaction with no common superscript are different ($P<0.05$).

[1] A probiotic mixture of *L. reuteri, B. animalis, E. faecium, and P. acidilacti.*

[2] Mean of 10 replicate cages. Non-injected are non-injected eggs in a separate hatcher; probiotic are eggs injected with 1ml of probiotic inoculations at 10^6cfu/ml in a separate hatcher.

[3] Control are birds fed diet containing 100% of NRC Ca and P requirements; deficient are birds fed diet formulated to contain 80% of NRC Ca and P requirements.

[4] Apparent total tract digestibility of Ca, P, and energy as a percentage of total amount in ration

Table 3.7: The main effects of control or Ca and P deficient diets and *in ovo* probiotic[1] injection at 25d of incubation on tibial and femoral morphology of DOH, 7 and 21d old poults fed

	Injection[2]			Diet[3]		
Parameter[4]	Mean ± SE	Mean ± SE	*P*-value	Mean ± SE	Mean ± SE	*P*-value
	In ovo control	Probiotic		Control	Deficient	
Tibia Length (mm)						
DOH	36.11[a]±0.42	34.09[b]±0.40	<0.001	34.82±0.4	35.38±0.56	0.32
d7	46.90[a]±0.34	44.16[b]±0.34	<0.001	45.29±0.33	45.28±0.42	0.28
d21	78.31[a]±0.78	75.98[a]±0.66	<0.001	75.97±0.71	75.93±0.82	0.96
Tibia Width (mm)						
DOH	1.98±0.02	1.94±0.02	0.09	1.95±0.02	1.97±0.02	0.59
d7	2.56[a]±0.03	2.42[b]±0.03	0.002	2.50±0.03	2.48±0.03	0.61
d21	4.82[a]±0.05	4.44[b]±0.06	<0.001	4.65±0.06	4.60±0.06	0.58
Femur Length (mm)						
DOH	23.99±0.27	23.24±0.29	0.06	23.74±0.28	23.49±0.28	0.53
d7	31.77[a]±0.19	29.95[b]±0.22	<0.001	31.04±0.2	30.68±0.21	0.21
d21	51.93[a]±0.37	48.82[b]±0.49	<0.001	50.41±0.42	50.34±0.45	0.91
Femur Width (mm)						
DOH	1.93±0.02	1.90±0.02	0.11	1.91±0.02	1.91±0.02	0.98
d7	2.58[a]±0.03	2.39[b]±0.03	<0.001	2.52±0.03	2.46±0.03	0.11
d21	4.66[a]±0.05	4.34[b]±0.07	<0.001	4.55±0.06	4.45±0.06	0.24

[a,b] Means and standard errors within row for each treatment with no common superscript are different ($P<0.05$).

[1] A probiotic mixture of *L. reuteri, B. animalis, E. faecium, and P. acidilacti.*

[2] Mean of 10 replicate pens. Non-injected are non-injected eggs in a separate hatcher; probiotic are eggs injected with 1ml of probiotic inoculations at 10^6cfu/ml in a separate hatcher

[3] Control are birds fed diet containing 100% of NRC (1994) Ca and P requirements; deficient are birds fed diets formulated to contain 80% of NRC Ca and P requirements

Figure 3.1: Tibial and femoral morphology of DOH, 7 and 21d old poults fed either control or Ca and P deficient diets with or without *in ovo* probiotic[1] injection at 25d of incubation. A.) Femur length. B.) Tibia length. C.) Femur width. D.) Tibia width. Non-injected are non-injected eggs in a separate hatcher; probiotic are eggs injected with 1 ml of probiotic inoculations at 10^6cfu/ml in a separate hatcher. Control are birds fed diet containing 100% of NRC (1994) Ca and P requirements; deficient are birds fed diet formulated to contain 80% of NRC (1994) Ca and P requirements. Results are expressed the means ± SE of 10 replicate pens. [a,b,c] Bars with no common letter at same age are different (P<0.05).

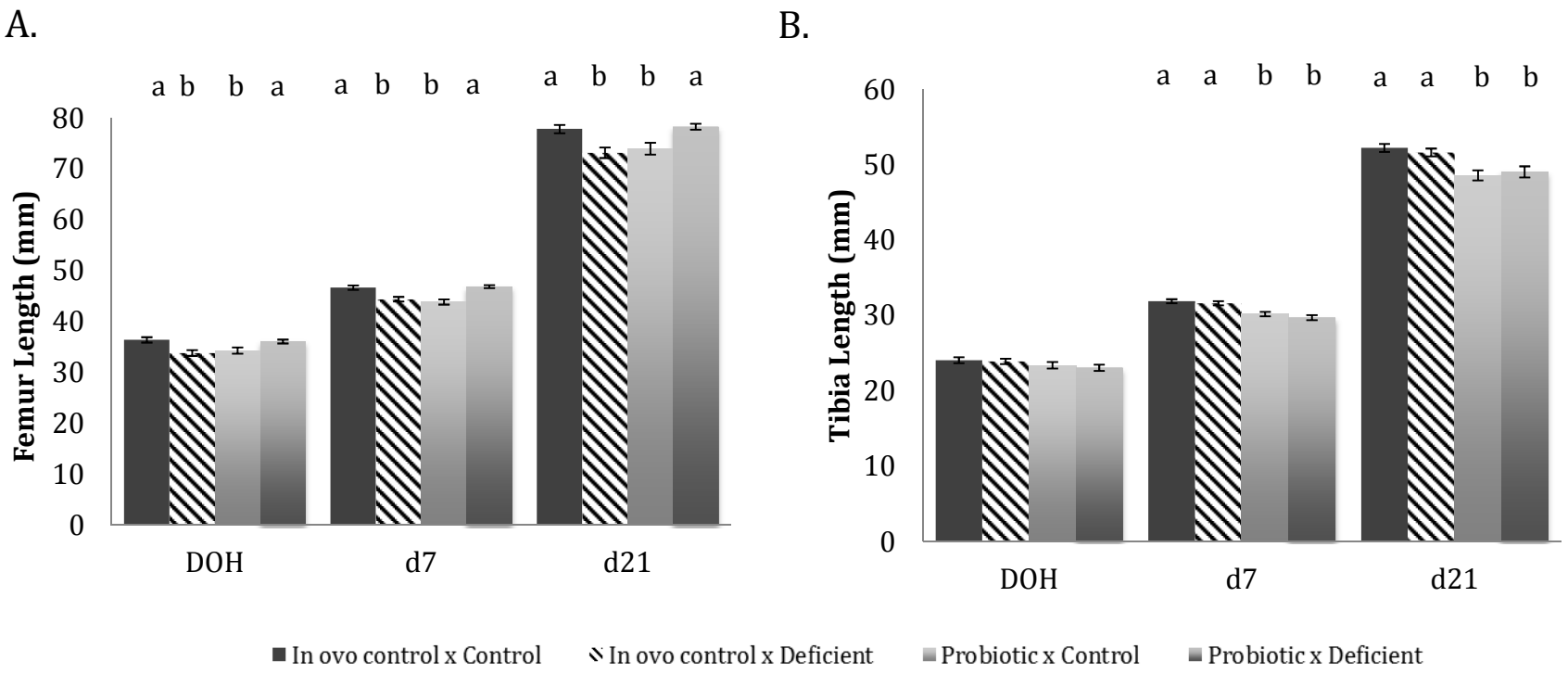


Figure 3.1 (cont'd)

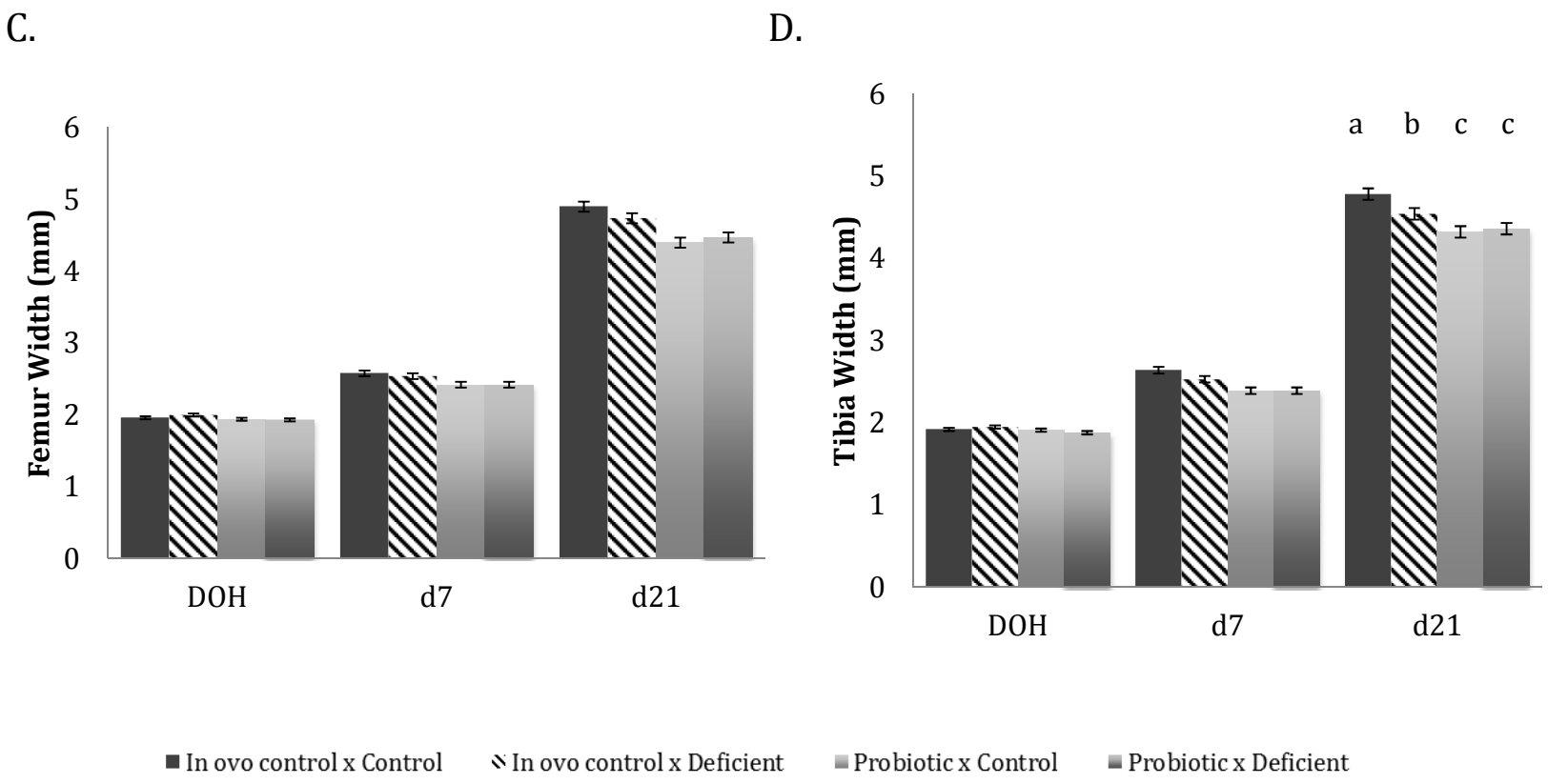
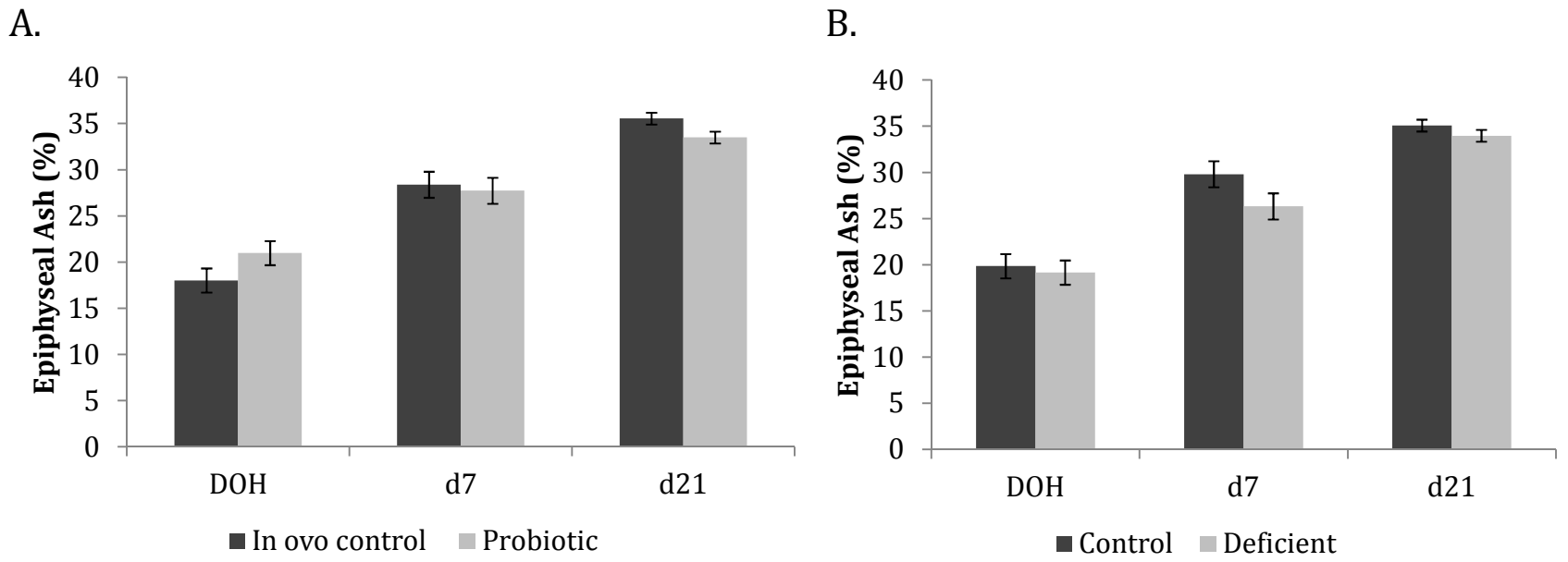


Figure 3.2: The main effect of *in ovo* probiotic injection and either standard or deficient nutrient diets on tibial ash (%) in turkey DOH, d7 and d21 turkey poults. A.) The effect of probiotics administered at 25d of incubation on the tibial epiphyseal ash (25% of each end of the tibia) of turkeys. B.) The effect of standard or a nutrient deficient diet on the tibial epiphyseal ash (25% of each end of the tibia) of turkeys. C.) The effect of probiotics administered at 25d of incubation on the tibial diaphyseal ash (middle 50% of the bone) of turkeys. D.) The effect of standard or a nutrient deficient diet on the tibial diaphyseal ash (middle 50% of the bone) of turkeys. Non-injected are non-injected eggs in a separate hatcher; probiotic are eggs injected with 1 ml of probiotic inoculations at 10^6cfu/ml in a separate hatcher. Control are birds fed diet containing 100% of NRC (1994) Ca and P requirements; deficient are birds fed diet formulated to contain 80% of NRC (1994) Ca and P requirements. Results are expressed the means ± SE of 10 replicate pens. [a,b] Bars with no common letter at same age are different (P<0.05).



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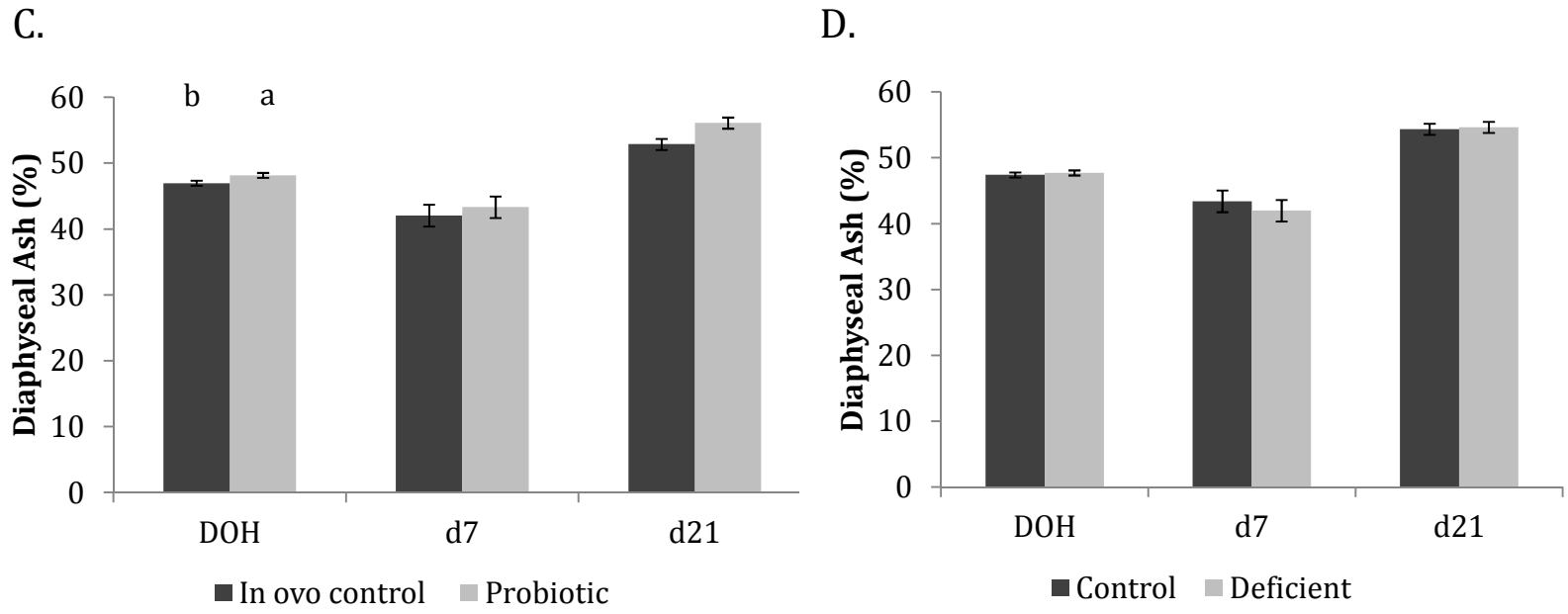


Figure 3.3: The main effect of *in ovo* probiotic injection and either standard or deficient nutrient diets on femoral ash (%) in turkey DOH, d7 and d21 turkey poults. A.) The effect of probiotics administered at 25d of incubation on the femoral epiphyseal ash (25% of each end of the tibia) of turkeys. B.) The effect of standard or a nutrient deficient diet on the femoral epiphyseal ash (25% of each end of the tibia) of turkeys. C.) The effect of probiotics administered at 25d of incubation on the femoral diaphyseal ash (middle 50% of the bone) of turkeys. D.) The effect of standard or a nutrient deficient diet on the femoral diaphyseal ash (middle 50% of the bone) of turkeys. Non-injected are non-injected eggs in a separate hatcher; probiotic are eggs injected with 1 ml of probiotic inoculations at 10^6cfu/ml in a separate hatcher. Control are birds fed diet containing 100% of NRC (1994) Ca and P requirements; deficient are birds fed diet formulated to contain 80% of NRC (1994) Ca and P requirements. Results are expressed the means ± SE of 10 replicate pens.[a,b] Bars with no common letter at same age are different (P<0.05).

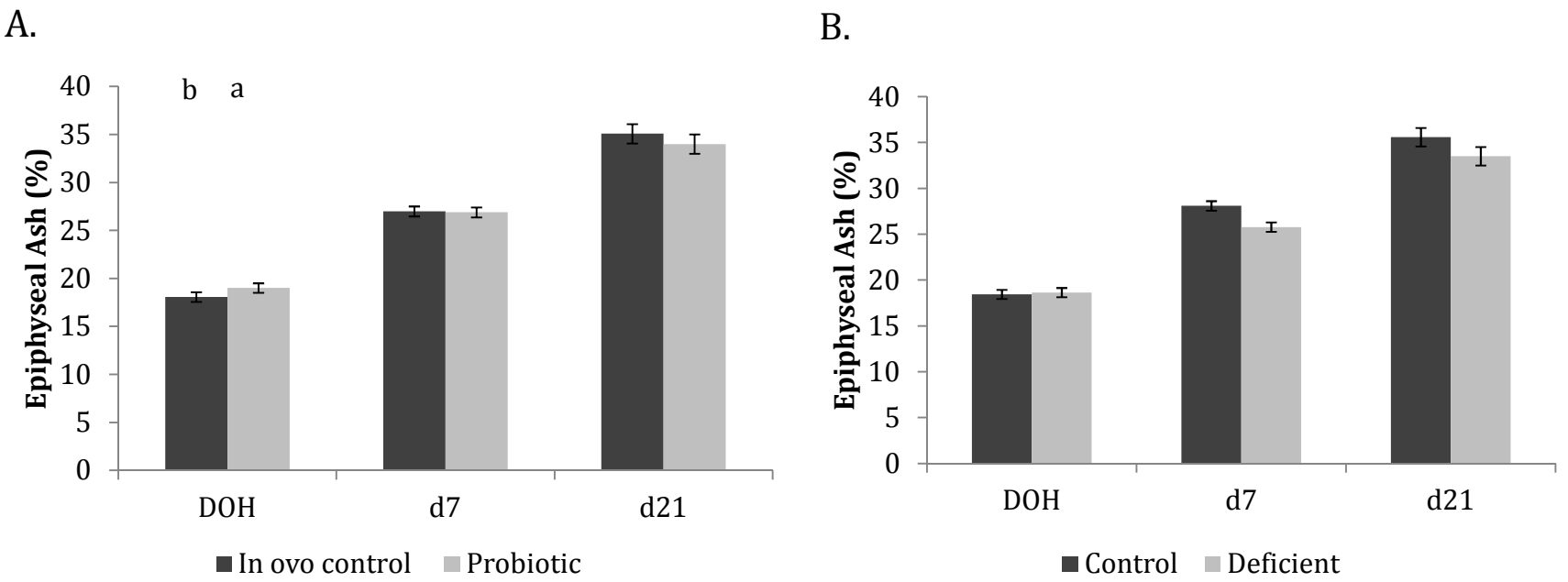


Figure 3.3 (cont'd)

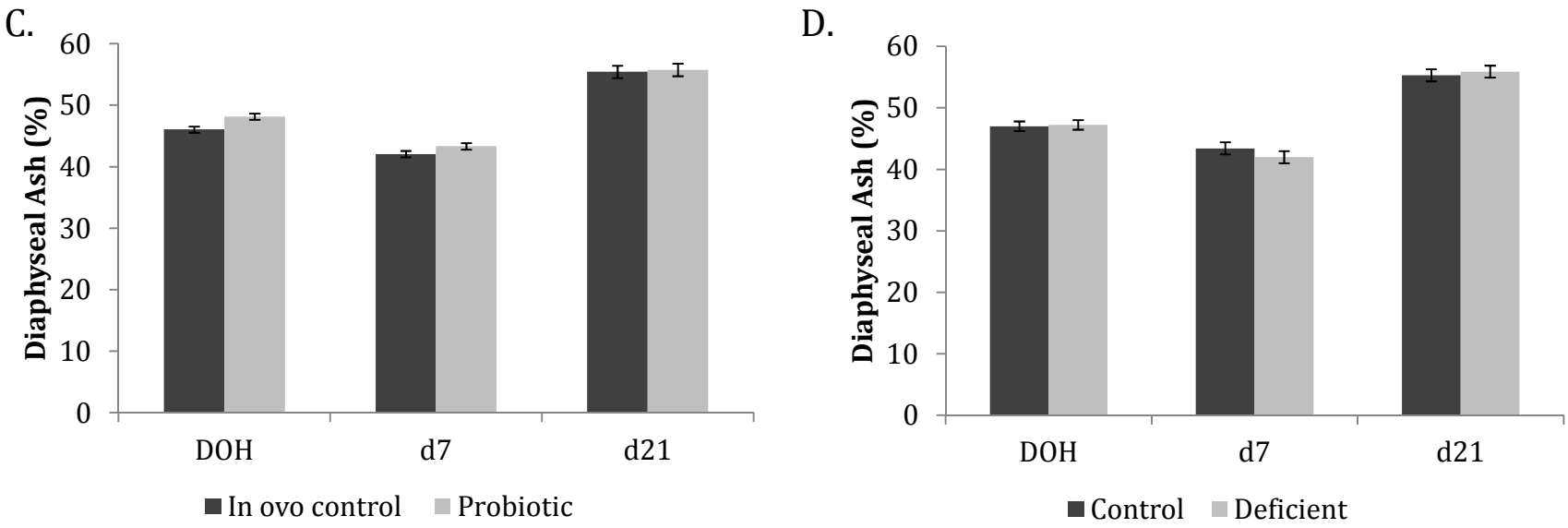
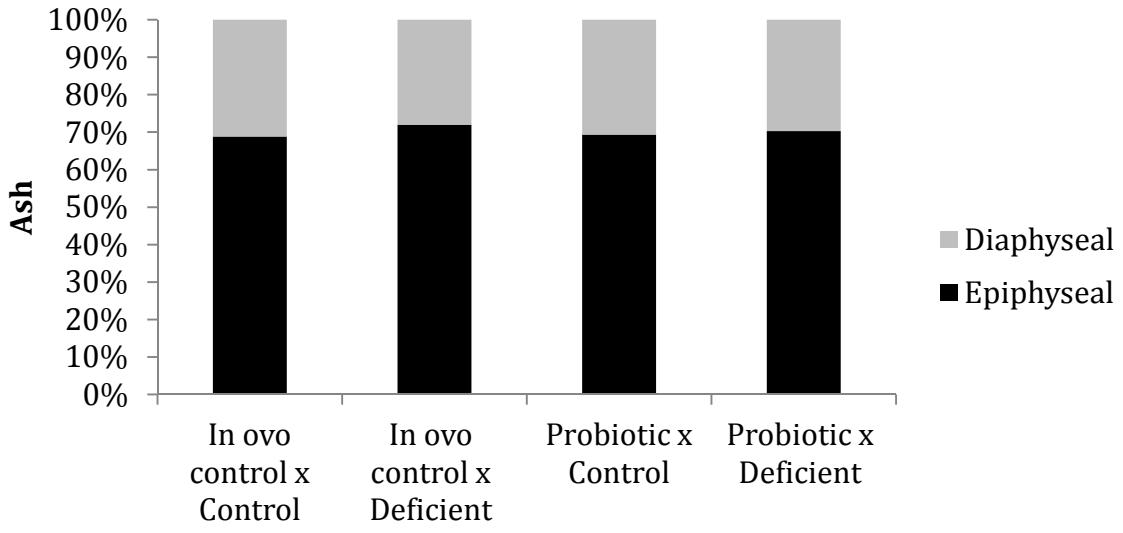


Figure 3.4: The interaction of *in ovo* probiotic injection at 25d of incubation and either standard or deficient nutrient diets on (A) tibial ash from DOH turkeys, (B) tibial ash from 7d old turkeys and (C) tibial ash from 21d old turkeys. Epiphyseal is 25% of each end of the tibia. Diaphyseal is the middle 50% of the bone. Non-injected are non-injected eggs in a separate hatcher; probiotic are eggs injected with 1 ml of probiotic inoculations at 10^6cfu/ml in a separate hatcher. Control are birds fed diet containing 100% of NRC (1994) Ca and P requirements; deficient are birds fed diet formulated to contain 80% of NRC (1994) Ca and P requirements.

A.



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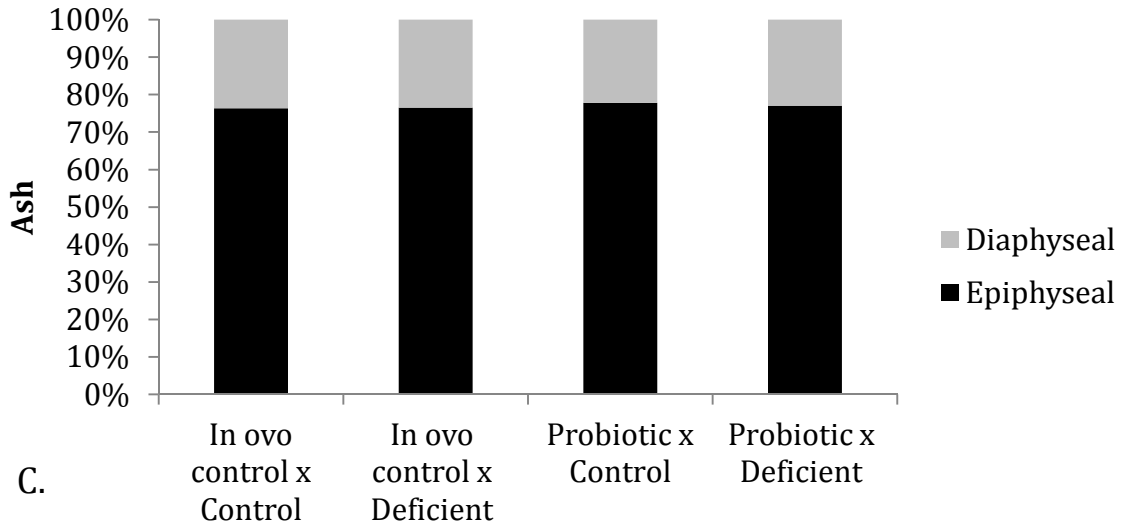
B.



C.

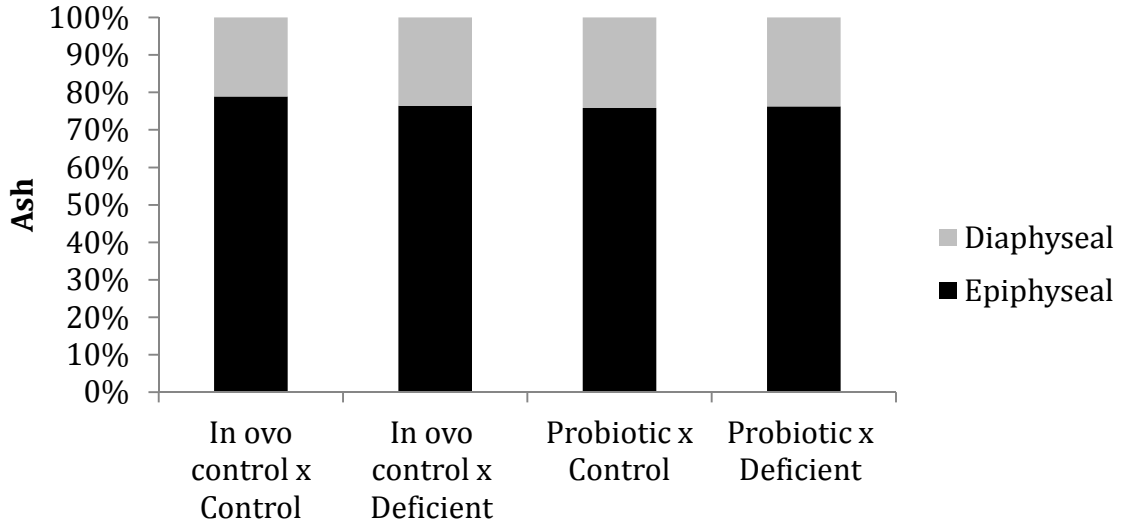


Figure 3.5:. The interaction of *in ovo* probiotic injection at 25d of incubation and either standard or deficient nutrient diets on (A) femoral ash from DOH turkeys, (B) femoral ash from 7d old turkeys and (C) femoral ash from 21d old. Epiphyseal is 25% of each end of the femur. Diaphyseal is the middle 50% of the bone. Non-injected are non-injected eggs in a separate hatcher; probiotic are eggs injected with 1 ml of probiotic inoculations at 10^6cfu/ml in a separate hatcher. Control are birds fed diet containing 100% of NRC (1994) Ca and P requirements; deficient are birds fed diet formulated to contain 80% of NRC (1994) Ca and P requirements.

A.

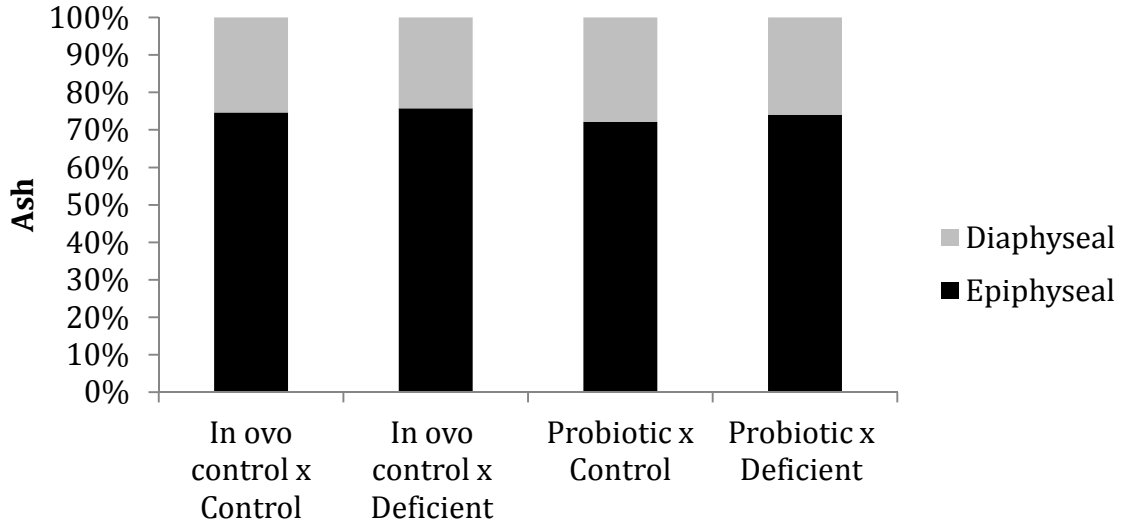
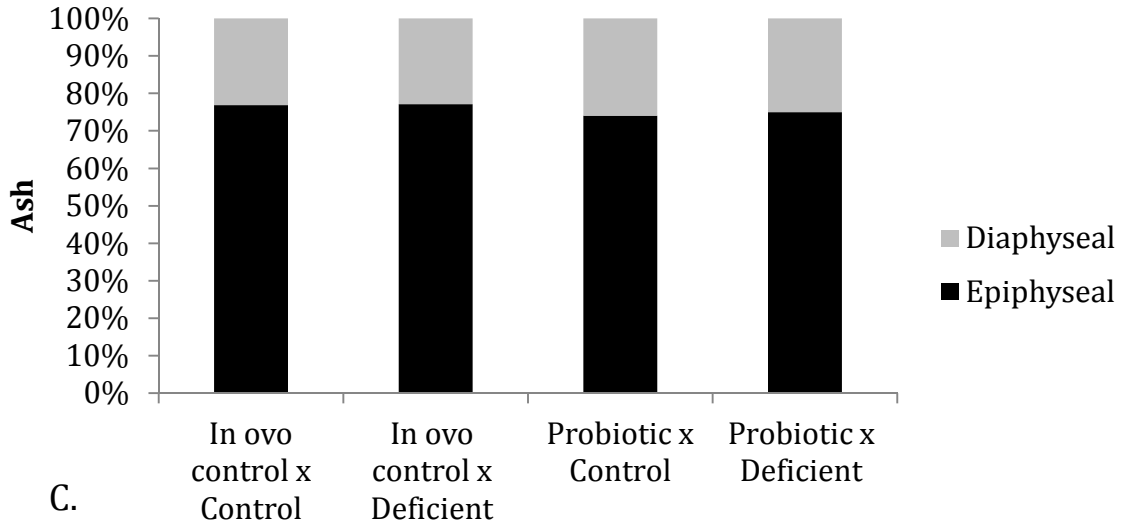


Figure 3.5 (cont'd)

B.



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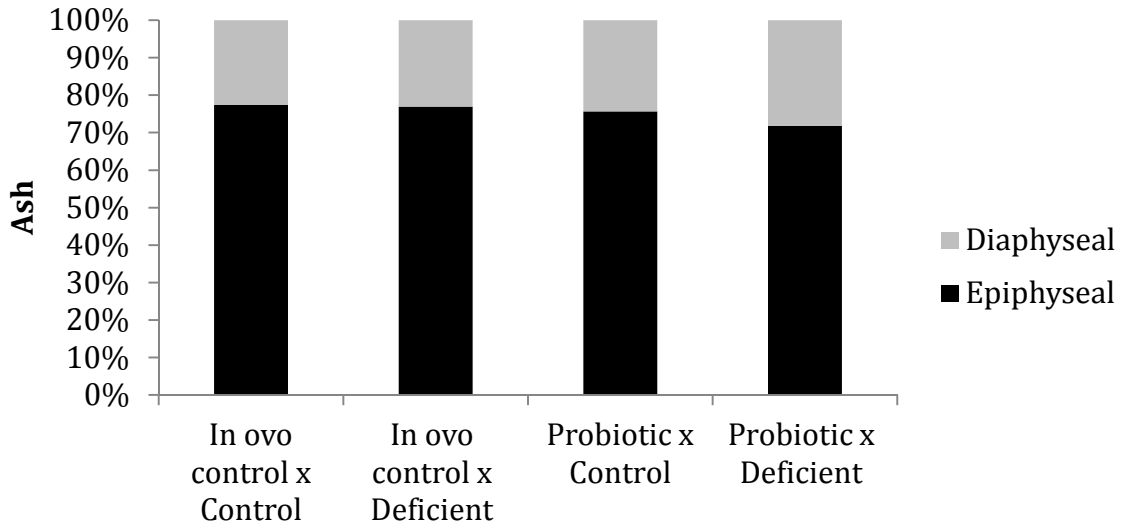

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SUMMARY AND FUTURE STUDIES

The current studies focused on the effect of *in ovo* probiotic injection on early life development of the poult. *In ovo* probiotics can be injected at approximately 10^6 cfu without altering hatchability. The injection improved FCR in turkey poults that were fed NRC recommended diets and increased diaphyseal ash levels in the femur early in life. The studies had some conflicting results as well; the injection of 10^6 cfu reduced hatchability and livability in the third study. Though the injections had the same amount of bacteria in the egg, there is some possible mechanism or unmeasured condition in the bird or the egg that allowed the amount of bacteria in the intestine to be higher. Any studies in the future should try to tease out the mechanism of growth of the bacteria in the egg. The current studies demonstrated that the effect of probiotic injections appears to last approximately three days. What is not clear is how this alteration can influence the birds later in life, specifically the increase in bone mineralization.

Alternative strains

The performed studies examined a single commercially available probiotic that was a mixture of several strains of bacteria. If the beneficial effects were a result of a single strain or a combination of all of the strains is unclear. There are also multiple commercial strains available, and these variable strains are expected to yield different results. Futures studies could identify the bacteria that are most beneficial to turkeys and which ones have little effect even though they are a part of normal gut microflora population.

Delivery method

Future studies should compare *in ovo* delivery to more common delivery methods, such as spray or drip delivery at the hatchery. These commonly used delivery methods may work to improve the effect of *in ovo* probiotics if used in concert with one another. Delivery in the diet or drinking water may assist *in ovo* injections as well. Turkeys showed improved FCR in study two, and the birds may have continued to improve FCR if prolonged dosage of bacteria were used. *In ovo* probiotics may increase the benefits of probiotics post hatch.

Combination with current vaccinations

Vaccines are injected into millions of eggs each year. Vaccines are given at a different location and a different dosage than the probiotic injections of the current studies. Producers are normally hesitant to invest in expensive new equipment. This makes the importance of studying how *in ovo* probiotics can work with current vaccination techniques. The egg can only hold a finite volume of liquid, normally believed to be one to one and one half milliliters. If injection machines can be repurposed to inject both vaccines and bacteria, the practically of the bacterial injections improves.

In ovo probiotics can be a benefit to turkeys, but are a long way from commercial application. The technology exists to inject the eggs. If several of the proposed studies were completed and shown to be beneficial, *in ovo* probiotics could become and important industrial practice in the future.