The transition from a lipid-rich in-ovo environment to a carbohydrate- and protein-rich diet is perhaps the biggest challenge that hatchlings must overcome during early life. Because amniotic fluid swallowing naturally prepares the embryonic digestive tract for post-hatch feed intake, amniotic fluid supplementation of the late-term avian embryo (i.e. in-ovo feeding – IOF) has been shown to stimulate enteric maturation of poultry and alleviate the stress of hatching. Moreover, rapidly dividing cells (e.g. intestinal epithelial cells) preferentially metabolize preformed nucleotides; hence, dietary nucleotide supplementation can improve post-hatch enteric maturation. Thus, our working hypothesis was that nutritional strategies influencing in-ovo nutrition and post-hatch dietary feed intake enhance the enteric development in perinatal turkey embryos and poults. Our study was among the first to characterize the ultra-structural changes in the small intestinal mucosa of the perinatal turkey embryo and poult (Chapter II). Furthermore, electron microscopy revealed that microbial colonization in the avian digestive tract begins prior to hatch, as early as 17E. Molecular profiling of these bacteria confirmed that some of the species include Bacillus, Lactobacilli, Pseudomonas and several uncultured bacteria (Chapter III). We showed that IOF accelerates the ultra-structural maturation of the jejunum mucosa of turkey embryos and poults around the time of hatch, and these effects are associated with elevated plasma triiodothyronine (T₃) levels, a potent stimulator of morphological development of the mucosa (Chapter IV). Finally, we showed that dietary nucleotide supplementation to IOF turkey poults enhances the enteric maturation after hatch and the positive effects of nucleotide supplementation appear to be associated with stimulation of feed intake post-hatch. Therefore, we concluded that: (1) profound ultra-structural adaptations of the intestinal mucosa coincide with the swallowing of the amniotic fluid by the embryo; (2) microflora colonization of the avian gut begins well before hatch, (3) IOF stimulates ultra-structural maturation of the small intestine at hatch and these effects involve up-regulation of genes mediating epithelial cell proliferation as well as higher T₃ levels. (4) Dietary nucleotide supplementation of IOF turkey poults enhances enteric maturation post-hatch, which coincide with a consistent stimulation of poults appetite by dietary nucleotide supplementation.
Nutritional Influences on the Ultra-structural Development of the Small Intestinal Epithelium of the Perinatal Turkey Embryo and Poult

by

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DEDICATION

To

My parents Rogelio and Carlota

My wife Elaine and my son
BIOGRAPHY

Diego V. Bohórquez (born on March 5, 1983, in Baeza, Napo) Ecuadorian Nutritional Physiologist. The youngest son of Rogelio Bohórquez and Carlota Montero, he began his carrier in the field of life sciences at the Pan-American Agricultural School, Zamorano in Honduras. During his junior year, he was awarded a travel grant to participate in the 2003 International Student Festival in Trondheim, Norway. This meeting opened great opportunities for subsequent conferences, but most importantly, for him to become part of a global network of students that believe in social development through education and equality. Soon after college graduation, Diego was granted a scholarship to continue graduate studies in Nutrition at North Carolina State University. This opportunity was made available by Dr. Abel Gernat and Dr. Peter Ferket, for which Diego is truly grateful. During his M.S. program he met his significant other Elaine Barbara Brooks. Diego and Elaine got married on July 21, 2007. Diego continued to pursue a Ph.D. in Nutrition under Dr. Peter R. Ferket’s supervision. His area of expertise focuses on the perinatal development of the digestive tract and the influence of early nutrition on its maturation and health. Upon completion of his Ph.D., Diego will continue on to a post-doctoral fellowship at the Gastroenterology Division of the Duke University School of Medicine. Diego and Elaine are expecting their first child in April 2010.
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CHAPTER I

LITERATURE REVIEW
1.1. INTRODUCTION

As the world population has doubled from 3 to 6 billion during the last four decades, the global demand for safe and affordable sources of protein has increased linearly during this period (USCB, 2009). In an effort to satisfy the increasing demand for meat products, the growth and feed efficiency of farm animals have reached limits that not long ago were considered unthinkable. A clear example of such progress is the advances in poultry performance. Today, modern turkeys need about 18 weeks to reach the commercial weight of 15kg whereas, 40 years ago, commercial turkeys of the same age would not have reached 8kg at this age (Havenstein et al., 2007). However, as consumers become more aware of production practices, these advances are becoming more restrained by issues of social conscience, such as biosecurity, food safety, environmental stewardship, and animal welfare (Ferket, 2008).

Current trends in growth rates and meat yield in poultry have been driven by a continuous genetic selection for traits of commercial importance, such as body weights and meat yield, leaving unattended traits of major physiological importance that often compromise livability. This issue has particularly affected modern commercial turkeys. Selection for fast-growing, high breast meat yield has changed the overall body structure and conformation of the birds. As a consequence, the incidence of lameness and skeletal abnormalities in turkeys can exceed 15% (Lilburn, 1994; Vaillancourt et al., 1999). Considering that the US alone produces about 8 billion pounds of turkey per year, skeletal abnormalities lead to losses in the hundreds of millions of dollars through mortality and condemnation losses (NASS, 2009; Uni and Ferket, 2004).

Although the symptoms of lameness and bone fractures frequently are observed towards the end of the growing period, the actual bone developmental abnormalities that lead to these events often are programmed early in life (Dibner et al., 2007; Ferket et al., 2009; Vaillancourt et al., 1999). Abnormal incubation temperatures or delayed placement are stressors that can further exacerbate these issues. Moreover, as fast-growing strains of turkeys require less time than their ancestors to achieve a market weight, the period of embryonic development becomes a greater proportion of the bird’s life (Ferket, 2008). Today, the 4 weeks of incubation and the first 2 weeks of life post-hatch
account for about 30% of the commercial life of a 20kg turkey. Consequently, modern turkeys are more susceptible to aberrations during early development than their ancestors because their metabolic demands for growth are much greater.

In turkeys as well as in other meat birds, energy available for growth is partly limited by the size of the digestive tract (a supply organ) and early investment of growth resources for the development of this organ favors a subsequently high growth rate capacity (Mitchell and Smith, 1991). Genetic selection for growth has altered the gastrointestinal tract (GIT) of poultry to such extend that digestive processes are not fully developed at hatch (Croom et al., 1999; Sell et al., 1991). Hence, an insufficient capacity of the intestinal tract for nutrient absorption negatively affects performance and survival. Mortality in turkey poults is particularly high among poultry, rates can reach up to 5% during the first week post-hatch, the symptoms appear to originate from an insufficient enteric capacity for nutrient absorption to support accelerated tissue growth (Uni and Ferket, 2004). Because the intestine is the primary nutrient supply organ, maturation of enteric function increases the resistance to pathogenic invasion and allows the bird to properly utilize dietary nutrients for growth and feed efficiency (Dibner et al., 1998; Uni and Ferket, 2004; Uni et al., 1999). Immediate access to dietary nutrients post-hatch is a well-known stimulus that ensures proper morphological and physiological development of the GIT in young hatchlings (Noy and Sklan, 1997; Potturi et al., 2005; Sklan, 2001; Turner et al., 1999; Uni et al., 1999). Facilitating enteric development during the post-hatch period is critical as the poult rapidly switches from a lipid-rich in-ovo nutrient supply to a carbohydrate- and protein-rich diet post-hatch. Although the effects of early feeding strategies on the development of the GIT of poultry have been described previously (Corless and Sell, 1999; Sklan, 2001; Uni et al., 1999), little work has been done to understand the morphological adaptations of the embryonic GIT that occur in preparation for post-hatch life and the potential of in-ovo nutrition to accelerate this transition.

Although the development of the gut occurs throughout incubation (Romanoff, 1960), its functionality only begins when the turkey embryo has swallowed most of the amniotic fluid at about 23 days of incubation. In mammals, oral imbibing of the amniotic fluid provides the embryo with its first meal, which is an indispensable event in the normal development of the GIT (Cleveland et al., 1991;
Wagner, 2002). Deficiencies in this essential fluid during fetal development are highly correlated with a list of perinatal maladies, some of which are strongly related to gastrointestinal tract dysfunction (El-Haddad et al., 2004). For example, restriction of fetal swallowing in sheep fetuses causes small intestinal atresia and impairs the internalization of the digestive tube into the abdominal cavity, a condition known as gastroschisis (Cleveland et al., 1991; Underwood and Sherman, 2006; Wagner, 2002). In contrast, supplementation of the amniotic fluid of chicken and turkey embryos can accelerate the morphological development and digestive capacity of the intestinal mucosa of birds (de Oliveira, 2007; Foye et al., 2003; Tako et al., 2004). Although the effects of amniotic fluid swallowing in the GIT development have been fairly well described in mammals, there is little information on the effects of amniotic fluid swallowing on the development of the digestive capacity in poultry.

With the advancement of probiotics, prebiotics and other functional foods that may modulate gut health, there is a need to understand the ultra-structural characteristics of the intestinal mucosa that precede colonization of symbiotic and pathogenic microflora. Understanding how imbibition of amniotic fluid by the late-term embryo prepares the intestinal mucosa for post-hatch feeding will allow us to design early feeding strategies that are more effective in sustaining intestinal development in rapidly growing birds and improving overall intestinal health. This review of the literature will cover some aspects of the perinatal development of the digestive tract including morphological adaptations, mucus blanket function, and the appearance of the gut microbiota. The review will also discuss the role of amniotic fluid swallowing on the ultra-structural maturation of the gut epithelium and the potential of early feeding strategies, such as in-ovo feeding and dietary nucleotide supplementation, to enhance intestinal development and health of poultry.

1.2. PERINATAL DEVELOPMENT OF THE AVIAN DIGESTIVE TRACT

From an evolutionary and developmental perspective, the gastrointestinal tract is an early innovation, and some scientists argue that this organ early in evolution was the first primitive brain of multicellular animals. This theory is supported by the fact that the enteric nervous system that forms the third branch of the autonomic nervous system may harbor as many or more neurons as the spinal
cord (Gershon, 1999). Besides maintaining close communication with the brain, the gut also forms a selective and permeable barrier to allow the entrance of nutrients into circulation and maintain harmful microorganisms out of circulation. This is a titanic task considering that the GIT may harbor about 10 times more bacterial cells than the total number of eukaryotic cells in the entire body (Eckburg et al., 2005; Guarner and Malagelada, 2003). In defense against this microbial population, the intestinal epithelium has its own immune surveillance system, known as gut associated lymphoid tissue (GALT), which contains more than 70% of the immune cells found in the body (Kagnoff, 1993). All these systems and their interconnections within the gut must be ready at the time of hatch when an influx of exogenous dietary nutrients along with microorganisms and other strange molecules, come in contact with the gut epithelium. Thus, the digestive tract must be functionally and morphologically “ready” to accommodate the rapid developmental transition that occurs during the first few days after hatch. Although there have been a number of studies on the gross and microscopic anatomy of the avian digestive tract with regard to its influences in feed utilization, little is known about the ultra-structural changes that accompany this transitional period. Below, is a description of the major histological characteristics of the avian GIT (Figure 1), as well as some observations about the ultra-structural organization of its epithelium drawn from light and electron micrographs.

1.2.1. General aspects of the perinatal digestive tract in birds

The avian digestive tract starts with a keratinous beak that surrounds the oral cavity where the tongue is found. Although very little is known in terms of developmental characteristics of the avian tongue, the signaling cascades and genes mediating its development are highly conserved among vertebrates and invertebrates (Huang et al., 2001). Contrary to mammals, the tongue in turkeys and chickens is a triangular-shaped bone and contains a synovial joint with articulated cartilages (Figure 2A). This morphological adaption serves to sort particles based on size and shape and to scoop feed into the gullet (Denbow, 2000). The surface is covered with heavily keratinized stratified squamous epithelium (SSE), giving the tongue a characteristic coarse texture (Figures 2-A1, 2-A2).
Figure 1. The avian digestive tract: It is limited cranially at the oral cavity where the tongue (Ton) is found. The upper esophagus (upEso) follows, and immediately a localized dilation occurs which is known as the crop. Particles are stored in the crop and released into the proventriculus (Pro) or glandular stomach. Here, feed particles are exposed to gastric juices and are subsequently ground in the gizzard (G) or muscular stomach (Romanoff, 1960) to reduce its size and increase the surface area exposed to digestive enzymes. The liver along with the gall bladder (not shown) participates primarily in the digestion and absorption of carbohydrates and lipids. The pancreas (Pan) is surrounded by the duodenal loop (Duo) and is primarily responsible for hormone (e.g. insulin) secretion that coordinates gut motility and nutrient absorption. The jejunum (Jej) extends from the end of the duodenum to the Meckel’s diverticulum (Meckel’s Div). This is a reminiscent of the yolk sac connection to the small intestine. The ileum (Ile) continues from the Meckel’s diverticulum to the ileo-cecal junction where both ceca (Cec) are connected. The two horn-like ceca (Cec) are the major factory of intestinal microflora anaerobic reproduction. After the ceca, the colon (i.e. large intestine) is found at the end of the tract. The bursa of Fabricius (Bur) is situated on the dorsal end of the colon. The alimentary canal terminates in a chamber known as the cloaca (Clo), into which open the genital and urinary ducts (Romanoff, 1960; Turk, 1982).
Under the electron microscope, (at 10,000X or above) a series of microfolds can be distinguished on the surface of the cells that form the SSE (Figure 2-A2). Although the contribution of the tongue to the digestion of carbohydrates, lipids and proteins is not significant (Jerrett and Goodge, 1973; Krogdahl, 1985), it is a major site for taste sensory ability. On the tongue of the day-old chick, about 316 taste buds can be found (Ganchrow and Ganchrow, 1985). Unlike mammals, the taste buds in chickens develop well before hatch, around 17 days of incubation (Ganchrow and Ganchrow, 1987). Interestingly, the appearance of the taste buds first occurs around the time that the embryo swallows the amniotic fluid. However, it is not known whether amniotic fluid swallowing initiates the ability to sense taste, or if it programs taste recognition in chickens.

Subsequent to the oral cavity, the esophagus has a number of longitudinal folds that allow it to distend as the food bolus is conveyed to the stomach by peristaltic contractions. Except for a few species of birds, such as ostriches, a localized dilation of the esophagus forms a crop (Denbow, 2000), which functions primarily for food storage. The crop may also play a role in regulation of feed intake and the colonization of enteric microflora (Denbow, 1994; Fuller and Brooker, 1974). For example, the crop of the Amazonian wild bird Hoatzin (Opisthocomus hoazin) is an active center of foregut fermentation similar to that in ruminants. This bird is an obligate folivorous with an enlarged crop and bulky esophagus, where bacterial fermentation of otherwise toxic plants occurs (Grajal et al., 1989). Similar to the tongue, the esophagus and crop are lined with incompletely keratinized SSE into which open numerous mucous glands (Figure 2B, 2-B1) (Denbow, 2000). These glands secrete abundant mucus at the time of hatch (Figure 2B), which serves to lubricate the food bolus that is propelled down the proventriculus by peristaltic contractions.

Whereas in mammals the stomach consists of a single chamber (except for ruminants), in birds, the stomach is a two-chamber organ: a glandular stomach known as proventriculus and a muscular stomach commonly called gizzard (Denbow, 2000). Food particles entering the proventriculus are exposed to a very acidic environment rich in digestive enzymes and mucin that protects the epithelium from self-digestion (Figure 3A). The thickened mucosa of the proventriculus contains several glands (Figure 3A&C), which have their duct openings into papillae scattered over the luminal
Figure 2. Ultrastructural arrangement of the tongue (A) and esophagus (B) of the turkey poult. Light micrographs reveal a lining of stratified squamous epithelium (SSE) that covers the surfaces of the tongue, esophagus and crop. The SSE (A1) in the tongue is heavily keratinized and each cell is covered by a series of microfolds (A2). The epithelium in the esophagus (B) is folded and each fold forms ridges where some cell projections occur (B1). This epithelium holds a series of secretory pits (B2) that actively produce mucins to maintain lubricated the inside of the esophagus. Light micrographs are a composite of several frames taken from tissue slides stained with Alcian Blue.
surface (Figure 3B) (Turk, 1982). Secretory cells lining these glands in birds perform similar functions
to the chief and parietal cells in mammals (Figure 3B), and in the chicken embryo these cells begin to
secrete acid after day 13 of incubation, in response to swallowing of albumen (Toner, 1965).

Once feed particles have been exposed to enzymatic digestion, they move into the gizzard where
mechanic digestion further reduces the particle size and increases the surface area exposed to
digestive enzymes (Romanoff, 1960). The gizzard has a powerful circular muscle and a rudimentary
longitudinal muscle. These layers of muscles produce rhythmical contractions that grind and mix the
particles with the digestive fluids. Although to the naked eye the lining of the gizzard’s lumen appears
smooth, a series of tubular glands can be distinguished by electron microscopy (Figure 4A-C). These
glands secrete the material forming the thick horny layer that protects the lumen of the gizzard from
abrasion (Turk, 1982). When particle size has been sufficiently reduced, the chyme passes into the
small intestine where most of the nutrient absorption is accomplished (Denbow, 2000; Turk, 1982).
The small intestine is commonly divided into duodenum, jejunum and ileum. Surrounded by the
duodenal loop is found the pancreas, which is a major player in the regulation of feed intake and
nutrient digestion through endocrine and exocrine mechanisms. On one hand, the pancreas
endocrine function is to release into circulation a series of hormones that include insulin, glucagon,
somatostatin pancreatic peptide and others, that regulate feed intake and gut motility, primarily
through feedback mechanisms (Denbow, 2000; Henderson et al., 1981). On the other hand, the
pancreas exocrine function is to secrete buffer (e.g. bicarbonate) and a series of enzymes for
digestion of lipids (e.g. pancreatic lipase), carbohydrates (e.g. pancreatic amylase) and proteins (e.g.
chymotrypsin) into the intestinal lumen to increase the pH of the feed bolus and to digest large
molecules into single nutrients that can be recognized and absorbed by epithelial cells (Jin et al.,
1998; Krogdahl, 1985). The liver also contributes to digestion and absorption of lipids in the small
intestine. The bile secreted by the liver is stored in the gall bladder prior to secretion into the
duodenum and contains NaCl, NaHCO3, Ca, bilirubin, bile salts, cholesterol, lecithin, fat soluble
vitamins (A, D, E, K), some toxic products conjugated for secretion as well as some heavy metals
such as Zinc (Denbow, 2000). The jejunum follows the duodenum and extends to the Meckel’s
Figure 3. Cross-section of the proventriculus or glandular stomach of the day-old turkey poult. The proventriculus contains several glands (A&C) that have their duct openings (B) into papillae scattered over the luminal surface. Secretory cells that perform similar functions to the chief and parietal cells in mammals line these glands (B). These secretions help in the digestion and also protect the epithelium from acid damage.
diverticulum, which is the remnant of the yolk sac connection to the small intestine. The ileum completes the small intestine and extends from the Meckel’s diverticulum to the ileo-cecal junction, where both cecal tonsils are connected.

The intestinal wall is covered by finger-like projections or villi that greatly increase the surface exposed to the digested nutrients. A single layer of epithelial cells lines the surface of these villi. Absorptive enterocytes comprise the majority of the epithelia lining each villus; however, only mature cells on the apical end have the most active digestive and absorptive capabilities (Moran Jr, 1985). Microvilli lining the apical end of enterocytes further enhance the luminal surface area. Extensions of actin bundles that form the core of microvilli extend to the lumen forming a terminal web or microcalix (Hirokawa and Heuser, 1981). This is the attachment site for mucins that form an unstirred water layer where digestive enzymes, immunoglobulins and other important molecules (e.g. bacterial attachment sites) are harbored.

Digested nutrients are recognized by transporters and internalized into enterocytes before entering circulation. Although, the absorptive capacity of the epithelium decreases towards the end of the small intestine, this is a very dynamic process, influenced by the presence and concentration of nutrients, gut microbiota and environmental factors. For instance, Hulan and Bird (1972), reported that the concentration of \( \alpha \)-amylase in the secreted pancreatic juice of chicks varies according to the amount of dietary starch concentration in the digesta. In addition, morphological adaptations such as villi lengthening or shortening can occur depending on the availability and nutrient density of food (Moran Jr, 1985). Villi may also lengthen in response to “competition” for nutrients with normal gut microbiota (Cook and Bird, 1973). Although there are several reports in the avian literature about the ultra-structural adaptations of the small intestinal epithelium in response to dietary and environmental changes, little is known about the ultra-structural changes that take place prior to hatch. There is a need to delineate the micro-anatomical baseline of the small intestinal mucosa prior to hatch in order to better understand the influence of diet and stressors post-hatch. This issue is addressed in the second chapter of this dissertation.
Figure 4. Cross-section of the gizzard of the day-old turkey poult. The gizzard is formed by a several layers of circular and longitudinal smooth muscle (A&C) that forcefully contract to mechanically digest feed particles entering from the proventriculus. Alcian blue staining (A) reveals a uniform layer of mucus that underlies the cuticle of the gizzard. This cuticle is densely populated with tubular glands, which secrete a mucus-like material that forms the thick horny layer that protects the lumen of the gizzard from abrasion.
Immediately after the small intestine, the ceca follow. There are several important functions that occur in this organ including: a) microbial fermentation of undigested substrates (e.g. cellulose, non-starch oligosaccharides, resistant starch and endogenous secretions) (Duke et al., 1984; Juzefiak et al., 2004; Vispo and Karasov, 1997); b) absorption of water and nitrogenous components (Chaplin, 1989); and c) production of immunoglobulins and antibodies (Lebacq-Verheyden et al., 1972). The morphological structure of this organ is as diverse as its function. In some species, the ceca are completely absent (e.g. hummingbirds) or rudimentary (e.g. pigeons), whereas in other species the ceca are either paired (e.g. parrots), singular (e.g. herons), or consist of a double pair (secretary bird) (Denbow, 2000). In poultry, the ceca are divided in 3 distinct areas. At the ileo-cecal junction, the villi are broad and contain crevices and goblet cell openings. As the junction area widens, the villi give way to ridges of epithelial cells. In the mid section, the muscular and mucosal layers become thinner and the crests of epithelium are longitudinally aligned along the length of the ceca. Towards the end, the crests flatten out and the surface becomes smooth (Clench and Mathias, 1995). The number of goblet cells decreases from mouth to the apex of the ceca, whereas the lymphoid tissue increases (Turk, 1982). These morphological adaptations allow the fluids and fine particles of undigested matter that enter the ceca to be retained for microbial fermentation. Active motility mixes the cecal contents and maintains a constant flow of fluids that continually fill and evacuate the organ (Clench and Mathias, 1995).

Finally, The large intestine or colon extends from the ileo-cecal junction to the cloaca, into which open the genital and urinary ducts (Turk, 1982). In poultry, this is a relatively short segment of the digestive tract as compared to that of mammalian species. Short ridges of villi line the mucosal wall of the colon in hatching fowl, although these become flatten with age (Romanoff, 1960). The bursa is conveniently located on the dorsal end of the colon, at the cloaca. Because the colon empties into the cloaca, this is an active site of immune surveillance. Antigens are sampled not only from normal flux but also from reverse peristalsis that moves contents back from the cloaca or even from outside the vent into the ceca (Dibner et al., 1998). In the process, B-cells in the bursa (Figure 6) are presented with antigens that direct the synthesis and release of antibodies (Denbow, 2000).
Figure 5. The ultrastructure of the small intestinal villi and enterocytes reveals the composition of one of the most elegant and elaborated structures in nature. Each villus (B&C) in the small intestine has a circulatory core (Vc) surrounded by a single layer of epithelial cells, which are predominantly absorptive enterocytes (En) and mucins secreting goblet cells (Gc). This can be specially appreciated in inset B, which is an scanning electron micrograph of a villi, which has unintentionally lost its tip during tissue preparation revealing the micro-arrangement of cells inside the villi. The mucins secreted by goblet cells are very hydrophilic polymers that are capable of binding and retaining large amounts of water. When combined with water, these mucin glycoproteins form an unstirred water layer or mucus blanket (Mb) that surrounds the villi and protects the epithelium. The mucus blanket also harbors immunoglobulins and digestive enzymes that help to break down feed particles into single-molecule nutrients. These nutrients are absorbed by enterocytes (D) via transporters located on the apical end where the brush border (A) or microvilli (Mv) is found. Enterocytes are “stitched” to each other by means of several different types of junctional proteins (e.g. tight junctions (Tj)) that also act as a protective and selective sieve. Transmission electron microscopic analysis of the microvilli reveals a series of actin polymers that extend from each microvilli and form a network of polymers known as glycocalix (Gc). Actin bundles that give structure to microvilli form a terminal web (Tw), which is visible at the base of the microvilli. This is an area an area densely populated by mitochondria (Mt) which provide energy molecules to support active transport of nutrients and ions. Note: The light micrograph in inset C was taken using the differential interference contrast (DIC) feature in a Leica DMIRB inverted microscope. The scanning electron micrograph in inset D is a composite of 4 micrographs taken at 10000X with a JEOL-5900 scanning electron microscope.
Figure 6. The ceca, colon and bursa of the hatchling turkey poult. The morphology of the epithelium lining the inside of the ceca varies tremendously at different points of the sacs. The ceca villi (Cv) are flatten towards the tip (A) and are arranged in ridges that run longitudinally with the sacs. The ceca are a major site of microbial activity specially that of anaerobic bacteria. At hatch (B), but before placement, several microcolonies of bacteria (Ba) can be observed in close interaction with the ceca villi (Cv). (C) The villi in the cecal pouches are short blunt projections arranged on top of crest-like formations. (D) Unlike the colon in adult fowl, the colon at hatch is lined with crests of villi. (E) These villi have a perfect finger-like shape and are lined by colonocytes. (F) The bursa found on the dorsal end of the colon is lined with a series of structures that resemble the tentacles of an octopus (F). These structures have a visible dome (G) where many different cell types are found. This is demonstrated by the variability of microvilli (Mva, Mvb, Mvc) found on the surface of the domes (H).
1.2.2. Regulation of the maturation of the gut epithelium prior to hatch

Genetic selection for faster growth rate and greater meat yield has changed the allometric growth characteristics of broilers and turkeys, and the relative proportion of visceral and vital organs has decreased accordingly (Havenstein et al., 2003a; Havenstein et al., 2003b). In perinatal broilers, the maturation of the gut epithelium is characterized by an increase in enteric villi size without affecting crypt depths (1990). This is a particular characteristic of birds, where proliferation of epithelial cells is not confined to the crypts but can also occur along the villi, at least during the first 10 days post-hatch (Geyra et al., 2001a; Uni et al., 1998). This process demands a coordinated supply of nutrients from the egg and an immediate dietary intake post-hatch to maintain the morphological development of the intestinal epithelium (Geyra et al., 2001a; Uni et al., 1995). In addition to the presence of specific nutrients (e.g. amino acids, vitamin A, zinc) essential to maintain cell proliferation and villi growth, a concerted influence of several different hormones (e.g. thyroid hormones, cortisol) and growth factors (e.g. epidermal growth factor, tumor necrosis factor alpha/beta) is required to maximize growth without compromising microanatomy of the gut epithelium. Below are discussed some of the factors that can influence morphological differentiation of the avian gut epithelium.

**Nutrients**

Because the epithelial cells of the digestive tract are the main interface between luminal nutrients and the bloodstream, these cells directly depend on both luminal and bloodstream sources for their nutrition (Duggan et al., 2002). Surprisingly few studies have been done to understand the specific role of nutrients on the embryonic GIT development in poultry, despite the advantages that the avian embryo provides to study nutrient influences in the absence of maternal input. The egg is rich in nutrients that likely play a role in the development of the epithelium (Yamamoto et al., 1997). The egg yolk and albumen contain an unsurpassable balance of amino acids, some of which are obligatory for maintaining intestinal mucosal mass and integrity through the synthesis of glutathione peroxidase, nitric oxide, polyamines, and purine and pyrimidine nucleotides (Wu, 1998; Yamamoto et al., 1997). Interestingly, the small intestinal mucosa catabolizes glutamine, arginine, proline and branched-chain
amino acids in the diet to such extend that 30 to 50% of these dietary amino acids are not available to extra-intestinal tissues (Windmueller, 1982; Wu, 1998). For instance, treatment of rats subject to ischemia reperfusion with glutamine, a major precursor for nucleotide biosynthesis, preserves mucosal glutathione concentrations and decrease lipid peroxidation (Harward et al., 1994). The protective properties of glutamine are of major importance in rapidly dividing cells where the production of free radicals can cause abnormal growth and accelerate cell apoptosis (Duggan et al., 2002). Likewise, arginine is an important precursor of nitric oxide (major signaling molecule) and polyamine synthesis. In particular, the polyamines (putrescine, spermidine and spermine) appear to mediate cell proliferation by providing a readily available energy source (e.g. succinate) (Bardocz et al., 1998), and by regulating specific genes involved in cell growth and apoptosis (Luk, 1990). Serine and threonine may also play an important role during the perinatal development of the GIT mucosa. These amino acids form up to 30% of the mucin glycoproteins, which are the structural networks of the mucus blanket that protects the epithelium (Corfield et al., 2000). Some of these mucins (e.g. MUC1 and MUC3) are transmembrane mucins that act also as intermediaries of growth factors (Nataro et al., 2005).

Besides amino acids, vitamin A has been shown to influence intestinal growth (Duggan et al., 2002). Its deficiency causes an overall reduction of epithelial cell proliferation and in particular, a reduced number of goblet cells in the crypt and villus (Rojanapo et al., 1980; Warden et al., 1996). Zinc, which is a central component of metalloenzymes and DNA/RNA polymerases, plays an important antioxidant and regulatory function in rapidly dividing cells (Filipe et al., 1995). In chicken embryos, it has been shown that intra-amniotic administration of Zinc-methionine enhances the expression of brush border enzymes and intestinal transporters, as well as the villus surface area (Tako et al., 2005). Although there may be several other nutrients (e.g. oligosaccharides found in amniotic fluid) that likely play a role directly or indirectly on intestinal maturation of the avian embryo and hatchling, more research is needed to understand their specific functions.
**Hormones**

Mammalian models have been used extensively to study the role of hormones in the regulation of the gut mucosal development. Glucocorticoids and thyroid hormones have been particularly well studied because of their major regulatory effects (Bernt and Walker, 1999; Thomson and Keelan, 1985). Glucocorticoids are major enteric modulators of structural and functional maturation that affect mucosal growth, acid secretion, digestive-enzyme induction and gastrin secretion (Thomson and Keelan, 1985). In fetal sheep, cortisol accelerates the rate of enterocytes migration, increases villus height and density, and reduces the thickness of the muscularis wall in the small intestine (Fowden et al., 2007; Thomson and Keelan, 1985; Trahair et al., 1987a; Trahair et al., 1987b). Moreover, adrenalectomizing the fetus prevents the onset of such changes in gut development (Trahair et al., 1987b). In addition, cortisol, a major glucocorticoid, induces changes in the glycosylation pattern of the microvillus membrane in the intestine (Mahmood and Torres-Pinedo, 1985). These changes are thought to influence the pattern of non-pathogenic bacteria colonization in the gut (Chu et al., 1989). Because intestinal barrier immaturity is the primary cause of necrotizing enterocolitis in mammals and birds, the regulatory mechanisms of cortisol on the maturation of the intestinal barrier are of special interest (Bernt and Walker, 1999; Helmboldt and Bryant, 1971).

Thyroid hormones are also major permissive regulators of the gut epithelium development during the perinatal period. In poultry embryos, triiodothyronine (T₃) remains stable during mid-incubation; but, it increases rapidly prior to hatch around the time the embryo begins lung respiration and the mucosal epithelium is preparing for an external influx of nutrients (Christensen et al., 1982). Thyroxine (T₄) levels reach high levels and stay high during amnion consumption, and decreases after hatch (Lu et al., 2007). There is strong evidence from mammalian and avian research that thyroid hormones are necessary to ensure normal maturation in intestinal mucosal cells (Black, 1978; Moog, 1979). Both total and free T₄ are considered to activate developmental changes in the gut around the perinatal period (D'Agostino and Henning, 1982; Thomson and Keelan, 1985). If the thyroid gland is removed in the immature rat, for example, the small intestine fails to mature normally and growth of the crypts in the intestinal mucosa is impaired (Brown et al., 1941; Carriere, 1966;
Middleton, 1971). Additionally, it has been shown that hypothyroid rats have delayed intestinal maturation and administering physiological amounts of T₃ or T₄ reverses these effects (Middleton, 1971). Black (1978) has shown that T₄ is required for the morphological maturation of the small intestinal epithelium in jejunal cultures from embryos treated with a T₄ blocker. Thyroid hormones also indirectly affect the mucosal development of the avian GIT by stimulating yolk utilization and promoting several metabolic processes necessary for hatching (Christensen et al., 1982; Christensen et al., 2003). Although there are other hormones (e.g. growth hormone, prolactin, gastrin) that likely play a role in the maturation of the avian small intestine, the literature available is limited.

**Growth factors**

Growth factors, such as insulin-like growth factors (IGF I and II), epidermal growth factor (EGF) and transforming-like growth factor alpha (TGF-α) play localized roles on the development of the gut epithelium during the perinatal period. Experiments in oesophageal ligated fetal sheep have demonstrated the positive effects of orally administered IGFs on the maturation of the intestinal mucosa prior to birth (Kimble et al., 1999). IGFs have been found in mammalian and avian amniotic fluid, and there is evidence of the influence of these growth factors in cell proliferation and villi growth in the gut during after amniotic fluid swallowing (Foye et al., 2006; Karcher et al., 2005; Lu et al., 2007). In addition, receptors for both IGF I and II have been found to be distributed along the gastrointestinal tract (Laburthe et al., 1988).

Another important family of growth factors involved in intestinal maturation is the EGF family. This is a large group of peptides characterized by their similarities in structure, biological function and receptor ligation (Harris et al., 2003; Playford et al., 1996). Members of this family include EGF and TGF-α, which bind to a highly homogenous receptor of the EGFr family found throughout the digestive tract. EGF plays a predominant role in the early development and maturation of the intestinal mucosa because its receptor located in the basolateral membrane is still accessible during the perinatal period (Playford et al., 1996; Scheving et al., 1989). Administration of EGF to pregnant mice increases alkaline phosphatase and trehalase activity in the brush border membrane of 18-
day-gestation offspring (Calvert et al., 1982). These effects are presumed to be similar in poultry where an EGFr highly homologous to the human isoform has been reported (Lax et al., 1988).

Other factors

There are several other hormones and growth factors that may be required for normal intestinal maturation, such as insulin and gastrointestinal hormones (e.g. gastrin, vip, cck), but there are no reports that support these claims in avian models. Besides conventional hormone mediators, such as hormones, growth factors, other endocrine signals, and some nutrients may also influence perinatal development of the gut epithelium. The contribution of these nutritional factors is discussed later in this review egarding embryo imbibition of amniotic fluid. Understanding the mechanisms of these endocrine regulators and maternal influence will further the knowledge about perinatal development of the digestive tract in poultry. Unlike mammals, oviparous females rely on specific endocrine signals that are deposited inside of the egg prior to oviposition to influence the growth of their offspring prior to and after hatching (Christensen and Davis, 2001; Schwabl, 1993; Schwabl, 1996). Some of the hormones that have been found thus far in avian eggs include: testosterone, estradiol (Schwabl, 1993), androstenedione (Schwabl, 1997), several thyroid hormones (Christensen and Davis, 2001; McNabb and Wilson, 1997), progesterone (Lipar et al., 1999) and corticosterone (Saino et al., 2005). These hormones vertically transmitted from the hen to the egg, are strategically distributed among the egg compartments and their concentrations vary within locations. In the yolk of freshly laid eggs, steroid hormones are allocated in a radial gradient reflecting the concentric layering of the yolk (Hackl et al., 2003; Lipar et al., 1999). This distribution suggests that hormones in the outer layers of the yolk may be taken up at different stages of embryo development as compared to those in the center of the yolk, and thereby serve different functions. Moreover, because the yolk sac is internalized into the embryonic abdominal cavity prior to hatch to support the maturation of the small intestine and other organs (Romanoff, 1960), identifying the distribution and release of these hormones in the yolk will improve our understanding of maternal influence of organ development in commercial poultry.
### 1.2.3. Goblet cells and the mucus blanket

The GIT epithelium is the largest surface in contact with the external environment and it is the place where a wide variety of microorganisms interact with the host (Nataro et al., 2005). This surface is coated with a thin layer of mucus, otherwise known as the mucus blanket or mucus layer (Figure 5C and 7A). Mucin glycoproteins provide the structural network for the mucus blanket, and these are produced, stored and secreted by specialized goblet cells (Figure 7C). When secreted (Figure 7B), mucins bind to large quantities of water and form a mucus layer that consists of two distinctive sublayers: (1) an inner layer or adherent mucus, which is in intimate contact with the epithelium and thereby comprise the actual mucus barrier; and (2) an outer layer, also known as the nonadherent mucus layer, that is responsible for the lubricating the epithelium and trapping bacteria (Corfield et al., 2001; Corfield et al., 2000).

Mucins can be divided into two main families: (1) transmembrane mucins that act primarily as intermediaries of growth factors, ultimately responsible for cell renewal; and (2) secreted mucins found on the cell surface where they form a viscous layer (Liévin-Le Moal and Servin, 2006; Nataro et al., 2005). In humans, there are at least 9 genes encoding membrane-bound mucins and another 7 that encode for secreted mucins. These glycoproteins are generally characterized by a protein core, rich in serine, proline and threonine, which serve as a docking site for many carbohydrate side chains. Together, all these components provide the viscoelastic properties of the mucus barrier (Corfield et al., 2001; Söderholm and Perdue, 2006). The sugar groups are attached to the luminal end of the protein, providing pseudo-attachment sites for bacteria and preventing direct attachment to the epithelium. This is the source of the protective properties of the mucus blanket that forms the interface between the microbiota and the host epithelium (Nataro et al., 2005). Thus, the ontogenesis of the mucus blanket has been the subject of several research reports across species (Liévin-Le Moal and Servin, 2006; Uni et al., 2003a). Because young animals lack the surveillance of a mature immune system, the appearance of goblet cells and the onset of mucin release are of particular importance in the early development and health of the intestinal epithelium.

Goblet cells arise by differentiation from pluripotential stem cells located at the base of the crypt.
or from poorly differentiated cells in the lower crypt referred to as oligomucous cells (Cheng and Leblond, 1974; Merzel and Leblond, 1969). In chickens, the appearance of fully developed goblet cells in the small intestine can be observed as early as 3 days before hatch (Uni et al., 2003a). These cells migrate toward the villus tip before sloughing off into the lumen, a process that takes 2 to 3 days (Geyra et al., 2001b). After hatch, goblet cells are observed to gradually increase in density along the duodenal-ileal axis (Uni et al., 2003a). Such pattern appears to be highly conserved across species (Merzel and Leblond, 1969; Specian and Oliver, 1991) protecting the epithelium from a bacterial load that increases by 8 fold from the proximal small intestine to the colon (Savage, 1977). However, the actual distribution and function of goblet cells is influenced by several factors, including diet (de los Santos et al., 2007), starvation (Smirnov et al., 2004), and stress (Castagliuolo et al., 1996).

### 1.2.4. Microbial ecology of the gut

The endogenous gastrointestinal microbial flora of any animal is essential in mediating nutrient metabolism, regulating epithelial development, defining the mucosal microanatomy and programming the immune system; however, the microbial ecology of the gut remains poorly described across species (Eckburg et al., 2005). Most of the initial knowledge about enteric microflora was built using gnotobiotic (a.k.a. known biology) or “germ free” animal models and the isolation and culture of bacterial colonies from the gut. These techniques have considerable limitations, considering that more than 80% of the microorganisms found in the gut are unculturable and at least 60% of the total bacterial phylotypes have never before been reported (Eckburg et al., 2005; Hayashi et al., 2002; Lan et al., 2002). Moreover, these bacteria surveys do not take into account that the gut microflora also include fungi and protozoa that contribute to the tremendous complexity of the microbial ecosystem of the gut.

Recent advances in molecular sequencing technologies have partially revealed the species diversity of the gut microbial ecosystem, and how its composition may impact enteric development and health. For instance, the human intestinal microbiota is composed of $10^{13}$ to $10^{14}$ microorganisms whose collective genome ("microbiome") contains at least 100 times as many genes as its own
Figure 7. Goblet cells and the mucus blanket. (A) Goblet cells (Gc) are one of the four main types of cells found in the single layer of epithelial cells that line the luminal end of the gastrointestinal tract. Commonly escorted by enterocytes (En), goblet cells secrete mucin glycoproteins that avidly bind water to form the mucus blanket (Mb). (Vc=villus core). (B) Scanning electron micrograph depicting the secretion of mucin glycoproteins (Ms) through the goblet cell opening (Gco). Note the filamentous nature of the mucins. (C) Scanning electron micrograph of a mature goblet cell next to the microvilli (Mv) brush border. The villus tip was sloughed off during processing, exposing the inner arrangement of epithelial cells. Hence, a vast array of mucin granules (Mg) tightly packaged can be clearly distinguished inside of this goblet cell. Note the differences in the size and shape of the mucin granules. Different mucin types are commonly packaged and stored in different granules. The goblet cell nucleus (Gcn) also is observed at the bottom.
genome (Gill et al., 2006). Considering that livestock species are generally exposed to higher loads of microorganisms, one may speculate that the microbial ecosystem in livestock species is perhaps more complex and abundant than in humans.

**Do the guests feed the host? Symbiotic functions of gut microbiota**

Microorganisms in the gut serve numerous important functions for their hosts, including: stimulation of angiogenesis, modulation of the mucosal microanatomy, regulation of immune development, regulation of nutrient processing and fat storage, and competitive exclusion of pathogens (Xu and Gordon, 2003). In mammals, it has been demonstrated that postnatal colonization of the gut epithelium is essential for immune tolerance to a wide variety of microbial by-products (Xu and Gordon, 2003). This sort of imprinting appears to reduce allergic responses to food or environmental antigens (Braun-Fahrlander et al., 2002).

A great example of the effects of colonizing bacteria on enteric development and function is the postnatal appearance of segmented filamentous bacteria (SFB) associated with the developing intestinal epithelium. SFB are indigenous non-culturable bacteria that are highly conserved in the gut of several species, ranging from chickens to humans (Snel et al., 1995). SFB commonly appear after weaning in piglets and mice (Snel, 1997) and around the first week post-hatch (Figure 8 A-E) in poultry (Goodwin et al., 1991). SFB are known to influence in the colonization of other commensal bacteria by serving as an anchor between the epithelium and the lumen (Angert, 2005; Klaasen et al., 1992) (Fig 8 E). SFB also increase the mitotic activity and the ratio of the number of columnar cells to those of goblet cells in the small intestinal epithelial cells (Umesaki et al., 1995) dramatically modulating the ultratopography of the intestinal epithelium (Snel, 1997). Additionally, they have also been identified as potent stimulators of the immunoglobulin A release and maturation of the mucosal immune system (Snel, 1997; Talham et al., 1999).

Enteric microorganisms have a wide variety of effects on nutrient digestion and absorption ranging from vitamin synthesis (Conly et al., 1994; Hill, 1997) to modulation of trace mineral absorption (Miyazawa et al., 1996). However, the fermentation of non-digestible dietary residue and
endogenous mucus produced by the epithelium is perhaps the major metabolic task of enteric microflora (Roberfroid et al., 1995). Short-chain fatty acids (SCFA) are produced in the hindgut by the bacterial fermentation of non-digestible dietary components, including oligosaccharides, unabsorbed sugars and alcohols, and polysaccharides (resistant starches, cellulose, hemicellulose, pectins, and gums) (Topping and Clifton, 2001). These by-products of bacterial fermentation not only serve as a major energy source for epithelial cell metabolism but can also have trophic effects on these cells (Cook and Sellin, 1998). Enteric microflora can also influence the host’s ability to harvest energy from the diet (Ley et al., 2006; Turnbaugh et al., 2006) and its capacity for energy storage (Backhed et al., 2004). This function has tremendous potential for poultry production and is already being exploited through the use of antibiotics, direct-fed microbials (e.g. probiotics and symbiotics), and prebiotics.

Commensal microorganisms (Figure 9) also protect the gut epithelium from invasion of pathogenic bacteria, primarily by competing for available luminal nutrients and attachment sites on the brush border of epithelial cells (Bernet et al., 1994). Commensal microorganisms can also inhibit the growth of their competitors via the release of potent antimicrobial substances called bacteriocins (Ji et al., 1997). This ecological struggle between commensal and pathogenic bacteria colonization is known as competitive exclusion or “Nurmi concept”. Nurmi and Rantala gave origin to this term when they were able to control Salmonella colonization by inoculating newly hatched chicks with intestinal contents from adult chickens (Nurmi et al., 1992; Rantala and Nurmi, 1973). Commensal bacteria also protect the mucosa indirectly by stimulating goblet cell proliferation and mucus secretion, which is mediated by the local release of bioactive factors and the activation of host immune cells (Deplancke and Gaskins, 2001). The commensal microflora is an “organ” composed of a rich diversity of bacteria and other unidentified microorganisms (e.g. fungi, protozoa), which perform a wide range of important functions for the host.
Figure 8. Segmented filamentous bacteria (SFB) anchored to the ileum epithelium of turkey poults. SFB are indigenous non-culturable bacteria that are highly conserved in the gut of several species, ranging from chickens to humans (Snel et al., 1995). (A) SFB commonly appear latched to the intestinal epithelium few weeks after hatch or birth. Mature SFB are formed by several segments of different length (Sg). (Mv= microvilli, Fl=filaments) (B) The reproduction cycle (white arrows) of SFB can follow two fates: 1. An active differentiation mechanism that results in the immediate formation of a hold-fast protrusion that can latch immediately onto the intestinal epithelium; or 2. An inactive fate through the formation of two intracellular offspring cells that are encased in a common spore coat, which forms an endospore (Angert, 2005). Formation of an inactive endospore is an effective dispersal mechanism especially in harsh environments where cells must await the right conditions before colonization. In fact, exposure to airborne endospores alone can establish SFB populations in a naive host (Klaasen et al., 1992). (C) Judging from the electron micrographs, it appears that this process is triggered by “strangulation” of a mature segment by a microfilament (Fl), which opens the cell and allows spreading of the spores. (D) Each SFB originates as a single, holdfast-bearing cell that embeds itself among the microvilli on the epithelial cell surface. Attachment is directly onto epithelial cells and not to goblet cell openings (Gco). (E) SFB are known to influence the maturation of the intestinal epithelium partially by harboring other commensal bacteria that colonize the epithelium (Klaasen et al., 1992).
Figure 9. Commensal bacteria in the digestive tract. (A) Round shaped bacteria can be seen patrolling the duodenum epithelium in turkey poults starting at 11 days post-hatch. (Lm=lumen, Mv= microvilli, En= enterocytes, Tw= terminal web). (B) At the end of the first week post-hatch, the small intestinal villi (Vi) are surrounded by a dense population of microorganisms (Bac) embedded in the mucus layer. (C) Bacteria are commonly seen in association (white arrows) to mucus (Mc), which is arranged as layers. (D) Bacteria commonly form microcolonies formed by several different types of organisms (white arrows). In this micrograph at least 4 different shapes of bacteria can be distinguished.
Microbial colonization of the gut: EMBRYONIC GUT MICROBIOTA?

The diversity and complexity of the gut microbial ecosystems is known to increase with the age of the host (Amit-Romach et al., 2004; Wielen et al., 2002) (Figure 9). However, the first bacteria to enter the gut are known to modulate expression of genes present in the intestinal epithelium cells to create a favorable habitat for themselves, and prevent growth of other bacteria introduced later in the ecosystem (Hooper et al., 2001). The initial colonization is therefore very relevant to the final composition of the permanent microflora in adult birds. Moreover, studying gut microbial ecosystems early in the host’s life offers the advantage of being relatively simple and less diverse. But how early does microbial colonization of the gut actually begin in avian species? Many scientists think that hatchlings are sterile or free from bacteria or other living microorganisms at the time of hatch (Amit-Romach et al., 2004; Lu et al., 2003; Wielen et al., 2002), and that microbial colonization begins at or soon after hatch. However, vertical contamination of fertile eggs during oviposition (Cox et al., 2005; Hong et al., 2003), as well as the presence in the embryonic gut of maternally derived immunoglobulins several days before hatch have been well described in the literature (Loeken and Roth, 1983; Rose et al., 1974). Such evidence raises the question of whether this is casual pathology or a conserved and regulated mechanism by which the hen has an input in the composition of the microbial ecosystem of its progeny.

Vertical transmission and embryonic colonization of bacteria is a process that has been thoroughly studied in certain invertebrates. For instance, the surface of embryos of the shrimp *Palaemon macrodactylus* is commonly colonized by *Alteromonas sp*. These aquatic bacteria protect the shrimp embryos from fungal infection (*Lagenidium callinectes*) by producing and liberating the antifungal metabolite 2,3-indolinedione (Gil-Turnes et al., 1989). Frequently, this bacterial-embryo association can lead to colonization of internal organs. The earthworm *Eisenia foetida* harbors specific and stable populations of Acidovorax-like bacteria within their excretory organs called the nephridia (McFall-Ngai and Ruby, 2000). The *E. foetida*-Acidovorax association has evolved mechanisms to ensure the transfer of the bacteria to the next generation. The *E. foetida* transmits the

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1 This hypothesis has been previously proposed in the thesis work of Bohorquez, D. 2007. Dietary and housing effects on growth performance, gut health and salmonella colonization of salmonella-challenged broilers. M.S. North Carolina State Raleigh.
nephridial bacteria directly into egg capsules where the embryos develop (Davidson and Stahl, 2006). In this way, Acidovorax bacteria and other multiple types of bacteria interact with the embryos externally and internally during the full course of development, and ultimately fill the gut lumen near the end of development prior to hatching (Davidson and Stahl, 2008). Thus, the juveniles emerge fully colonized by their specific Acidovorax-like symbionts. Similar mechanisms of mother-embryo vertical transmission to inherit microorganisms have also been reported in marine sponges (Ereskovsky et al., 2005), arthropods (O’Neill et al., 1997) and insects (Douglas, 1989). However, the existence of this mechanism in higher animals has yet to be proven.

Certain literature reports indicate that perhaps this mechanism of vertical transmission of microflora from dam to progeny can occur even in mammals. Pioneering microbiologists René Dubos states in his book “Man Adapting” (1980) “The fetus seem to be essentially free of bacteria before birth...There are some indications, however, that protoplast-like forms can be transferred from the mother to the fetus in utero”. Pedroso (2009) used fluorescence in situ hybridization (FISH) and PCR to identify Salmonella in the ceca contents of 18 day-old chicken embryos. Although the results have not been peer reviewed, the principle appears to point towards vertical transmission of bacteria prior to hatch. Certain bacteria, such as segmented filamentous bacteria, are known to produce dormant cells or endospores that survive harsh environmental conditions (Angert, 2005). During egg oviposition, the hen may deposit during egg oviposition bacteria spores that remain dormant until the right nutritional conditions exist for them to propagate and colonize the embryonic gut. Perhaps this is a conserved mechanism in birds to ensure the inheritance of commensal microorganisms. This hypothesis will be addressed in the third chapter of this dissertation.

1.3. AMNIOTIC FLUID: THE FIRST MEAL IS WHAT MATTERS

Amniotic fluid (AF), once thought of as a simple fetal cushion made out of fetal urine excretions, is now emerging as a fruitful area of research in several species including birds. The digestive tract of poultry undergoes dramatic changes in its ultra-structure prior to hatch and most of these changes

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2 A protoplast is a living plant, bacterial or fungal cell that had its cell wall completely or partially removed.
coincide with the swallowing of the amniotic fluid. For instance, the villus size and surface area increase about 3-fold between 23 days and 25 days of incubation in the turkey (Chapter II). These changes coincide with the imbibing of about 70% of the AF during these two days by the embryo. AF volume in turkey embryos decreases from 6 mL/egg at 22E to almost 0 mL/egg at 24E (de Oliveira, 2007). At least in mammals, disturbances in this essential fluid during fetal development are highly correlated with several perinatal maladies, including those related to gastrointestinal tract dysfunction. Below is discussed some of the relevant literature that relates the nutrient composition of AF to the development of the digestive tract.

1.3.1. Nutrients in the amniotic fluid that favor gut development

Although AF is not the nutritional mainstay of the fetus in mammals or late-term embryo in birds, it contributes up to 15% of fetal nutritional requirements and plays a significant role in gut development and maturation (Shah and Sanderson, 2000). Initially, the role of amniotic fluid in GIT development was studied by fetal esophageal ligation of animal models such as sheep and rabbits. These studies demonstrated that AF ingestion is required for normal fetal growth and development (Koski and Fergusson, 1992). For instance, esophageal ligation in-utero of fetal sheep causes fetal growth restriction that is reversible by intragastric feeding (Buchmiller et al., 1993; Kimble et al., 1999). In chickens, around the 13th day of incubation, the albumen is incorporated into the amnion through the sero-amniotic connection, resulting in significant increases in the protein content of the AF (Romanoff, 1960). At this point, the chicken embryo begins swallowing of the AF, a process that continues until day 18 of incubation (Lopez de Torre et al., 1992; Romanoff, 1960). Lopez de Torre et al. (1992) has shown that intestinal obstruction of the mid jejunum in the chicken embryo around the time of AF swallowing alters the metabolism of proteins in the bloodstream and causes severe malnutrition of the embryo. Hence, it is now assumed that AF swallowing by the embryos/fetus prepares the gastrointestinal tract for postnatal nutrition, just as milk in mammals continues to nourish enteric development. In fact, the nutritional composition of human AF and breast milk are very similar (Table 1), and these two essential fluids function together to aid in the fetal/neonatal transition in mammals.
(Wagner, 2002). Since birds lack postnatal maternal nutrition, imbibing of AF perhaps has a more prominent influence on the development of the GIT in preparation for post-hatch feed intake. However, limited information is available across species regarding which, and in what physiological amounts, macro- and micronutrients initiate present in AF the preparation of the embryonic intestines for postnatal life. Below are outlined some of the nutrients found in AF that likely play a role.

Table 1. Nutrient composition of human amniotic fluid versus human breast milk. Note that lack of complexity in AF nutrient composition is due to limited data available. However, its composition closely resembles that of milk (Picciano, 2001; Underwood and Sherman, 2006).

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Amniotic Fluid</th>
<th>Milk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein and amino acids</td>
<td>Lactoferrin, glutamine, arginine</td>
<td>Lactoglobulin, casein, lactalbumin, nucleotides</td>
</tr>
<tr>
<td>Vitamins</td>
<td>A, C, folate, B12</td>
<td>A, D, E, K, B1-3, 6,12, C Cobalt, copper, fluoride, iodine, iron, manganese, selenium, zinc</td>
</tr>
<tr>
<td>Minerals</td>
<td>Zinc, iron</td>
<td></td>
</tr>
<tr>
<td>Hormones and Growth</td>
<td>Growth hormone, prolactin, IGF-1, EGF</td>
<td>IGF I and II, HGF, EGF, growth hormone</td>
</tr>
</tbody>
</table>

**Macro- and micro-nutrients**

Although swallowing of AF containing excess glucose may enhance fetal weight in rats, there are no reports addressing the influence on gastrointestinal development. It is important to note that the amount of this carbohydrate in AF can be influenced by the mother’s diet (Koski and Fergusson, 1992). Mucin glycoproteins have also been identified in AF. These are rich in mannose sugars, and the glycans of the carbohydrate moiety are O-linked (Hanisch and Katalinic, 1992). Because of their affinity for water, O-linked mucins are a large structural component of the mucus blanket that protects the intestinal epithelia (Liévin-Le Moal and Servin, 2006). In addition, O-linked glycans are known to have potent antimicrobial activity (Wopereis et al., 2006). In poultry, bacterial populations have been identified in the egg, and their origins are thought to be due to vertical transmission from the hen ovaries to the egg during deposition (Cox et al., 2005). Hence, amniotic fluid swallowing could be a precursor or at least a major contributor to the establishment of gut microbiota.

Amino acids in AF have been reported during early, middle, and late periods of normal and abnormal human pregnancy (Schulman et al., 1972). The content of amino acids in AF is quite
sensitive to dietary and maternal health influences. For instance, diabetes, abnormal pregnancies and maternal caloric deprivation can alter profoundly amino acid composition in AF. Starvation elevates valine, leucine, isoleucine, and taurine concentrations and lowers alanine and citrulline concentrations (Felig et al., 1972). Glutamine and arginine present in AF are of particular interest in fetal intestinal growth and maturation. Glutamine is the major fuel of gut epithelial cells and is considered conditionally essential for normal gastrointestinal growth and function. In fetal sheep, glutamine in swallowed AF is absorbed by the intestine and used as a fuel in the intestine. The infusion of IGF-I into the AF appears to increase intestinal glutamine uptake (Klurfeld, 1999). Of interest, preterm infants who develop necrotizing enterocolitis have lower circulating levels of glutamine and arginine for 7 days before the development of symptoms (Underwood and Sherman, 2006). Arginine is the precursor of NO and polyamines. In the sheep model of fetal growth retardation, citrulline, the other product of NO synthesis, is reduced in the fetal intestine (Bloomfield et al., 2002). However, it is not known whether or not the fetal gut absorbs arginine nor if it can trigger the formation of these important molecules.

The polyamines putrescine, spermidine and spermine, are formed during arginine hydrolyzation. Polyamines are highly charged, multivalent cations that play a well-established role in epithelial restitution (Blikslager et al., 2007). Putrescine and spermidine levels increase in AF until the 30th week of human pregnancy, while spermine remains high throughout gestation (Russell et al., 1978). In preterm infants, arginine and polyamines are associated with intestinal growth and function. However, the role of arginine and polyamines in the growth of the fetal bowel is based on inferred evidence (Underwood and Sherman, 2006). More research is needed to understand the functions in the fetal gut of these and other molecules (e.g. vitamin E, zinc) that play a significant role in the homeostasis of the adult gut.

**Hormones and growth factors**

Growth factors and hormones initially were identified in human AF three decades ago (Chochinov et al., 1976), but only years later were the principal mediators of fetal intestinal and somatic growth in
AF delineated. AF contains many proteins, some of which are important hormones and growth regulators. For example, transferrin and lactoferrin are iron-binding glycoproteins present in AF toward the end of gestation, and these proteins are known to deliver ferric iron to the intestinal epithelia (Cleveland et al., 1991). Lactoferrin has growth factor-promoting qualities, and it accelerates the incorporation of thymidine into enterocytes (Cleveland et al., 1991). In addition, several hormones that are commonly associated with the regulation of intestinal growth are also found in AF. Growth hormone, prolactin, parathyroid hormone, and thyroid hormones have been identified in the AF (Chopra and Crandall, 1975; Schindler, 1982). Although it is very likely that these hormones are directly involved in the perinatal development of the GIT, there is little information about the hormones’ fate from AF and whether or not they play a role during AF swallowing by the embryo.

Hirai et al., (2002) performed elegant experiments to examine the trophic effects of AF, human milk, and several recombinant growth factors on human fetal small intestinal cell lines. These studies have demonstrated the importance of growth factors present in AF in normal development of the perinatal gut. Because of its role in epithelial division and differentiation, epidermal growth factor has been the focus of attention in recent publications. EGF in human AF increases significantly during the second trimester. In fetal rabbits, enteral administration of EGF reverses the effects of esophageal ligation. Amniotic injection of EGF also increases small intestinal length as well as the activities of lactase and maltase in rabbits (Cellini et al., 2004). EGF receptors are detected in the fetal gastric mucosa of humans starting at the 18th week of gestation (Tremblay et al., 1997). Transforming growth factor alpha (TGF-α) shares ~60% of its structure sequence with EGF and binds to the same receptor (EGFr). TGF-α is also produced in the fetal intestine and has been shown to elicit a synergistic trophic response on cultured intestinal cells when combined with EGF, insulin-like growth factor-1, fibroblastic growth factor, and hepatocyte growth factor. However, the trophic response is not as strong as either AF or breast milk (Hirai et al., 2002).

Other growth factors such as transforming growth factor beta-1 (TGF-β1) are also found in rat AF and human breast milk, although in human AF TGF-β1 is only present during the late stages of gestation. TGF-β1 may induce terminal differentiation of intestinal epithelial cells and to accelerate
the rate of healing of intestinal wounds by stimulating cell migration (Underwood et al., 2005). TGF-β1 may also stimulate IgA production. Hence, TGF-β1 could prepare the intestine during the perinatal transition to external environment. Insulin-like growth factor-I (IGF-I) is also found in human AF and when infused into the esophagus of fetal sheep, it improves somatic growth, spleen weight, and bowel wall thickness (Kimble et al., 1999). IGF-I and IGF-II receptors are found throughout the human neonatal gut. IGF-I in AF may also increase the uptake of swallowed glutamine in the mammalian gut (Underwood et al., 2005). Although several other growth factors (i.e. erythropoietin or hepatocyte growth factor) are found in human AF, limited information is available on their role in fetal gut maturation during swallowing of AF.

1.3.2. Embryo programming through amniotic fluid supplementation

Lima-Rogel et al. (2004) and Christensen et al. (2005) have proposed a novel approach to early feeding intolerance in very low-birth weight infants. To decrease the intestinal atrophy that occurs with prolonged fasting, an AF-like formula might be useful to enhance enteric maturation in these types of newborns. The AF-like formula used by Christensen et al. (2005) was sterile, isotonic, and noncaloric, and contained electrolytes, albumin, and two recombinant human growth factors, erythropoietin (EPO) and granulocyte-colony stimulating factor (G-CSF). The advantage of this solution is that it is resistant to digestion and the ingested growth factors can reach the small intestine, where it has been demonstrated that receptors for the two growth factors are localized. These receptors are commonly found on the luminal end of enterocytes, suggesting a localized effect of growth factors. This report shows the potential of AF-like formulas to boost the development of the premature tract.

Understanding the composition of AF in poultry can help to design suitable starting diets in poultry that can improve the growth and livability during the first week of life post-hatch. Furthermore, understanding the nutritional potential of AF may render new pathways to influence neonatal gut physiology and function. El-Haddad et al. (2004) have introduced the idea of fetal programming in humans. Thirst and appetite-mediated ingestive behavior develop and are likely programmed in-utero, thus preparing for newborn and adult ingestive behavior. An adverse intrauterine environment,
with altered fetal appetite factors during the critical developmental period of fetal life, may alter the
normal set points of appetitive behavior and potentially lead to programming of adulthood
hyperphagia and obesity (El-Haddad et al., 2004). In poultry, Uni and Ferket (2004) introduced the
concept of embryonic programming of the avian embryo almost a decade ago. The benefits of
nutrient administration into the amnion, or in-ovo feeding (IOF), of late-term poultry embryos on early
development of the digestive tract have been demonstrated by several experiments (Ferket and Uni,
2006). In each experiment, IOF showed advanced morphological development of the intestinal tract
and mucin barrier (Foye et al., 2003; Tako et al., 2004). In addition, IOF has also been shown to
enhance expression of genes for the brush border enzymes, sucrase-isomaltase and leucine
aminopeptidase (Foye et al., 2005a; Foye et al., 2005b). Evidently, there is a great potential to modify
the perinatal function and physiology of the intestine through amniotic fluid supplementation.

1.4. EARLY NUTRITIONAL STRATEGIES TO MODULATE ENTERIC DEVELOPMENT

1.4.1. The sooner the better: Early feeding concept

Because the embryonic gut must adapt rapidly to an external source of nutrients, the hatching
period constitutes a critical transition for the development and survival of commercial broilers and
turkeys (Moran Jr, 1985). The sooner the intestine reaches its functional capacity, the sooner the
hatchling bird can utilize dietary nutrients to achieve its genetic potential for growth (Uni and Ferket,
2004; Uni et al., 2003a; Uni et al., 2003b). Although the digestive capacity begins to develop a few
days before hatch, most of the development occurs post-hatch when the neonatal poult begins
consuming feed (Sklan, 2001). The small intestinal weight increases at a faster rate than the body
mass (Katanbaf et al., 1988; Sell et al., 1991; Sklan, 2001) due to rapid epithelial cell proliferation
(Geyra et al., 2001b) occurring at the crypts as well as along the villi (Uni et al., 1998). This pattern of
enteric growth continues at least up to the 10 days after hatch (Uni et al., 2000; Geyra et al., 2001b).
Consequently, the presence of dietary nutrients is critical to maintain proper development and
function of the digestive tract.

Previous studies have shown that feeding poultry immediately post-hatch accelerates the
morphological development of the small intestine (Noy and Sklan, 1998), while delayed access to 
external feed arrests the development of the small intestine mucosal layer (Uni et al., 1998; Geyra et 
al., 2001b; Uni et al., 2003b). Hatching chicks denied access to first feed for 24 hours post-hatch 
have decreased villi length (Yamauchi et al., 1996), decreased crypt size and number per villus, and 
lower enterocyte migration rate (Geyra et al., 2001a). In addition, delayed feed access for 48 hrs 
post-hatch dramatically alters goblet cell numbers and mucin secretion, exposing the intestinal 
epithelium to bacterial invasion and reducing its absorptive capacity (Uni et al., 2003a). Thus, early 
feeding accelerates the adaptation of hatching chicks and poults to an exogenous supply of nutrients 
and increases the probability of achieving their genetic potential for growth and feed utilization.

1.4.2. Amniotic fluid supplementation or in-ovo feeding of poultry embryos

Since access to feed soon after hatch is critical for the development of digestive capacity and 
muscle tissue, “feeding” the embryo around the time the AF is consumed can accelerate enteric 
development and its capacity to digest nutrients (Ferket, 2006). This procedure is known as in-ovo 
feeding (IOF) (Uni and Ferket, 2003) and its potential influence on early growth and development of 
poultry has been demonstrated by several experiments (Ferket and Uni, 2006). However, the degree 
of response to IOF may depend upon formulation of the solution, genetics of the breeders, hen age, 
egg size, and incubation conditions (Ferket, 2004). Several IOF formulas have been tested with 
positive effects and the list of nutrients in the solution includes: sucrose, maltose, and dextrin (Uni et 
al., 2005; Uni and Ferket, 2004), β-hydroxy-β-methyl butyrate (Tako et al., 2004), arginine (Foye et 
al., 2005a; Foye et al., 2005b), egg white protein (Foye et al., 2006), and zinc-methionine (Tako et al., 
2004). Although the effects on body weights at hatch appear to be somewhat inconsistent, IOF of 
chicken-, turkeys and duck embryos consistently accelerated the digestive and nutrient uptake 
capacity of the digestive tract around the perinatal period (Chen et al., 2009; de Oliveira, 2007; Foye, 
2005; Tako et al., 2004). For instance, IOF chicken embryos at 17.5 days of incubation enhances the 
villus surface area at hatch of the small intestinal epithelium (Smirnov et al., 2006; Tako et al., 2004) 
along with its digestive capacity, as shown by higher expression of digestive enzymes (sucrase-
isomaltase, leucine aminopeptidase) and nutrient transporters (SGLT-1, PEPT-1, and NaK ATPase) (Foye et al., 2005b; Tako et al., 2005). Changes in villus surface area, digestive enzyme activity and nutrient transporter have been shown to last through the first week post-hatch (Foye, 2005). Moreover, IOF may influence positively the protective function of the enteric mucosa by accelerating the onset of the mucus blanket. Smirnov et al. (2006) have reported that IOF increased the proportion of goblet cells containing acidic mucin by 50% over controls, which corresponded with enhanced expression of the mucin encoding genes. Although, the effects of IOF on the digestive capacity and nutrient uptake of the small intestine have been extensively demonstrated, its effects on the ultra-structural maturation of the small intestinal epithelium have not been explored so far. Because, morphological maturation of the small intestine precedes the onset of gut barrier function, digestive capacity and nutrient uptake, understanding the effects of IOF on the ultra-structural maturation of the intestinal epithelium of hatchlings is of particular importance. This hypothesis will be explored in Chapter IV of this dissertation. These results will further improve our understanding of the mechanisms by which in-ovo nutrition can enhance the maturation of the GIT in the perinatal turkey poult and chicken. Ultimately, these benefits will depend on the formulation used and may alleviate the growth constraints of modern fast-growing chickens and turkeys (Uni and Ferket, 2004).

1.4.3. Dietary nucleotide supplementation

It has been well documented that rapidly dividing cells, such as intestinal epithelial cells, have a voracious demand for nutrients and metabolic cofactors, in particular for nucleotides, the building blocks of DNA and RNA (He et al., 1993; Tanaka et al., 1996). Nucleotides are chemical compounds that consist of three molecular fragments: sugar, heterocyclic base and phosphate group (Horton et al., 2002). Nucleotides not only supply nucleic acids for DNA and RNA replication, but also are important secondary messengers that mediate the activities of peptide hormones responsible for growth, as well as provide energy (e.g. ATP) for enzymatic processes and muscle work (Saenger, 1984). Because synthesis and salvage of nucleotides consume considerable amounts of metabolic energy in the form of ATP, dietary nucleotides can reduce the metabolic cost of de novo nucleotide
The sources of dietary nucleotides include several feed ingredients of animal and plant origin containing cellular material, usually in the form of nucleoproteins (Mateo and Stein, 2004). The nucleotide content is particularly high in ingredients such as animal protein solubles, fish-meal, legumes and unicellular organisms such as yeasts extracts that are rich in RNA or DNA (Barness, 1994). The content, proportion and availability of nucleotides differ among ingredients. For instance, muscle protein is a poor source of nucleotides as they are mainly in the form of actin-myosin protein (Barness, 1994). Fish and animal protein solubles are highly digestible but they leach easily, affecting overall availability (Devresse, 2000). Yeast extract supplements have been preferentially used in livestock production because of their high content of readily digestible and available nucleotides and other nutritional components (e.g. protein, free amino acids, vitamins). In poultry, yeast-extracts products have been shown to improve growth performance and carcass traits for broilers and turkeys (Fegan, 2006).

Although it appears that tissues of the body preferentially use exogenous dietary sources, nucleotides can be synthesized directly or scavenged by salvage pathways in the body (Quan, 1992). Intestinal epithelial cells have limited capacity to synthesize nucleotides de novo, so they depend on nucleotides from other sources (Carver and Walker, 1995; Leleiko et al., 1983; Quan, 1992). In fact, when nucleotides are fed, there is a rapid increase in the expression of hypoxanthine guanine phosphoribosyl transferase in the gut epithelium, which ultimately favors the salvage of preformed nucleosides from the diet (Leleiko et al., 1983; Leleiko et al., 1987). In an effort to replicate the nutritional content of human breast milk (rich source of nucleotides) in infant formulas, the effects of dietary supplementation of nucleotides has been extensively studied in mammals (Carver and Walker, 1995; Pickering et al., 1998; Uauy et al., 1990). For instance, it has been shown that young infants fed nucleotide-supplemented formula have lower fecal gram-negative enterobacteria and higher fecal bifidobacteria and lactobacilli than formula fed infants (Gil et al., 1986). Moreover, villus height, crypt depth, total protein and DNA content of the proximal gut is higher in young rats supplemented with dietary nucleotide, compared to controls fed a nucleotide-free purified diet (Uauy...
et al., 1990). In piglets, it has also been shown that dietary nucleotide supplementation can ameliorate the erosion of the mucosa observed immediately after weaning and reduce the occurrence of diarrhea (Martinez-Puig et al., 2007). Although the positive effects of dietary nucleotide supplementation in mammalian newborn have been extensively demonstrated, little has been done to apply this knowledge in poultry. Recently, we have shown that dietary supplementation with a yeast-extract product rich in nucleotides (up to 6% of dry matter) increases the villi surface area of turkey poults by 20% one week after hatch and increases their body weights up to 3 weeks of age, compared to controls fed a basal corn-soy bean meal diet (Bohórquez et al., 2008). Consequently, dietary supplementation of nucleotides in hatchlings may stimulate enteric development at a time when rapid cell proliferation appears to be limited by dietary constrains. Moreover, because IOF appears to consistently stimulate enteric development, perhaps its potential can be enhanced by post-hatch dietary supplementation of enteric modulators such as nucleotides. This hypothesis will be the focus of Chapter V in this dissertation.

1.5. HYPOTHESES AND RESEARCH OBJECTIVES

The growth performance and meat yield of commercial poultry have improved linearly during the last four decades (Ferket, 2004; Havenstein et al., 2003a). This trend will likely continue in the future as new technologies in genetics, biotechnology, and developmental biology are introduced and adopted by the poultry industry (Ferket, 2008). However, as selection for greater growth rate continues in commercial meat birds, the pattern of external growth as well as that of vital internal organs such as that of the gastrointestinal tract has been altered (Smith et al., 1990). Feeding poultry immediately post-hatch is critical for normal development of the digestive tract as well as for the growth of the animals (Geyra et al., 2001a; Sklan, 2001; Uni et al., 1999). Moreover, “feeding” the embryo during amniotic fluid consumption can accelerate enteric development and its capacity to digest nutrients prior to hatch (Ferket, 2006). Under normal conditions, the development of the GIT is characterized by rapid cell division and differentiation, which rely on nucleotide availability for DNA and RNA synthesis (Carver and Walker, 1995). Because in-ovo feeding consistently accelerates
intestinal maturation, dietary supplementation of nucleotides post-hatch may contribute to tissue nucleotide pools and further improve the benefits of feeding in-ovo.

Therefore, our working hypothesis is that early nutritional strategies in-ovo and post-hatch can enhance the morphological development of the intestinal mucosa in perinatal turkey poult. Since there are no previous reports that delineate the ultra-structural changes in the small intestinal mucosa of the perinatal turkey poult, it is useful to visualize the micro-anatomical adaptations that accompany the maturation of the intestinal mucosa in the turkey embryo in preparation for life post-hatch before attempting its manipulation. To accomplish our working hypothesis, our research objectives have been divided into one specific aim and three specific hypotheses described below:

CHAPTER 2

Specific Aim 1. To characterize the morphological and ultra-structural maturation of the intestinal mucosa in the perinatal (12 days prior to and 12 days post-hatch) turkey embryo and poult.

Objective 1: To measure histo-morphometrical changes in the small intestinal villi from 20 days of embryo to 10 days post-hatch in turkeys.

Objective 2: To analyze ultra-structural changes in the villi topography from 20 days of embryo to 10 days post-hatch in turkeys using scanning and transmission electron microscopy.

CHAPTER 3

Specific hypothesis 1. Bacteria colonize the gut of the turkey embryo before hatch.

Objective 1: To determine the presence of microbial colonies in the ceca of turkey embryos from 15 days of incubation (E) up to day of hatch using scanning electron microscopy.

Objective 2: To characterize the potential bacteria types present in the luminal contents of late-term turkey embryos (25E) using 16SrRNA bacterial profiling and terminal restriction fragment length polymorphism

CHAPTER 4

Specific hypothesis 2. In-ovo feeding stimulates plasma triiodothyronine (T₃) levels and enhances
the ultra-structural development of the small intestinal mucosa in the perinatal turkey embryo and poult.

**Objective 1.** To measure the effects of *in-ovo* feeding on \( T_3 \) activity levels from 25 days of incubation to 12 days post-hatch using serum radioimmunoassays.

**Objective 2:** To evaluate the effects of *in-ovo* feeding on the histo-morphometrical and ultra-structural development of the small intestinal villi from 25 days of incubation to 11 days post-hatch using light and electron microscopy.

**Objective 3.** To survey the effects of *in-ovo* feeding on the expression of genes related to maturation of the small intestinal mucosal in turkey poultTs using gene microarrays.

**Objective 4.** To evaluate the effects of *in-ovo* feeding on the growth performance and feed efficiency of turkey poultTs from hatch to 11 days of age.

**CHAPTER 5**

**Specific hypothesis 3.** Post-hatch dietary supplementation of *in-ovo* fed turkey poultTs with dietary nucleotides stimulates the morphological development of the small intestinal mucosa after hatch.

**Objective 1.** To evaluate the effects of dietary inclusion of a nucleotide-rich yeast extract (Nupro\(^\text{®}\)) on the histo-morphometrical characteristics of the jejunum villi in *in-ovo* fed and conventional turkey poultTs up to 12 days of age.

**Objective 2.** To evaluate the effects of effects of *in-ovo* feeding and subsequent dietary inclusion of a nucleotide-rich yeast extract (Nupro\(^\text{®}\)) on the expression of genes involved in mucosal maturation (*egfr*, *igfbp-1* and *muc2*) from 25 days of incubation to 12 days of age.

**Objective 3.** To evaluate the effects of dietary inclusion of a nucleotide-rich yeast extract (Nupro\(^\text{®}\)) on the growth performance and feed efficiency of *in-ovo* fed and conventional turkey poultTs from hatch to 12 days of age.
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CHAPTER II

ULTRA-STRUCTURAL DEVELOPMENT OF THE SMALL INTESTINAL MUCOSA IN THE PERINATAL TURKEY EMBRYO AND POULT: A LIGHT AND ELECTRON MICROSCOPY STUDY
2.1 ABSTRACT

The morphology of the intestinal mucosa changes dramatically in the perinatal turkey as the embryo shifts from a lipid-rich in-ovo nutrient supply to a carbohydrate- and protein-rich diet after hatch. These changes in mucosal morphology and the associated gut function can be influenced by dietary or microbial factors. Scanning electron microscopy (SEM), transmission electron microscopy (TEM), and light microscopy (LM) observations were conducted to study the morphological development of the duodenum, jejunum, and ileum mucosa from turkey embryos at 15E, 17E, 19E, 21E, 23E, 25E, 27E and pouls at hatch, 4, 8 and 12d. SEM and TEM observations were corroborated by histomorphometrical analysis made by LM. Longitudinal pre-villus ridges at 15E gradually form zigzag patterns that lead to the formation of two parallel lines of villi by 21E. Villi topography and size profoundly changed between 23E and 25E, perhaps fueled by amniotic fluid swallowed by the embryo. Villi surface area increase about 3.5 fold from 23E to 25E. Subsequently, villi surface area continued to double at each time point (27E, Hatch, 4d) until plateau at 8d. Villi morphology changed from finger-like projections before hatch to leaf-like projections by 12D. Microbial colonization of the gut is evident during the first 12 days post-hatch, and some organisms, such as segmented filamentous bacteria, likely influence villi microanatomy development. Profound morphological adaptations occur in the gut epithelium during perinatal development of the turkey embryo or poult, which contribute to the unique specialization and compartmentalization of the small intestine in preparation for an influx of exogenous nutrients.
2.2 INTRODUCTION

The digestive tract epithelium of poultry undergoes ultra-structure during late-term incubation as the embryo in prepares for precocial post-hatch life. During the last 3 days of incubation, the relative weight of intestine in the broiler chicken embryo increases from approximately 1% on day 17 of embryonic age (E) to 3.5% of body weight at hatch (Uni et al., 2003). These changes coincide with the imbibing of about 70% of the amniotic fluid (AF) by the embryo during this period (Romanoff and Hayward, 1943). In turkeys, the embryo swallows about 6mL of AF fluid from 22E to 24E, completely depleting the vital fluid (de Oliveira, 2007). During this time, the proliferation of epithelial cells in the primordial ridges that lined the base of the gut increases significantly, giving way to villi formation. In chicken embryos, most of the epithelial cells on the ridges are barely defined and their apical surfaces are flattened at 16E, but at 18E definitive villi with microvilli protruding on the apical end of the epithelial cells are clearly distinguished in the proximal small intestine (Lim and Low, 1977). Hence, as observed in mammals (Buchmiller et al., 1993; Kimble et al., 1999; Koski and Fergusson, 1992), disturbances associated with amniotic fluid swallowing in avian embryos (Lopez de Torre et al., 1992) may be highly correlated with perinatal maladies related to gastrointestinal tract dysfunction.

Developmental changes in the mucosal epithelium proceeds at an accelerated rate around the time of hatch when the avian embryo must endure several physiological and metabolic stress conditions. The nutrient supply shifts abruptly from yolk lipids in-ovo to a carbohydrate- and protein-rich exogenous diet post-hatch (Moran Jr, 1985; Sklan, 2001). During this critical perinatal period, the intestinal mucosa undergoes dramatic morphological and functional changes. Intestinal tissue grows rapidly until about 10 days post-hatch because of the incessant proliferation of absorptive cells near the crypts and along the villi, resulting in a several fold increase in villi volume and height (Geyra et al., 2001; Uni et al., 1995; Uni et al., 1998b). During this early period of growth, the avian small intestine increases in weight more quickly than the actual whole body mass (Sell et al., 1991; Sklan, 2001). Also at hatch, microorganisms from the external environment rapidly invade the pristine luminal environment and colonize the epithelium, where they play important functions in the structural maturation of the epithelium. For instance, segmented filamentous bacteria are known to increase the
mitotic activity and influence epithelial cell differentiation (Umesaki et al., 1995), thus dramatically modulating the ultratopography of the intestinal epithelium (Snel, 1997). Although the gut develops at a slower rate in turkeys than in chickens, this accelerated pattern of mucosal growth is conserved among poultry (Pinchasov and Noy, 1994; Sell et al., 1991; Uni et al., 1999).

Timely morphological adaptations of the gut epithelium are required to accommodate functional brush-border enzymes that mediate the ingestion of carbohydrate- and protein-rich diets after hatch (Sklan, 2001; Uni et al., 2003). Hence, any stressor that disrupts the normal progress of the morphological evolution of the gut mucosa frequently compromises the overall performance of the animal. For instance, withholding feed intake in day-old broiler chickens for 36 hours significantly reduces the crypt depths and the villi heights up to 10 days post-hatch (Uni et al., 1998a). Villi along the small intestine undergo extensive invagination and epithelial cell erosion in fasted chicks (Bayer et al., 1981). These morphological changes in feed restricted chicks are associated with compromised growth performance of birds later in life (Bigot et al., 2003; Noy et al., 2001). Hence, researchers commonly measure the histo-morphometric structure of intestinal mucosa to evaluate the influences of feed additives and microbial challenges in gut function and health (Chichlowski et al., 2007; Madden and Ruff, 1979; Rahimi et al., 2009).

Documenting the ultra-structural characteristics that define the gut mucosa around the time of hatch is of preponderant interest to understand the influence of stressors and nutrients on gut health. Although previous studies have examined the morphological and ultra-structural development of the gut in the perinatal broiler chicken and embryo (Lim and Low, 1977; Uni et al., 1998a), little is known about the morphological development of the gut epithelium in the perinatal turkey embryo and poult. The use of scanning electron microscopy (SEM) allows for the assessment of the surface of the gut epithelium in three dimensions. When combined with histo-morphometrics, SEM provides a clear assessment of the gut mucosal microanatomy. The objective of this study was to characterize the micro-anatomical features associated with the transition of the small intestinal epithelium of turkeys from 15 days of incubation (E) through 12 days of age post-hatch (D).
2.3 MATERIALS AND METHODS

**Bird husbandry and sampling procedure**

Fertile Nicholas turkey eggs were obtained from a commercial hatchery and incubated in a Chickmaster® research incubator3 at 37.5°C and 52% relative humidity. After hatch, poultse were reared up to 12 days of age in Alternative Design4 battery cages with ad libitum access to feed and water. Four embryos or poultse were euthanized by cervical dislocation at each time point (15E, 17E, 21E, 23E, 25E, 27E, Hatch, 4d, 8d and 12d) and free yolk embryo or poult weights were recorded at each time point. In addition, amniotic fluid volume was determined in six eggs per time point from 15E to 25E. For the histomorphometrical analysis, a 3cm segment of the proximal jejunum, starting at the end of duodenal loop, was removed and immediately fixed in 10% neutral buffered formalin for at least 72 hours prior to processing. For electron microscopic analysis, a 1cm segment was removed from the proximal jejunum and distal-ileum. Four tissue blocks (~3x3mm each) per segment were then dissected immediately on dental wax while submerged in a drop of 0.1M sodium phosphate buffer, pH 7.2, (SPB), to avoid drying of the mucosa. Tissue blocks were fixed with cold (~4°C) 3% glutaraldehyde in SPB for at least 72 hours.

**Histomorphometrical analysis**

Three sections (~3 mm) were obtained from each fixed segment, placed into tissue cassettes and submerged into 10% neutral buffered formalin. Cassettes were then sent to the North Carolina State University College of Veterinary Medicine histology lab for preparation of slides and hematoxylin and eosin staining. Light microscopy photographs were taken of the transverse sections and analyzed using UTHSCSA Image Tool5 software. The following measurements were performed on 8 villi per sampled embryo or poult: villus height, villus apical width at the villus tip, villus basal width at the crypt-villus junction, crypt depth, and muscularis depth. Apparent villus surface area was estimated by the following mathematical formula: $[((villus \, tip+villus \, base)/2) \times villus \, height]$. The relationship between villus surface area and basal metabolic rate or body surface area (body weight$^{0.75}$) was plotted to identify the time point at which villus surface area peaks in relationship to total body surface area.

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3 Chick Master Incubator Co. Medina, OH
4 Alternative Design Manufacturing and Supply, Inc. Siloam Springs, AR
5 Department of Dental Diagnostic Science at The University of Texas Health Science Center, San Antonio, Texas
Also, villus height:crypt depth ratio was calculated as an estimation of epithelial cell renewal.

To illustrate an accurate profile of villi development in the small intestine, a series of high resolution micrographs (20X) were obtained from representative villi at each time point using a Leica DM IRB research microscope equipped with a Q-IMAGING Micropublisher cooled CCD color camera. The slides used for this purpose were stained with Alcian blue dye to facilitate the appearance of goblet cells in the developing villi. To enhance the contrast between absorptive epithelial cells and goblet cells, micrographs were taken using difference interference contrast microscopy. Micrographs were then merged using the Photomerge option of Adobe Photoshop CS4 Extended.

**Electron microscopy analysis**

Tissues were prepared for examination at the center for electron microscopy of the NCSU Microbiology Department according to the protocol described below. Tissue blocks fixed on 3% glutaraldehyde were washed 3 times in SPB, each time for 20 minutes at 4°C. Then, tissue blocks were post-fixed with 1% osmium tetroxide (OsO₄), for 1 hour at 4°C. Tissue blocks were then rinsed in SPB and dehydrated through a graded series of alcohol (30%, 50%, 70%, 95% and 100% each time for 30 minutes). At this point some blocks were separated for transmission electron microscopy (TEM) analysis. Scanning electron microscopy (SEM) samples were critical point dried in a Samdri PVT-2 using liquid carbon dioxide. Specimens were mounted on aluminum stubs with silver paint and coated with a total of 50-60kÅ of gold/palladium in a Hummer 6.2 Sputtering Device. SEM was performed in a JEOL 5900LV at 20 kV accelerating voltage.

Tissue blocks separated for TEM analysis were infiltrated two times (1:1 with 100% ethanol and 3:1, 100% ethanol) with Spurr embedding media for 6 hours each time and followed by three changes in 100% Spurr (no ethanol) for 6 hours each. Finally, tissue blocks were embedded overnight at 70°C in fresh 100% Spurr using flat embedding molds (for correct orientation). Blocks were trimmed with razor blades and sectioned at 70-80 nm on a diamond knife using an LKB NOVA Ultramicrotome. Sections were stained on-grid with 4% uranyl acetate for 1 hour at room temperature in the dark, followed by 3 water rinses, and 4 minutes in Reynolds’ lead citrate with 3 water rinses. Grids were

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6 Adobe Systems Incorporated, San Jose CA
analyzed using a JEOL JEM100S Transmission Electron Microscope at 80 kV accelerating voltage.

**Animal ethics**

Embryos and birds were managed according to normal husbandry practices and the experiments were conducted according to the experimental protocol approved by the Institutional Animal Care and Use Committee (IACUC) of North Carolina State University.

### 2.4 RESULTS AND DISCUSSION

Although the development of the gut occurs throughout incubation (Romanoff, 1960), the emergence of villi only begins during the second half of incubation when a series of complex mucosal changes take place in preparation for life ex-ovo (Figure 1). In order to facilitate the discussion, the observations have been divided into four temporal stages based on the main villi structural adaptations that occur during this period: (1) the period from the appearance of longitudinally pre-villus ridges (15E) until the formation of villi (23E), (2) the rapid changes in villi ultra-structure that take place after amniotic fluid swallowing until hatch, (3) the structural adaptations of the small intestinal epithelium post-hatch (up to 12D) in response to dietary intake, (4) the contribution of endogenous gut microorganisms to the morphological adaptations of the mucosa after hatch.

At 15E, only pre-villus ridges are visible lining the base of the gut wall (Figure 4a-b). These mucosal structures commonly appear as mucosal folds, during the second trimester of incubation or gestation (Hilton, 1902) and this developmental event is conserved across species (Trahair and Sangild, 2002). In the duodenum of the chicken embryo, ridges arise opposite to each other as early as 9E (Bell and Williams, 1982; Burgess, 1975; Coulombre and Coulombre, 1958; Lim and Low, 1977) as a consequence of active microfilaments constrictions in epithelial cells to form longitudinal folds along the gut wall (Burgess, 1975). In the 15E turkey embryo, primitive enterocytes exhibit a bubbly appearance (Figure 6a) and microvilli are seen as small and sparse protrusions at the apical end (Figure 6a-b). This appearance is characteristic of the early stages of epithelial cell development in the gut (Lim and Low, 1977). The height of the pre-villus ridges remains practically unaffected until 23E. There is only a 32 µm differential in the height of the pre-villus ridges height from 15E to 23E.
(98µm vs 130µm, respectively), while the crypts are not evident at least before 25E (Table 1, Figure 1). In the chicken, crypts are not evident until hatch (Uni et al., 2000). However, there are rapid changes in the microanatomy of the turkey mucosa during this period. Longitudinal pre-villus ridges at 15E (Figure 4c-d) become a serpentine-like structure by 17E (Figure 4e-f). By 21E, the serpentine-like structure splits into two lines of alternating villi that clearly resemble a zigzag pattern (Figure 5a-b). In the chicken embryo, this pattern is observed around 14E (Burgess, 1975; Hilton, 1902). By 23E, two parallel lines of mature villi are distinguished (Figure 5c-d).

The morphological changes of the mucosa up to this time coincide with the dynamics of amniotic fluid (AF). Data regarding the accumulation, composition and swallowing of AF in turkeys is scarce (de Oliveira, 2007; Romanoff and Hayward, 1943). Here we observed AF to reach a peak volume of 14mL at 19E (Figure 2). Romanoff and Hayward (1943) reported a similar pattern of accumulation and disappearance of AF in turkeys; however, the peak volume reported at the time was ~8mL. The strain of turkeys studied by Romanoff and Hayward was the White Holland, which is not a commercial strain anymore. Although the dynamics of AF volume among late-term embryos of current commercial strains of turkeys (Nicholas or Hybrid turkeys) appear similar to the previous Holland White strain, the volume (and perhaps the composition) has changed. From 19E, AF volume rapidly decreases as the embryo swallows it, such that there is little or no fluid left by 25E. Although the mucosa of the 23E embryo resembles that of a poult at hatch, it is only after the embryo has completely imbibed the amniotic fluid (AF) that the onset of villi digestive and absorption capacity takes place (Moran Jr, 1985; Uni et al., 2003). Because AF is rich in proteins, amino acids, growth factors and hormones, swallowing, it is literally the bird’s first meal, primarily used to support rapidly dividing cells of the intestinal epithelium (Cleveland et al., 1991; Wagner, 2002). The morphological changes observed immediately after this AF swallowing is indicative of the potential for the nutritional supplementation of the AF via in-ovo feeding (Uni and Ferket, 2003; Uni and Ferket, 2004).

Between 23E and 25E, there is a clear inflexion in the rate of change in villi height observed at low magnifications (Figure 1). Also during this period, epithelial cells actively differentiate, as is evident by the appearance of prominent goblet cells along the villi (Figure 1). There is more than a 2-
fold increase in villus height (130 µm at 23E vs 295 µm at 25E), perhaps sustained by an increase in cell proliferation and a decrease in the rate of epithelial cell turnover as indicated by a 2-fold increase in the villus-height:crypt-depth ratio (2.9 at 23E vs 5.2 at 25E). Consequently, a 3.5-fold increase in apparent villus surface area occurs from 23E to 25E (Table 1, Figure 5e), which continues to increase linearly until hatch. When expressed relative to metabolic body mass (Body Weight$^{0.75}$), the fastest expansion of villus surface area in relationship to that of the whole body occurs between 25E and hatch (Figure 3). During this period, the villi are typically round in shape and composed primarily of absorptive epithelial cells with a distinctive columnar shape (Figure 5f). However, some villi do not form the characteristic finger-like projections and appear as “balloon towers” (Figure 5e-inset) with few microvilli (data not shown). Similar balloon-shaped villi become more prevalent in the small intestine of fetal sheep that have undergone esophageal ligation to prevent amniotic fluid swallowing (Trahair and Sangild, 2002).

High magnification cross-sections revealed that cells are more closely joined at 25E (Figure 6e) by different cell junctions (e.g. tight junctions, desmosomes) than at 23E (Figure 6d) and much more so than at 19E (Figure 6c). The higher permeability of the gut during embryonic development allows for the absorption of macromolecules (Shah and Sanderson, 2000; Vukavic, 1984). However, across species, cell permeability must be tightly regulated immediately after birth to prevent the entrance of harmful agents (e.g. toxins, microorganisms), while allowing the influx of essential nutrients and other molecules (Ozden, 2004; Ozden, 2009). A comparable process, known as gut closure, has been widely studied in the perinatal and newborn mammals, in particular with regard to immunoglobulin absorption and the onset of the immune system (Hardy et al., 1971; Lecce and Broughton, 1973; Vukavic, 1984).

By hatch, the apical ends of the enterocytes form a distinctive hexagonal shape with long filamentous microvilli projecting into the lumen (Figure 6f-inset, 7a). The single layer of epithelial cells is gracefully arranged around the villus core (Figure 8a-c), which holds a vast array of blood arterioles and vessels where nutrients and other molecules can be exchanged (Kessel and Kardon, 1979). Enterocytes are long columnar epithelial cells with wider ends at the brush border than at the basal
membrane attachment (Figure 7a). The distance between cells varies from apical to basal end
(Ozden, 2004; Ozden, 2009). At the apical end, tight junctions bring epithelial cells in intimate contact
to each other (Figure 7c), where as cell junctions appear loose at the basal end (Figure 7d), perhaps
to allow the transport of large molecules (e.g. chylomicrons) to pass into circulation (Figure 7e). At
hatch, the mucosa is exposed to a rapid influx of exogenous nutrient particles and bacteria invading
the gut lumen. Although the villi are still arranged in parallel lines, the formation has started to lose
their organized identity, and extrusion zones at the apical end of individual villus are visible (Figure
9a-b). Figure 9a also shows villi from parallel lines have variable heights at hatch; some appear as
small villi stubs while others are longer more defined villi structures. After hatch, epithelial cells are
actively exfoliated at the extrusion zones (Skrzypek et al., 2005). The active cell turnover is
compensated by increased epithelial cell mitosis associated with a large increase in crypt depths
(49\( \mu \)m at E27 vs 101\( \mu \)m at hatch). Microvilli height also increased 2-fold from 0.97\( \mu \)m at 27E to
1.90\( \mu \)m at hatch, which likely contributes to the expansion of the luminal surface required for the rapid
increase in digestive capacity observed post-hatch (Moran Jr, 1985; Sell et al., 1991; Sklan, 2001;
Uni et al., 1998a; Uni et al., 1995).

The small intestine is constantly exposed to a changing intraluminal environment and has the
capacity to adapt its structure and function to variations in the diet (Moran Jr, 2007; Shah and
Sanderson, 2000; Thomson and Keelan, 1985; Uni et al., 1998a). For instance, starvation can rapidly
decrease the density of villi, reduce villus length and villus surface area, cause microvilli clustering
and accelerate epithelial cell turnover, thus compromising epithelial cell barrier and growth
progression of the animal (Bayer et al., 1981; Butzner and Gall, 1988; Noy et al., 2001; Uni et al.,
1998a). Although stressors usually affect the progression of mucosal microanatomy, luminal contents
from feed intake are also important modulators of mucosal microanatomy (Dibner et al., 1996; Shah
and Sanderson, 2000). In the turkey poult, these changes are clearly visible by 12 days post-hatch
when the small intestinal villi adopt a distinctive tongue-like shape, flat and extended (Figure 9e). By
this time, the villi height and villus surface area appear to reach a plateau (Figure 1, Table 1). In
chickens, this plateau has been reported to occur at the end of the first week after hatch (Uni et al., 1998a; Uni et al., 1995).

Finally, gut microbiota cannot be overlooked as major modulator of the microanatomical adaptations of the mucosa after hatch. Although some protozoa and unidentified bacteria can be observed prior to hatch (Chapter III), only after hatch do bacterial colonies thrive to form a complex microbial ecosystem. By 12 days post-hatch microorganisms are abundant in the distal small intestine of the turkey poult (Figure 10d). Microbial colonies are commonly associated with the mucus blanket (Figure 10e) (Liévin-Le Moal and Servin, 2006). Based on observations in germ-free animals, postnatal colonization of the gut epithelium by microorganisms is necessary for normal gut development (Xu and Gordon, 2003). The colonization of segmented filamentous bacteria (SFB) serves as a good example of these symbiotic effects. SFB are indigenous non-culturableViewable bacteria that are highly conserved in the gut of several species, ranging from chickens to humans (Snel et al., 1995). These bacteria commonly appear after weaning in piglets and mice (Snel, 1997), and after the first week post-hatch in birds (Goodwin et al., 1991). Likewise, we observed SFB colonizing the distal small intestine of turkey poult as early as the second week post-hatch (Figure 10a-c). This type of bacteria attach directly onto the epithelium (Figure 35), not into goblet cell openings (Figure 36) (Angert, 2005), and trigger a cascade of events that affect villi morphology and the developing immune system (Braun-Fahrlander et al., 2002). SFB are also known to influence the colonization of other commensal bacteria by serving as an anchor between the epithelium and the lumen (Angert, 2005; Klaasen et al., 1992), allowing other bacteria to interact with the mucus blanket (Figure 10c). SFB have also been associated with higher mitotic activity in the small intestinal epithelial cells (Umesaki et al., 1995). Although SFB are among the microorganisms that may influence the normal development of the intestinal epithelium, there are several other microorganisms within the complex gut microbial ecosystems that also influence enteric maturation. A large proportion (~85%) of microorganisms associated with the gut have not been previously described (Xu and Gordon, 2003). This estimation is based on the human gut microbiome, where most of the research has been performed.
In summary, villi emerge from pre-villus ridges between 15 and 23E. The embryo swallows the majority of amniotic fluid by 23E, after which there is an accelerated morphological development of the small intestinal mucosa. Villus surface area relative to metabolic body weight reaches its peak by hatch. Mature columnar epithelial cells with hexagonal tips are observed lining the villus core at this time. Dietary nutrients have a major influence on the villi morphological adaptations. Finger-like villi at hatch become elongated tongue-shaped projections by 12d. Finally, microbial colonization contributes to the morphological adaptations of the villi post-hatch, and this is exemplified by the interaction of segmented filamentous bacteria with the mucosal epithelium.

ACKNOWLEDGMENTS

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2.5 TABLES AND FIGURES

Table 1. Histomorphometrical analysis of jejunum villi in turkey embryos and poults from 15 days of incubation (E) to 12 days post-hatch (d).

<table>
<thead>
<tr>
<th>Embryo or Poul Age, days(^{\dagger})</th>
<th>Body Weight, (g^2)</th>
<th>Tip, (\mu m^3)</th>
<th>Height, (\mu m)</th>
<th>Base, (\mu m)</th>
<th>Crypt, (\mu m)</th>
<th>Villi:Crypt, (\mu m: \mu m)</th>
<th>Muscularis, (\mu m)</th>
<th>Villus Surface, (\mu m^4)</th>
<th>Microvilli, (\mu m^4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15E</td>
<td>7.4+/-.6</td>
<td>27.0+/-.2.8</td>
<td>98.0+/-.7.3</td>
<td>42.1+/-.3.2</td>
<td>-</td>
<td>2.04+/-.16</td>
<td>84.0+/-.4.9</td>
<td>3435+/-.479</td>
<td>NA</td>
</tr>
<tr>
<td>17E</td>
<td>13.0+/-.1.3</td>
<td>23.7+/-.3.6</td>
<td>108.9+/-.9.7</td>
<td>47.2+/-.3.9</td>
<td>-</td>
<td>2.32+/-.0.24</td>
<td>90.2+/-.7.1</td>
<td>3836+/-.769</td>
<td>NA</td>
</tr>
<tr>
<td>19E</td>
<td>20.5+/-.0.7</td>
<td>35.7+/-.6.3</td>
<td>116.6+/-.6.2</td>
<td>54.7+/-.12.7</td>
<td>-</td>
<td>2.58+/-.0.14</td>
<td>93.7+/-.15.4</td>
<td>5227+/-.820</td>
<td>0.27+/-.0.06</td>
</tr>
<tr>
<td>21E</td>
<td>30.4+/-.2.2</td>
<td>38.4+/-.5.2</td>
<td>148.0+/-.15.8</td>
<td>66.3+/-.10.1</td>
<td>-</td>
<td>3.19+/-.0.42</td>
<td>125.8+/-.8.4</td>
<td>7753+/-.1168</td>
<td>0.43+/-.0.07</td>
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<tr>
<td>23E</td>
<td>40.2+/-.2.9</td>
<td>37.2+/-.1.6</td>
<td>130.4+/-.16.7</td>
<td>47.7+/-.8.2</td>
<td>-</td>
<td>2.90+/-.0.46</td>
<td>131.2+/-.1.5</td>
<td>5505+/-.530</td>
<td>0.67+/-.0.17</td>
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<tr>
<td>25E</td>
<td>51.8+/-.3.6</td>
<td>53.8+/-.3.0</td>
<td>295.1+/-.35.7</td>
<td>75.5+/-.17.3</td>
<td>57.2+/-.2.7</td>
<td>5.24+/-.0.81</td>
<td>162.8+/-.17.4</td>
<td>19541+/-.5277</td>
<td>0.52+/-.0.06</td>
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<tr>
<td>27E</td>
<td>57.6+/-.2.9</td>
<td>62.0+/-.1.3</td>
<td>407.3+/-.83.8</td>
<td>91.9+/-.11.6</td>
<td>49.2+/-.4.7</td>
<td>8.27+/-.0.90</td>
<td>192.8+/-.13.9</td>
<td>32752+/-.7543</td>
<td>0.97+/-.0.04</td>
</tr>
<tr>
<td>Hatch</td>
<td>59.8+/-.4.0</td>
<td>79.3+/-.5.1</td>
<td>615.4+/-.55.4</td>
<td>113.4+/-.15.0</td>
<td>101.2+/-.8.7</td>
<td>6.08+/-.0.45</td>
<td>154.4+/-.21.5</td>
<td>59029+/-.5458</td>
<td>1.90+/-.0.13</td>
</tr>
<tr>
<td>4d</td>
<td>87.2+/-.1.2</td>
<td>106.8+/-.17.2</td>
<td>739.6+/-.95.0</td>
<td>117.9+/-.17.9</td>
<td>139.8+/-.11.3</td>
<td>5.29+/-.0.49</td>
<td>186.6+/-.34.0</td>
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<td>8d</td>
<td>154.1+/-.5.1</td>
<td>115.0+/-.11.8</td>
<td>883.6+/-.52.2</td>
<td>126.8+/-.22.5</td>
<td>133.7+/-.4.7</td>
<td>7.14+/-.1.41</td>
<td>243.8+/-.61.4</td>
<td>98264+/-.7678</td>
<td>NM</td>
</tr>
<tr>
<td>12d</td>
<td>272.3+/-.5.9</td>
<td>117.1+/-.7.0</td>
<td>910.3+/-.64.3</td>
<td>130.6+/-.10.2</td>
<td>148.7+/-.19.4</td>
<td>6.25+/-.1.21</td>
<td>395.6+/-.67.2</td>
<td>112725+/-.8939</td>
<td>3.11+/-.0.14</td>
</tr>
</tbody>
</table>

\(^{\dagger}\)Embryo age (E) is presented as days of incubation and turkey poult age (d) is presented as days post-hatch

\(^{3}\)Values represent the average of four yolk free embryos or poults.

\(^{4}\)Values represent the average of measurements in four embryos or poults per time point. Ten mature villi were measured per embryo/poult.

\(^{*}\)Villus surface area was calculated as: [(villus tip + villus base)/2]*villus height

\(^{\ast}\)Values represent the average of at least 40 microvilli per time point. NA = Not available microvilli. NM = No measurements performed on this time point.
**Figure 1.** Histomorphological and histomorphometrical (inset) development of proximal jejunum villi in the turkey embryo and poult from 15 days of incubation (E) to 12 days post-hatch (d). ‡Values represent the average of measurements in four embryos or poults per time point. Ten mature villi were measured per embryo/poult. Goblet cells revealed by Alcian blue dye staining.
Figure 2. Changes in amniotic fluid volume of Nicholas turkey embryos from 15 to 25 days of incubation (E). Each value represents the average of six observations per time point plus/minus the standard deviation of the mean.
Figure 3. Villus surface area relative to metabolic body weight (Body Weight^{0.75}) in the perinatal turkey embryo and poult from 15 days of incubation to 12 days post-hatch (d). Each value represents the average of four observations per time point plus/minus the standard deviation of the mean.
Figures 4. Ultra-structural arrangement of small intestinal villi in the turkey embryo from 15 days of incubation to 19 days of incubation. (a-b) Longitudinal pre-villus ridges in the proximal jejunum of turkey embryos at 15 days of incubation. (c-d) Formation of zigzag patterns by the pre-villus ridges in the proximal jejunum of turkey embryos at 17 days of incubation. Inset shows a lateral view of the zigzag pattern of the ridges. Note the bubbly appearance of enterocytes. (e-f) Zigzag pattern of previllus-ridges in the proximal jejunum of turkey embryos at 19 days of incubation.
Figure 5. Ultra-structural arrangement of small intestinal villi in the turkey embryo from 21 days of incubation to 25 days of incubation. (a-b) Formation of individual villus in the proximal jejunum of turkey embryos at 21 days of incubation. (c-d) Parallel arrangement of villi lanes in the proximal jejunum of turkey embryos at 23 days of incubation. Note that each lane has an adjacent line of new villi that joins the formation. (e-f) Villi in the proximal jejunum of turkey poults at 25 days of incubation. Finger-like villi with perfectly round apical ends. However, some villi failed to differentiate and form shapes that resemble a balloon on a column (inset).
Figure 6. Microvilli ultra-structure in the perinatal turkey poult. (a) Three dimensional appearance of enterocytes and microvilli in the proximal jejunum of turkey embryos at 15 days of incubation. Note that some cells have a distinctive bubbly appearance, which is characteristic of the early stages of epithelial cells. (b) Cross-sectional appearance of enterocytes and microvilli in the proximal jejunum of turkey embryos at 15 days of incubation. Note that microvilli emerge as small protrusions at the apical end of epithelial cells. (c) Cell junctions (e.g. tight junctions – white arrow) in the proximal jejunum epithelium of turkey embryos at 19 days of incubation. (d) Cell junctions (e.g. tight junctions – white arrow) in the proximal jejunum epithelium of turkey embryos at 23 days of incubation. (e) Cell junctions (e.g. tight junctions – white arrow) in the proximal jejunum epithelium of turkey embryos at 25 days of incubation. (f) Hexagonal arrangement of epithelial cells (mostly enterocytes) in the apical end of villus from the proximal jejunum of turkey pouls at hatch. By this time, the microvilli that form the brush border are fully developed (inset).
Figure 7. Ultra-structural arrangement of small intestinal enterocytes. (a) Small intestinal enterocytes. Note the unique arrangement of cell junctions along the enterocytes from the brush border to the basal lamina. (b) Apical view of mature enterocytes from the proximal small intestine of turkey poult at hatch. (c) Apical cell junctions (e.g. tight junctions – white arrows) of mature enterocytes from the proximal small intestine of turkey poult at hatch. (d) Cell junctions at the basal lamina of mature enterocytes from the proximal small intestine of turkey poult at hatch. (e) Basal end of enterocytes at the villi core. Note the process of molecule extrusion (arrow) from the basal end of enterocytes to the villus core occurring through openings at the basal lamina known as fenestrations.
Figure 8. Ultra-structural topography of small intestinal villi. (a) The open villus. Note the tight arrangement of different types of epithelial cells (e.g. goblet cells, enterocytes) around the villus core formed by an arrangement of afferent and efferent blood vessels that facilitate counter-current exchange of molecules. (b) Three-dimensional structure of mature villus in the proximal jejunum of a 4-day-old turkey poult. Note the distinctive finger-like shape. (c) Two-dimensional structure of mature villus in the proximal jejunum of a turkey poult at hatch. Note the active mucus secretion by goblet cells (blue stained) that rapidly forms a mucus blanket around the epithelium.
Figure 9. Ultra-structural arrangement of small intestinal villi in the turkey poult from day of hatch to 12 days of age. (a-b) Villi in the proximal jejunum of turkey poults at hatch. Note that the previous parallel arrangement of villi begins to dissipate and extrusion zones are evident on the apical ends. (c-d) Villi in the proximal jejunum of turkey poults at 8 days of age. Note that villi begin to adopt a flat configuration on the tips. (e) Villi in the proximal jejunum of turkey poults at 12 days of age. Note the characteristic tongue-like shape of villi at this age. Inset shows a lower magnification of the same view.
Figure 10. Gut microbiota in the 12 day-old turkey poult. (a) Segmented filamentous bacteria attached to villi in the distal-ileum of 12 day-old turkey poult. Note the major structural influence of these organisms on the intestinal villi. (b) Young segmented filamentous bacteria preferentially penetrate the epithelium instead of attaching onto goblet cell openings (arrow). (c) Segmented filamentous bacteria (black arrow) harbor other bacilli-like bacteria (white arrow). This mechanism helps other bacteria to colonize the mucosal epithelium without direct interaction. (d) Complex microbial ecosystem surrounding villi in the distal-ileum of turkey poult at 12 days of age. (e) Microorganisms (white arrows) in association with mucus structures (black arrow).
2.6 REFERENCES


Ozden, O. 2004. Developmental profile of claudin-3, claudin-5, and claudin-16 tight junction proteins in the chick intestine. MS. North Carolina State University, Raleigh, NC.


CHAPTER III

GUT MICROBIOTA IN THE TURKEY EMBRYO
3.1 ABSTRACT

The digestive tract of avian embryos is assumed to be sterile at hatch; however, the occurrence of bacterial vertical transmission from the hen to the egg during oviposition has been reported in poultry (Cox et al., 2005; Hong et al., 2003). In some oviparous species of invertebrates, in-ovo transmission of microflora is a common mechanism to ensure the inheritance of commensal microorganisms from one generation to the next (Davidson and Stahl, 2006; Davidson and Stahl, 2008; McFall-Ngai and Ruby, 2000). Hence, we hypothesized that microbial colonization of the avian gut begins in-ovo prior to hatch. This hypothesis was tested in three separate experiments using different incubation groups of Nicholas turkey embryos. In experiment 1, the distal ileum of 4 embryos was sampled at 23 days of incubation (E), 25E and 27E for scanning electron microscopic (SEM) analysis. In experiment 2, ceca tissue from 4 embryos was sampled at 15E, 17E, 19E, 21E, 23E, 25E and 27E for SEM analysis. In experiment 3, luminal contents of the distal intestine and ceca from 24 embryos were aseptically collected at 25E, and brain tissue was collected as a negative control. DNA was extracted from each intestinal and brain sample for microbial profiling using 16S rDNA gene amplification and Terminal Restriction Fragment Length Polymorphism (TRFLP) analysis. SEM micrographs of the distal ileum revealed distinctive colonies of bacteria and protozoa associated with the epithelium at 27E. SEM analysis of ceca samples revealed micro-colonies of bacteria from 17E through 27E. These micro-colonies appeared to be composed of at least 3 different shapes of bacteria-like microorganisms, and the complexity of these micro-colonies seemed to increase with age. Amplification using a set of universal primers of the 16S rDNA gene was accomplished only from intestinal samples. Evidence of 16S rDNA presence, a unique component of the small prokaryotic ribosomal subunit (30S), confirms microbial colonization in the distal gut of embryos. TRFLP analysis confirmed the presence of a few bacterial species in the ceca of 25E embryos. The potential species include *Bacillus*, *Lactobacilli*, *Pseudomonas* and several uncultured bacteria. This microscopic and molecular biological evidence confirms that microbial colonization of the gut of turkeys begins during embryonic development several days prior to hatch.
3.2 INTRODUCTION

When does microbial colonization of the gut actually begin in avian species? Many scientists consider that the intestinal tract of hatchlings to be sterile or free of microorganisms at the time of hatch (Amit-Romach et al., 2004; Lu et al., 2003; Mead, 1997; Wielen et al., 2002), and they think microbial colonization occurs during or soon after hatch (Dibner et al., 2008; Lev and Briggs, 1956; Mead, 1997; Vispo and Karasov, 1997). Nonetheless, vertical contamination of fertile eggs during oviposition has been documented before (Cox et al., 2005; Hong et al., 2003). Moreover, there are reports of maternally-derived immunoglobulins present in the lumen of the embryonic gut several days before hatch (Loeken and Roth, 1983; Rose et al., 1974). This evidence raises the question of whether this early microflora colonization is a casual pathology or a conserved and regulated mechanism by which the hen has an input in the composition of the microbial ecosystem of its progeny.

Vertical transmission and embryonic colonization of bacteria are processes that have been thoroughly studied in oviparous invertebrates. For instance, adult earthworms (Eisenia fetida) have evolved mechanisms to ensure the transfer of specific Acidovorax-like bacteria associated with their excretory organ (i.e. nephridia) to the next generation (McFall-Ngai and Ruby, 2000). Nephridial bacteria are transmitted directly into fertile egg capsules of Eisenia fetida (Davidson and Stahl, 2006). In this way, bacteria interact with the embryo internally and externally during its development and, right before hatch, bacteria invade the embryonic gut lumen so that juveniles emerge fully colonized by their specific Acidovorax-like symbionts (Davidson and Stahl, 2008). Similar mechanisms of mother-embryo vertical inheritance of microorganisms have also been reported in marine sponges (Ereskovsky et al., 2005), arthropods (O'Neill et al., 1997), shrimp larvae (Gil-Turnes et al., 1989) and insects (Douglas, 1989). However, the existence of this mechanism in higher animals has yet to be proven.

While working on an ultra-structural development profile of the small intestinal villi of the turkey embryo and poult (Chapter II) (Bohórquez et al., 2009), we observed some microbial-like shapes associated with the ileal epithelium right before hatch, prompting questions about the actual timeline
of gut microbial colonization (experiment 1). Therefore, we hypothesized that bacteria colonize the gut of the turkey embryo before hatch. To accomplish this hypothesis, we used scanning electron microscopy to survey the ceca epithelium of turkey embryos from 15E to 27E for the presence of bacteria. In addition, a molecular profile of the potential bacteria species present in the contents of the embryonic distal gut was developed using terminal restriction fragment length polymorphism (TRLFP) analysis of the 16S rDNA prokaryotic gene. We focused our quest on the distal gut because this is the site where the largest concentration of microorganisms is found in the digestive tract of more mature poultry (Salanitro et al., 1974; Zhu et al., 2002).

### 3.3 MATERIALS AND METHODS

**Tissue collection**

**Experiment 1**

Fertile Nicholas turkey eggs (600) were obtained from a commercial hatchery and incubated in a Chickmaster® research incubator at 37.5°C and 52% relative humidity. Four embryos were euthanized by cervical dislocation and sampled at 23E, 25E and 27E. A tissue segment was removed from the distal ileum and four tissue blocks (~3x3mm each) per segment were then dissected on dental wax while submerged in a drop of 0.1M sodium phosphate buffer, pH 7.2, (SPB) to avoid drying of the mucosa. Tissue blocks were immediately submerged and fixed in cold (~4°C) 3% glutaraldehyde for at least 72 hours before processing for scanning electron microscopy analysis.

**Experiment 2**

Fertile Nicholas turkey eggs (100) were obtained from a different commercial hatchery than those eggs in experiment 1 and incubated under similar conditions. Four embryos were euthanized by cervical dislocation and sampled at 15E, 17E, 19E, 21E, 23E, 25E and 27E. A tissue segment was removed from the tip of ceca horns and four tissue blocks (~3x3mm each) per segment were then dissected and fixed immediately for scanning electron microscopy analysis as described in experiment 1.

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1 Chick Master Incubator Co. Medina, OH
**Experiment 3**

Fertile Nicholas turkey eggs (30) were obtained from a commercial hatchery at 25E and transported to a lab equipped with a biological hood using a styrofoam portable incubator maintained at 37.5°C. Eggs were removed from the portable incubator one at a time and sprayed with 70% ethanol before sampling. Only embryos that had not internally pipped the air sac were sampled. Sampling procedures were performed under sterile conditions. Twenty-four embryos were euthanized by cervical dislocation, and the luminal contents within the distal ileum, ceca, and colon were collected from 8 embryos, pooled into a sterile cryogenic vial and placed in liquid N₂, for a total of 3 pooled sample replicates. Because the brain is known to be devoid of bacteria, brain tissue from three embryos was collected and pooled as a negative control. Samples were then stored at -80°C before DNA extraction for terminal restriction fragment length polymorphism (TRFLP) analysis of the prokaryotic gene 16S rDNA.

**Scanning electron microscopy (SEM)**

Tissues were prepared for examination at the center for electron microscopy of the NCSU Microbiology Department according to the protocol described below. Tissue blocks fixed on 3% glutaraldehyde were washed 3 times in SPB, each time for 20 minutes at 4°C. Then, tissue blocks were post-fixed with 1% osmium tetroxide (OsO₄), for 1 hour at 4°C. Tissue blocks were then rinsed in SPB and dehydrated through a graded series of alcohol (30%, 50%, 70%, 95% and 100% each time for 30 minutes). Then, samples were critical point dried, mounted on aluminum stubs with silver paint and coated with 50-60kÅ gold/palladium before observation in a JEOL-5900LV SEM at 20 kV.

**Terminal restriction fragment length polymorphism (TRFLP)**

**PCR of 16S rDNA and TRFLP analysis.** Intestinal contents and brain tissue samples collected in experiment 3 were processed at the UNC-Chapel Hill Microbiome Core Facility for 16S rDNA amplification and TRFLP analysis. Nucleic acid extraction and purification was performed using the DNeasy Blood & Tissue kit. Then, 100ng of DNA from intestinal content and brain samples were PCR amplified with the universal primers Eub8f-HEX (5’ HEX - AGA GTT TGA TCC TGG CTC AG -

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2 QIAGEN Inc., Valencia, CA
3') and Eub1113r (5' - GGG TTG CGC TCG TTG -3'). The forward primer was 5' labeled with hexachlorofluorescein (HEX), for TRFLP analysis. PCR conditions were: Initial step DNA polymerase activation at 95°C for 5 min; followed by 35 cycles of denaturation at 95°C for 45 sec; annealing at 58°C for 45 sec; extension at 72°C for 1 min, and the final extension step at 72°C for 10 min. The size of the expected amplification product was corroborated by gel electrophoresis. PCR products were digested using the following restriction enzymes: HhaI (GCG^C), MspI (C^CGG) and RsaI (GT^AC). A TRFLP profile was generated by analyzing digested PCR products with an ABI 3130xl Genetic Analyzer. TRFs had a reported fragment length, in nucleotides, and a reported peak area of fluorescently labeled product, in fluorescence units (FU) (Osborne et al., 2006).

**TRFLP Data Analysis.** The fluorescence integrated under any one peak is referred to as the area of that peak, and the total area for any one profile is the sum of the areas of all of the peaks excluding those generated by fragments of less than 50 nt or greater than 1000 nt (Osborne et al., 2006). The relative peak area ratio, Pi, was calculated by dividing each individual peak area by the total peak area of each profile. Pi values had to be equal or greater than 1% to be considered as a real peak to eliminate possible background noise (Li et al., 2007). GeneMapper generated profiles were tentatively assigned to bacterial operational taxonomic units (OTUs), using the RDP SSU 16S rDNA database generated by the Phylogenetic Assignment Tool (PAT). In addition, a phylogenetic tree was developed based on the peak area. The dendrogram (phylogenetic chart) was constructed using the hierarchical option and the Ward’s method of analysis of JMP (Blackwood et al., 2003).

### 3.4 RESULTS

**Experiment 1. Microorganisms in the ileal mucosa of turkey embryos at 27E:** SEM micrographs of the distal ileum of turkey embryos at 23E and 25E did not reveal any visual evidence of bacteria or other microorganisms associated with the epithelium. However, some structures with distinctive bacteria-like shapes and size were visible on the surface of ileal villi at 27E (Figure 1a). Some of these bacteria-like shapes (Figure 1a-inset) appeared to be associated with a structure similar to yolk
lipid-droplets (Bellairs, 1961). More evident was a protozoa-like organism directly associated with ileal villi (Figure 1a). A close-up micrograph of this microorganism revealed the serrated nature of one of its edges and its association with smaller bacteria-like shapes (Figure 1b). These data suggested that microbial colonization of the distal digestive tract, at least in the turkey embryo, began prior to hatch.

**Experiment 2. Microbial colonies in the ceca of turkey embryos from 17E to 27E.** The objective of experiment 2 was to survey the ceca horns in the developing embryo using SEM for the presence of microorganisms prior to hatch. SEM micrographs of ceca epithelium did not reveal evidence of microbial colonization at 15E. However, small micro-colonies of bacteria-like shapes associated with structures similar to lipid droplets, perhaps from internalized yolk (Bellairs, 1961), were observed at 17E (Figure 2a). Few of these micro-clusters were localized in between cecal ridges (Figure 2b). The complexity of these micro-colonies appeared to increase with age. At 19E, a cluster of bacteria-like microorganisms composed of structures from at least three shapes (rods, filaments and spirals) was localized on the epithelium surface (Figure 2d). This particular cluster was in association with some unidentified luminal structure (Figure 2c). Similar shapes of microorganisms were observed at 21E, 23E, 25E and 27E (Figures 2e-f, 3a-f). Complex associations of micro-colonies were observed at 25E on top of cecal ridges (Figure 3c-d), and by 27E some filamentous-like bacteria were piercing the ceca epithelium (Figure 3f).

**Experiment 3. TRFLP Bacterial profile of gut contents from turkey embryos at 25E.** The objective of experiment 3 was to develop a molecular profile of potential bacterial species found in the distal gut of turkey embryos. Gel electrophoresis of PCR products from gut contents showed a band corresponding to the molecular weight of 16S rDNA gene (Figure 4); however, the PCR product from brain tissue samples, used as the negative (-) control, did not yield any visible band in the gel. In addition, 5ng of *Lactobacillus casei* DNA was used as a positive control. TRFLP analysis of PCR products digested with restriction endonucleases (*HhaI, MspI* and *RsaI*) yielded a list of potential bacterial species that are present in the gut lumen of turkey embryos at 25E (Table 1). These bacteria have been associated in 3 main clusters as shown by the phylogenetic analysis (Figure 5). The results show a predominance of bacteria from the firmicutes and proteobacteria phyla, as well as a
considerable number of previously uncultured bacteria. *Lactobacilli, Bacilli* and *Pseudomonas* are some of the bacteria genera of primary interest from a gut microbial ecology perspective.

### 3.5 DISCUSSION

Although the diversity and complexity of the gut microbial ecosystems is known to increase with the age of the host (Amit-Romach et al., 2004; Wielen et al., 2002; Zhu et al., 2002), it is the first bacteria to enter the gut that modulate the expression of epithelial cell genes to create a more favorable environment for themselves and prevent the growth of other incoming bacteria (Hooper et al., 2001; Nurmi et al., 1992; Nurmi and Rantala, 1973). Moreover, the endogenous intestinal microbiota is essential in mediating the host’s nutrient metabolism (Turnbaugh et al., 2006), regulating epithelial cell development (Gordon et al., 1997; Stappenbeck et al., 2002), modulating mucosal microanatomy (Snel, 1997), and programming the immune system (Eckburg et al., 2005; Klaasen et al., 1992). Therefore, understanding the timing and composition of the initial colonization is very relevant to the final gut microbial composition of adult fowl (Lev and Briggs, 1956; Lu et al., 2003; Tannock, 2005; Vispo and Karasov, 1997).

Contrary to the common belief that the avian embryo is sterile at hatch (Dibner et al., 2008; Mead, 1997; Zhu et al., 2002), here we present some visual and molecular evidence supporting the hypothesis that bacteria colonize the gut of the turkey embryo before hatch. While working on a developmental profile of the small intestinal villi of the turkey embryo (Chapter II) (Bohórquez et al., 2009), we observed bacteria- and protozoa-like shapes associated with the epithelium at 27E. These data prompted us to survey the ceca of turkey embryos for the presence of microbial organisms starting at 15E. SEM micrographs revealed a few micro-colonies of bacteria in between ridges of the ceca (Figure 2a-b) by 17E. Perhaps microorganisms can be found earlier in incubation but the difficulty of obtaining representative samples from the scanty embryonic gut mucosa limits their identification. The complexity of these micro-colonies in terms of numbers and shapes of microorganisms appeared to increase with age, which correlates with gut microbial maturation post-hatch (Amit-Romach et al., 2004; Mead, 1997; Wielen et al., 2002; Zhu et al., 2002). SEM
micrographs also showed that some filamentous bacteria are embedded into the epithelium of the 27E turkey embryo (Figure 3f). This type of microorganism-epithelium interaction was also observed in the distal ileum at 27E (Figure 1a-b), although the occurrence of this association appeared to be less frequent than in the ceca. Direct interaction of bacteria with the epithelium is known to be an important modulator of intestinal maturation (Hooper, 2004). For instance, segmented filamentous bacteria (SFB), indigenous unculturable bacteria of the gut (Klaasen et al., 1993), commonly latch directly onto gut epithelial cells and increases the mitotic activity and the ratio of the number of columnar cells to those of goblet cells in the small intestinal epithelial cells (Umesaki et al., 1995). In this way, SFB influence the ultra-structure of the intestinal epithelium (Snel, 1997), and the maturation of the immune system (Angert, 2005; Braun-Fahrlander et al., 2002).

After the presence of microorganisms in the ceca of the turkey embryo was confirmed using SEM, we then attempted to identify some of the bacterial species present before hatch using 16S rDNA molecular profiling. For this purpose we collected the contents of the distal gut of turkey embryos at 25E. Our rationale behind using 25E was based on the fact that internal pipping has not begun at this time (Christensen et al., 2000), thus the embryo remains isolated from direct contact to external environment. Yet, we knew from Chapter II that the mucosa of the embryo increases dramatically in size by 25E (Bohórquez et al., 2009), which facilitates the collection of gut luminal contents. Electrophoretic analysis of 16S rRNA PCR products from gut luminal contents revealed the expression of a band corresponding to the molecular weight of that in the positive control (Lactobacillus casei). To our favor, brain tissue used as a negative control failed to show the expression of a band, demonstrating the lack of cross contamination during sampling. TRFLP profiling of DNA products digested with restriction endonucleases, yielded a list of potential bacterial species present in the gut contents of turkey embryos at 25E (Table 1). These data showed a predominance of the genera Lactobacilli, Bacilli and Pseudomonas from the firmicutes and proteobacteria phyla. Hierarchical association of the phylogenetic tree analysis grouped these bacteria in 3 main clusters (Figure 5). Some of these genera of bacteria have been previously identified in the 3- and 4-day old broiler chicken (Amit-Romach et al., 2004; Lu et al., 2003).
addition, a large proportion of the potential bacteria matches generated by TRFLP belong to previously uncultured bacteria. These results are in agreement with conservative estimates from human data, which suggest that about 80% of the gut microorganisms cannot be cultured in vitro (Eckburg et al., 2005; Gill et al., 2006; Hayashi et al., 2002; Lan et al., 2002).

Pedroso (2009) has also reported the presence of *Salmonella* in the ceca contents of 18E chicken embryos using fluorescence in situ hybridization (FISH) and PCR assays. This report further supports the hypothesis that the avian gut is not sterile prior to hatch. In fact, based on research in insects, arthropods and other oviparous invertebrates (Davidson and Stahl, 2006; Davidson and Stahl, 2008; Douglas, 1989; Ereskovsky et al., 2005; Gil-Turnes et al., 1989; O'Neill et al., 1997), perhaps this is a conserved mechanism in birds to ensure the inheritance of commensal microorganisms to their progeny. In light of the visual and molecular evidence presented herein, we conclude that microbial organisms are present in the distal gut of turkey embryos as early as 17 days of incubation and that some of these bacteria belong to *Lactobacilli*, *Bacilli* and *Pseudomonas* genera as well as other uncultured bacteria.

ACKNOWLEDGMENTS

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### 3.6 TABLES AND FIGURES

**Table 1.** Terminal restriction fragment length polymorphisms (TRLFP) profile of bacteria present in the luminal contents of turkey embryos at 25 days of incubation.

<table>
<thead>
<tr>
<th>Species match</th>
<th>Fragment(^{12})</th>
<th>Fragment Length, (bp)(^{*})</th>
<th>Peak Area(^{*})</th>
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<tr>
<td></td>
<td>(HhaI) (GCG(^{+}C))</td>
<td>(MspI) (C(^{+}CGG))</td>
<td>(RsaI) (GT(^{+}AC))</td>
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<tr>
<td><strong>Bacillus halodurans.</strong></td>
<td>(G,25)</td>
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<td><strong>Comamonas sp.(^{1})</strong></td>
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<td>(G,19)</td>
<td>(G,6)</td>
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<tr>
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<td>(G,17)</td>
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<td><strong>Delftia spp.(^{2})</strong></td>
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<td>(G,30)</td>
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Table 1. Continuation

| Sp = There are more than one strain of bacteria contained in this genus or the specific strain cannot be distinguished. |
| Spp = There are more than one strain of bacteria contained in this genus. |
**Figure 1.** Gut microbiota in the small intestine of turkey embryos at 27 days of incubation. (a) Microorganisms on the ileal epithelium of turkey embryos at 27 days of incubation. Inset is a magnification of area enclosed in black dashed lines that shows some bacteria-like shapes (white arrows) associated with lipid-like droplets (black arrow). Area enclosed in white dashed lines is magnified in Figure 1b. (b) Protozoa-like microorganisms inserted into ileal villi of turkey embryos at 27 days of incubation. Close observation revealed serrated nature (white arrow) of the lower edge of the microorganisms and its association with some bacteria-like shape (black arrow).
Figure 2. Gut microbiota in the ceca of turkey embryos from 17 days of incubation to 21 days of incubation. (a) Microorganisms located in between ceca ridges of turkey embryos at 17 days of incubation. Area enclosed in dashed lines is magnified in Figure 2b. (b) Micro-colony of microorganisms (white arrows) in the ceca of turkey embryos at 17 days of incubation. (c) Microorganisms in the ceca of turkey embryos at 19 days of incubation. Area enclosed in dashed lines is magnified in Figure 6d. (d) Magnified cluster of microorganisms in the ceca of turkey embryos at 19 days of incubation. White arrows point to at least three different shapes of microorganisms (rods, filaments and spirals). (e) Microorganisms in the ceca of turkey embryos at 21 days of incubation. Area enclosed in dashed lines is magnified in Figure 2f. (f) Magnified cluster of microorganisms in the ceca of turkey embryos at 21 days of incubation.
Figure 3. Gut microbiota in the ceca of turkey embryos from 23 days of incubation to 27 days of incubation. (a) Microorganisms localized on the ceca epithelium (arrows) of turkey embryos at 23 days of incubation. (b) Magnified colony of microorganisms in the ceca of turkey embryos at 23 days of incubation. There are some protozoa-like structures (arrow) in some of the clusters. (c) Microorganisms localized on the ceca epithelium (arrows) of turkey embryos at 25 days of incubation. (d) Magnified colony of microorganisms in the ceca of turkey embryos at 25 days of incubation. Note the complexity in terms of numbers and shapes of this micro-colony compared to that in Figure 3. (e) Microorganisms localized on the ceca epithelium (arrows) of turkey embryos at 27 days of incubation. (f) Filamentous bacteria (arrow) inserted into the ceca epithelium of turkey embryos at 27 days of incubation.
Figure 4. Gel electrophoresis of 16S rDNA PCR assay of bacterial DNA from intestinal contents of turkey embryos at 25 days of incubation. One hundred ng of DNA from intestinal content and brain samples were amplified with the universal primers Eub8f-HEX and Eub1113r. The forward primer was labeled with hexachlorofluorescein (Hex), for TRFLP analysis. Lane 1-3: 100ng of DNA from the intestinal contents of 24 embryos (8 embryos per replicate); lane 4: 100ng of brain sample as a (-) control; lane 5: 5ng of Lactobacillus casei DNA, used as positive control; lane 6: molecular grade water. The brightness of the picture was increased in order to see the expected bands only in the ceca samples, plus in the positive control. The molecular marker used was a DNA ladder of 1 Kb (Invitrogen Corp., Carlsbad, CA).
Figure 5. Hierarchical dendrogram of terminal restriction fragment length polymorphism (TRFLP) analysis of bacteria from intestinal contents of turkey embryos at 25 days of incubation. Colors (red, green, and blue) denote bacteria from different clusters.
* Bacillus halodurans
* iron-reducing bacterium enrichment culture clone HN4
* uncultured bacterium
* uncultured bacterium
* uncultured bacterium
* uncultured Burkholderiales bacterium
* uncultured bacterium
* uncultured bacterium
* Lactobacillus spp. 1
* Lactobacillus spp. 2
* uncultured bacterium
* uncultured bacterium
+ Comamonas sp.
+ Pseudomonas sp. enrichment culture clone Guo9
+ uncultured bacterium
+ uncultured bacterium
+ Delftia spp.
+ uncultured bacterium
+ Nostoc spp.
+ uncultured bacterium
+ uncultured bacterium
+ uncultured bacterium
+ uncultured bacterium
× Comamonas testosteroni
× uncultured bacterium
× uncultured bacterium

Option: Hierarchical
Method: Ward
3.7 REFERENCES


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CHAPTER IV

THE EFFECTS OF IN-OVO FEEDING ON THE MORPHOLOGICAL MATURATION OF THE SMALL INTESTINAL MUCOSA IN THE PERINATAL TURKEY EMBRYO AND POULT
4.1 ABSTRACT

Nutrient supplementation of the amnion of late-term embryos or in-ovo feeding (IOF) has previously been shown to enhance digestive capacity, nutrient uptake and post-hatch growth of turkey poults (de Oliveira, 2007; Foye et al., 2003). Because morphological maturation of the intestinal epithelium is required for the onset of digestive capacity, we hypothesized that in-ovo feeding accelerates the ultra-structural maturation of the small intestinal epithelium of the perinatal turkey. We also explored the correlation of these effects with circulating triiodothyronine (T₃) levels. The hypothesis was tested in three different experiments. Experiment 1. Eggs were in-ovo fed (IOF) at 23 days of incubation (E) with 0.4mL of an IOF solution containing metabolic co-factors and compared with saline-injected controls. Upon hatch, male poults from both treatments were reared up to 11d. Jejunum histomorphometry, ultra-structure (electron microscopy), and growth performance were evaluated at hatch, 4d and 11d. Jejunum gene expression was surveyed at 4d using a focused microarray with 70-mer oligonucleotides selected from the chicken genome. Experiment 2. Eggs were IOF at 23E, with 0.8mL of an IOF solution containing metabolic co-factors plus carbohydrates and compared with saline-injected controls. Jejunum histomorphometry was analyzed at 25E, 27E and hatch. Plasma triiodothyronine (T₃) levels were evaluated from 25E to 12d using I²⁵ radioimmunoassay. Experiment 3. An indigestible carbohydrate (lactose at 9%) was IOF (1.5mL) at 22E and compared with non-injected controls. Jejunum histomorphometry was analyzed at hatch, and body weights were determined at hatch and 3d. IOF enhanced villi height and surface area in all treatments up to the time of hatch as compared to controls. Gene expression profiles showed down-regulation of smooth muscle growth and up-regulation of intestinal disaccharidases, epithelial cell growth, thyroid receptors, and innate immune response pathways in IOF poults. IOF poults had higher T₃ levels at 27E, hatch and 12d than the saline-injected controls. By 11d, IOF birds had 5% heavier (P<0.05) body weights and 6% (P<0.05) lower cumulative feed conversion ratios. In-ovo feeding of turkey embryos clearly accelerates the morphological maturation of the jejunal mucosa up to hatch. These effects involve localized up-regulation of genes mediating epithelial cell growth and
systemic increase of plasma T₃. This study provides evidence that amniotic fluid supplementation of turkey embryos can have a lasting impact on intestinal morphology and function.

4.2 INTRODUCTION

Modern fast-growing strains of commercial poultry are highly susceptible to enteric diseases (Yu et al., 2000), skeletal disorders (Cook, 2000; Oviedo-Rondon et al., 2006), and stunted growth (Angel et al., 1990) during the growing period. These aberrations are frequently due to immature development of the intestinal mucosa at hatch (Croom et al., 1999; Sell, 1996; Sell et al., 1991) and deficient thyroid function (Funderburk, 2006). Thyroid hormones are major stimulators and permissive regulators of intestinal growth (Black, 1978; Moog, 1979). Because the sole presence of nutrients in the gut lumen potently stimulates cell proliferation and intestinal growth (Shah and Sanderson, 2000), delayed feed access at hatch exacerbates growth aberrations (Corless and Sell, 1999; Noy et al., 2001; Uni et al., 1999b). It has been shown extensively that immediate access to dietary nutrients post-hatch enhances the morphological and physiological development of the gastrointestinal tract (GIT) in young hatchlings (Noy and Sklan, 1997; Potturi et al., 2005; Sklan, 2001; Turner et al., 1999; Uni et al., 1999).

The embryo naturally consumes the amniotic fluid during incubation because it constitutes the first meal to enter the embryonic digestive tract (Cleveland et al., 1991; Underwood et al., 2005; Wagner, 2002). Based on mammalian research data, that this complex embryonic liquid is composed of several nutrients (Underwood and Sherman, 2006), hormones (Schindler, 1982), growth factors (Karcher et al., 2005), immunoglobulins (Cleveland et al., 1991), antioxidants (Burlingame et al., 2003), and other molecules (Hanisch and Katalinic, 1992; Russell et al., 1978) that prepare the digestive tract for post-natal nutrition (Wagner, 2002). Thus, amniotic fluid supplementation or in-ovo feeding (IOF) (Uni and Ferket, 2001) accelerates the maturation of the digestive tract in turkeys and chicken embryos (Uni and Ferket, 2004). Two of the major advantages of IOF over conventional early feeding strategies are: 1) the maturation of the intestinal epithelium is influenced when the embryo naturally imbibes amniotic fluid and the intestinal epithelium plasticity is at its peak; and 2) the
flexibility of the stimulus depends upon nutrient content of the injected formula. For instance, Tako et al. (2004) demonstrated that IOF of carbohydrates or \( \beta \)-hydroxy-\( \beta \)-methyl-butyrate to chicken embryos 3 days prior to hatch results in over 30% greater villus surface area and about 50% greater activity of digestive enzymes by the time of hatch than controls. Similarly Foye et al. (2007) reported that turkey embryos IOF egg white protein, arginine and \( \beta \)-hydroxy-\( \beta \)-methyl-butyrate exhibited greater intestinal absorption of carbohydrates and amino acids at hatch than controls. Moreover, de Oliveira (2007) showed that genes mediating nutrient utilization are up-regulated around the time of hatch among IOF turkey embryos improving poult quality scores and post-hatch skeletal development. These reports demonstrate that the digestive capacity of IOF hatchlings is functionally similar to that of a conventionally-fed chick at 2 days of age (Tako et al., 2004; Uni and Ferket, 2004).

Morphological maturation of the digestive tract precedes the onset of digestive capacity and nutrient uptake, and this depends upon circulating \( T_3 \). Therefore, we hypothesized that \textit{in-ovo} feeding enhances the ultra-structural development of the intestinal mucosa in turkey poults, which correlates with increased circulating plasma triiodothyronine (\( T_3 \)) levels. This hypothesis was tested by the following objectives: (1) To determine the IOF effects on the morphological and ultra-structural maturation of the jejunum of the perinatal turkey poult; (2) to determine the effects of IOF on jejunum gene expression in the turkey poult after hatch; (3) to determine the effects of IOF on plasma \( T_3 \) levels in the perinatal turkey poult; and (4) to determine the effects of IOF on the growth performance and feed efficiency of turkey poults after hatch. These objectives were accomplished by three separate experiments of slightly different design. In experiment 1, we evaluated the effects of an IOF solution containing nutrients and metabolic co-factors (Table 2) on the ultra-structural (light and electron microscopy) development of the intestinal mucosa. Jejunum gene expression was also surveyed at 4d after hatch, using a focused 70-mer oligonucleotide microarray designed from the chicken genome (Druyan et al., 2008). The use of this microarray to analyze gene expression changes in response to treatment effects in the turkey has been validated and previously described by de Oliveira et al. (2009). Growth performance and feed efficiency were evaluated from hatch until 11d. In experiment 2, we evaluated the effects of an IOF solution containing nutrients and metabolic
co-factors plus carbohydrates (Table 2) on plasma T₃ levels from 25E to 12d using radioimmunoassays. The treatment effects on the histomorphometrical development of the intestinal mucosa prior to hatch were also evaluated. In experiment 3, we tested the effects of IOF lactose (9mg lactose hydrate/mL) as a non-digestible source of carbohydrate on the jejunum histomorphometry of hatchlings.

4.3 MATERIALS AND METHODS

Bird husbandry and tissue collection

Experiment 1

Two hundred and forty Nicholas day-old male turkey poults were randomly assigned among 24 battery cages¹, with 10 birds per cage. Half of those birds were injected (in-ovo fed) at 23 days of incubation (E) with 0.4mL of a solution containing nutrients and metabolic cofactors (Table 2-experiment 1) and the other half with 0.4mL of a saline-solution (control). The saline control was prepared as 4g of NaCl per litter of solution. The solutions were delivered into the amnion using an Inovoject² system (injection depth = 318mm). Birds were given ad libitum access to feed and water. The composition of the diet is presented in Table 1. Individual bird body weights (BW), bird feed intake (FI), cumulative feed:gain ratio (FCR), and mortality rates were determined at hatch, 4d and 11d. Growth performance data were analyzed by a t-test and means that differed significantly were separated by the least-square means procedure of JMP³ software at P≤0.05.

Histology samples. Twelve turkey poults per treatment were euthanized by cervical dislocation and sampled at hatch, 4d and 11d. A 2cm tissue segment was removed from the proximal jejunum and immediately fixed in 10% neutral buffered formalin for at least 72 hours prior to slide preparation.

Electron microscopy samples. A 1cm segment was excised from the proximal jejunum of 6 birds per treatment and four tissue blocks (~3x3mm each) per segment were dissected immediately on dental wax while submerged in a drop of 0.1M sodium phosphate buffer (SPB), pH 7.2, to avoid drying of the mucosa. Tissue blocks from same treatment were pooled into one vial containing ~5mL

¹ Alternative design manufacturing & supply Inc., Siloam Springs, AR
² Pfizer animal health, Inc. RTP, NC. USA.
³ SAS Inc. Cary, NC
of cold (~4°C) 3% glutaraldehyde and fixed for at least 72 hours before processing for scanning or transmission electron microscopy analysis.

**Gene microarrays samples.** A 2cm segment of the mid jejunum was excised from six poult's per treatment, flushed with saline buffer and placed individually in a vial containing 5mL of RNALater™. Samples were then stored at -80°C before RNA extraction.

**Experiment 2**

Fertile Nicholas turkey eggs (600) were obtained from a commercial hatchery and incubated at 37.5°C and 52% relative humidity. At 20E, all eggs were candled and assigned to two groups of equal weight distribution. At 23E, 250 eggs per group were injected with either 0.8mL of a solution containing nutrients and metabolic cofactors plus carbohydrates (in-ovo fed) (Table 2-experiment 2) or 0.8mL of a saline-solution (control). The saline control was a prepared as 4g of NaCl per litter of solution. The IOF volume injected per egg was increased relative to what was used in experiment 1 to accommodate the inclusion of carbohydrates without altering osmolality. This time, the solutions were manually injected into the amnion using a 23g needle5 (injection depth = 318mm). In addition, we determined the accuracy of amnion targeting by manual injection of 40 eggs with a solution containing 0.5% brilliant blue6. The accuracy of the technique was above 90%. Manual IOF injection procedures were performed according to the protocol described by Foye (2005) and de Oliveira (2007). Birds were grown up to 12d for T3 analysis and given ad libitum access to feed (similar to that presented in Table 1) and water from hatch to 12d.

**Histology samples.** Six turkey embryos per treatment were euthanized by cervical dislocation and sampled at 25E, 27E and hatch. A 1cm tissue segment was removed from the proximal jejunum and immediately fixed in 10% neutral buffered formalin for at least 72 hours prior to slides preparation.

**Plasma samples for T3 radioimmunoassay.** Blood samples from nine embryos per treatment were collected by decapitation and pooled into 3 BD Vacutainer™ blood collection tubes (EDTA coated) at 23E, 25E and 27E. At hatch, 4d, 8d and 12d, blood was drawn by heart puncture from 3 poult's per

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4 Applied biosystems / Ambion. Austin, TX
5 Becton, Dickinson and Company. Franklin Lakes, NJ
6 Fisher Scientific. Pittsburgh, PA
treatment and collected in individual Vacutainer® tubes. In this way, three 1mL replicates of blood samples per treatment were obtained at each time point. Samples were immediately centrifuged at 700xg for 15 minutes at 4°C. Blood plasma from each sample was then transferred into a new sterile vial and stored in -20°C before T₃ analysis.

**Experiment 3**

Fertile Nicholas turkey eggs were obtained from a commercial hatchery and incubated under commercial incubation conditions. At 22E, one hundred fertile eggs were injected with 1.5mL of a solution containing 9 mg lactose hydrate/mL of IOF solution (*in-ovo* fed), a non-digestible source of carbohydrates (Table 2-experiment 3). The solution was delivered into the amnion using an Inoject® system (injection depth = 318mm). One hundred non-injected fertile eggs served as controls. At hatch, birds from both groups were individually tagged and reared on a floor pen with ad libitum access to feed and water from 1 to 3d of age. Individual bird body weights from each treatment were recorded at hatch and 3d. Body weight data were analyzed by a t-test and means that differed significantly were separated by the least-square means procedure of JMP³ software at P≤0.05.

**Histology samples.** Ten turkey poults per treatment were euthanized by cervical dislocation and sampled at hatch. A 1cm tissue segment was removed from the distal ileum and immediately fixed in 10% neutral buffered formalin for at least 72 hours prior to slides preparation.

**Histomorphometrical analysis**

**Histology slides preparation.** Histological samples from all three experiments were processed according to the following protocol. Three sections (~3 mm) were obtained from each fixed segment, placed into tissue cassettes and submerged into 10% neutral buffered formalin. Cassettes were then sent to the North Carolina State University College of Veterinary Medicine histology lab for preparation of slides and hematoxylin and eosin staining.

**Image acquisition and histomorphometrical analysis.** Light microscopy photographs were taken of the transverse sections and analyzed using UTHSCSA Image Tool⁷ software. The following

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⁷ Department of Dental Diagnostic Science at The University of Texas Health Science Center, San Antonio, Texas
measurements were performed on all experiments on a blind basis on at least 10 villi per sampled bird: villus height, villus apical width at the villus tip, villus basal width at the crypt-villus junction, crypt depth, and muscularis depth. Apparent villus surface area was estimated by the mathematical formula: 
\[ \frac{((\text{villus tip}+\text{villus base})/2) \times \text{villus height}}{1000} \] . In experiment 1, goblet cell number per 100 µm² of villus surface was determined at hatch and 4d. Villus height: crypt depth ratio was also calculated as an estimation of epithelial cell renewal.

**Data analysis.** Ten villi measurements were averaged within each sampled bird and this number served as the experimental unit. Data were analyzed by a t-test and the means that differed significantly were separated by the least-squares means procedure at \( P \leq 0.05 \), using JMP3 software.

**Electron microscopic analysis**

Tissues were prepared for examination at the center for electron microscopy in the NCSU Microbiology Department according to the protocol described below. Tissue blocks fixed on 3% glutaraldehyde were washed 3 times in SPB, each time for 20 minutes at 4°C. Then, tissue blocks were post-fixed with 1% osmium tetroxide (OsO₄) for 1 hour at 4°C. Tissue blocks were then rinsed in SPB and dehydrated through a graded series of alcohol (30%, 50%, 70%, 95% and 100%, each time for 30 minutes). At this point, some blocks were separated for transmission electron microscopy (TEM) analysis. Scanning electron microscopy (SEM) samples were critical point dried in a Samdri PVT-2 using liquid carbon dioxide. Specimens were mounted on aluminum stubs with silver paint and coated with a total of 50-60kÅ of gold/palladium in a Hummer 6.2 Sputtering Device. SEM was performed in a JEOL 5900LV at 20 kV accelerating voltage.

Tissue blocks separated for TEM analysis were infiltrated two times (1:1 with 100% ethanol and 3:1, 100% ethanol) with Spurr embedding media for 6 hours each time. Then, three changes in 100% Spurr (no ethanol) for 6 hours each were performed. Finally, tissue blocks were embedded overnight at 70°C in fresh 100% Spurr using flat embedding molds (for correct orientation). Blocks were trimmed with razor blades and sectioned at 70-80nm on a diamond knife using an LKB NOVA Ultramicrotome. Sections were stained on-grid with 4% uranyl acetate for 1 hour at room temperature in the dark, followed by 3 water rinses, and 4 minutes in Reynolds’ lead citrate with 3 water rinses.
Grids were analyzed using a JEOL JEM100S Transmission Electron Microscope at 80kV accelerating voltage.

**Gene microarray analysis**

**RNA extraction and quality control.** Total RNA was extracted from 100mg of jejunum tissue per sample (6 samples per treatment) using the TRIzol® extraction method and according to the protocol previously described by de Oliveira (2007). Extracted RNA pellets were resuspended in 100µl of nuclease-free water and its concentration was determined using a NanoDrop spectrophotometer. Then, 20µg of total RNA from each sample were treated with TURBO™ DNase according to the manufacturer’s protocol. The integrity of RNA was verified by electrophoresis on 1.2% agarose gel and samples were stored at -80°C before synthesis of cDNA.

**Sample labeling and hybridization.** Sample preparation was performed according to the protocol previously reported by de Oliveira (2007). The array used was a focused 70-mer oligonucleotide microarray that was built in the NCSU-Domestic Animal Genomics Lab and has been described in detail by Druyan et al. (2008). The experimental design of the array was planned as an all-paired design (Figure 10-inset). Four samples of good quality ($A_{260}/A_{280} \geq 1.85$) RNA per treatment were used to produce aminoallyl-cDNA using the ChipShot™ indirect labeling and clean-up system. The aminoallyl-cDNA was either labeled with Cy3 (green) or Cy5 (red) fluorescent dyes according to the experimental design (Figure 10-inset). Pronto Plus! Microarray Hybridization Kit was used for pre-hybridization and hybridization of array slides. Pairs of labeled cDNA, ~50pmol per treatment sample, were mixed, dried, resuspended in hybridization solution and applied to pre-hybridized slide covered with a pre-cleaned glass coverslip. The slides were hybridized overnight in a water bath at 42°C in complete darkness. Hybridized slides were then scanned using a ScanArray GX PLUS Microarray Scanner.

**Data Analysis.** A detailed description of the data analysis for this particular 70-mer oligonucleotide

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8 Invitrogen corporation, Carlsbad, CA
9 Thermo Scientific – NanoDrop products. Wilmington, DE
10 Promega USA. Madison, WI
11 Amersham Biosciences Corp., Piscataway, NJ
12 Corning, Inc., Acton, MA
13 PerkinElmer Life and Analytical Sciences, Shelton, CT
microarray was previously reported by Druyan et al. (2008). Intensity raw data files were obtained from the scanned images using ScanAlyze Software (Eisen et al., 1998), transformed to a log base 2, and analyzed in JMP Genomics3. Transformed data for all spot measures were subjected to loess normalization. The residuals from this model were analyzed by mixed ANOVA, as described by Wolfinger et al. (2001). Mean intensities were compared using False Discovery Rate (FDR) at P<0.01. Results were used to produce clustering plots and parallel plots. In addition, potential metabolic pathways affected by the treatments were obtained using MetaCore™ software14.

**Plasma triiodothyronine radioimmunoassay**

Radioimmunoassay. Plasma samples were assayed for thriiodothyronine (T₃) activity using the technique described by Christensen and Davis (2001). All samples were analyzed using the same assay to avoid inter-assay variation. The kit used for the radioimmunoassay analysis was Total T3 Coat-A-Count®15. A stock solution of T₃ (350ng/mL) was serially diluted from 16 to 0.5 ng/mL for preparation of standards to test recovery. Each serially diluted concentration of T₃ was added to stripped turkey plasma for a total 1mL volume. In the meantime, samples were thawed in water at room temperature and centrifuged at 5000xg for 6min. One hundred µL of plasma from each sample was transferred twice to assay tubes provided by the kit. In this way, each sample was processed in duplicate. Then, 1mL of T₃ radioactive material was added to each tube. Tube samples were then individually covered with paraffin paper, gently mixed and incubated at 37°C (water bath) for 2 hours, followed by a second incubation at room temperature for 15min, before radioactivity counting.

Data analysis. Data from both treatments at each time point were analyzed by a t-test and the means that differed significantly (P≤0.05) were separated by the least-squares means procedure from JMP software3.

**Animal ethics**

Turkey embryos and poults were managed according to normal husbandry practices and the experiments were conducted according to the experimental protocol approved by the Institutional Animal Care and Use Committee (IACUC) of North Carolina State University.

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14 GeneGO. St. Joseph, MI
15 Siemens corporation. New York, NY
4.4 RESULTS

Experiment 1. Maturation of the jejunum mucosa at hatch and growth performance post-hatch is enhanced by in-ovo feeding metabolic cofactors. At hatch, villus height and apparent surface of proximal jejunum mucosa were significantly (P<0.1) higher among IOF poult (Table 3). In fact, villus surface area of IOF treated poult was ~16% higher than controls (66,280 vs. 57,297 µm²). Similarly, goblet cell population per 100 µm² was greater (P<0.001) in the jejunum of IOF poult than controls (11.5 vs. 6.5 cells/100µm²), which correlated with histological sections and transmission electron micrographs (TEM) (Figures 1a-b and 2c-d). In addition, scanning electron micrographs (SEM) revealed that jejunum villi were larger in IOF poult than controls poult (Figures 1c-d). At higher SEM magnifications, the microvilli on the apical end of individual villus of control samples were clumped, exposing the hexagonal-like shape of enterocytes (Figure 2a). In contrast, the apical end of villi among the IOF samples appeared more uniform and surrounded by active mucus secretion from goblet cells (Figure 2b). There were no significant differences observed among the other parameters measured at 4d and 11d. Although the IOF treatment clearly appeared to influence the morphological maturation of the jejunum mucosa at hatch, these histomorphometrical and ultra-structural differences were no longer observed at 4d or 11d. However, microarray analysis at 4d revealed that IOF up-regulates genes mediating carbohydrate and aminoacid metabolism (maltase-glucoamylase, glycogen synthase kinase, aminopeptidase 1), cell growth (EGFr, ILGF-BP1, thyroid receptors α1 & β1) and innate immune response (IL-8, TLR2-2, Fas-ligand), and down-regulates genes mediating smooth muscle development (F-actin capping protein, myosin light-chain 4 & 8) (Figure 3a-b). The fold change and P-values of gene expression significantly affected by treatments are presented in Table 6. A potential MetaCore™ pathway that summarizes the treatment effects on the metabolism of carbohydrates and proteins in the intestinal epithelium is presented in Figure 4. The average bird body weight (BW) was not affected by treatments at hatch or 4d; however, IOF treated poult were ~5% heavier (P<0.05) than controls at 11 days. The cumulative feed conversion ratio (cFCR) was 9% and 6% lower (P<0.1) among IOF treated poult points at 4d and 11d respectively, than among the
control poults (Figure 6). There were no differences in feed intake or mortality at any time point. We conclude from this experiment that *in-ovo* feeding metabolic cofactors stimulates the morphological maturation of the small intestinal mucosa around the time of hatch. Although the morphological differences appeared to dissipate with age, the effects of IOF persisted until 11d in terms of the up-regulation of metabolic pathways that mediate nutrient utilization and cell growth in the intestinal epithelium. The consequences of these effects are observed by improved growth performance and feed efficiency at 11d.

**Experiment 2.** *In-ovo* feeding metabolic cofactors and carbohydrates enhances the morphological maturation of the jejunum mucosa and increases plasma $T_3$ levels around the hatching period. Villus height and villus surface area among the IOF treatment group were higher ($P<0.05$) at 25E, 27E, and hatch, than the control group. However, the marginal difference between the treatment groups decreased over time: the differences in villus height were 25%, 18% and 11% at 25E, 27E, and hatch, respectively. Villus crypt depth was greater ($P=0.102$) in IOF than controls poults only at 27E. There was no significant treatment effects on other histomorphometrical variables (e.g. villus tip, muscularis, etc.) evaluated at any time point. There were no significant treatment effects on plasma $T_3$ levels at 25E, but plasma $T_3$ levels were significantly ($P<0.01$) higher in the IOF group than control group at 27E and hatch. At this time, plasma $T_3$ levels spiked in both treatment groups, which coincides with active mobilization of metabolic resources required during the hatching process. Although treatment differences in plasma $T_3$ levels were not observed at 4d and 8d plasma $T_3$ levels of the IOF treated poults were higher ($P<0.1$) than controls at 12d. We conclude from this experiment that *in-ovo* feeding metabolic cofactors and carbohydrates stimulates morphological maturation of the small intestinal mucosa up to the day of hatch, and that these localized effects correlate with systemic up-regulation of plasma $T_3$ levels around the time of hatch.

**Experiment 3.** *In-ovo* feeding lactose effects on the morphological maturation of the ileum in the hatchling turkey poult. Body weights of turkey poults at hatch were not affected by IOF treatment. Yet, at 3 days of age, IOF poults were significantly heavier (65.7 vs 64.1g, $P<0.05$) than controls. Villus crypt depth, villus tip width, villus base width, and muscularis width did not differ significantly between
treatments at hatch. However, villus height, villus surface area, and villus height:crypt depth ratio were significantly (P<0.01) higher among IOF poults than controls. Similar to what was observed in the IOF treatment groups in experiments 1 and 2, villus height and villus surface area were about 18% and 22% higher, respectively, after IOF of lactose than controls. We concluded from this experiment that lactose, an indigestible carbohydrate in birds, could influence the maturation of the distal intestinal epithelium at hatch. Speculation of the potential mechanism mediating this response is discussed below.

4.5 DISCUSSION

The stress of the hatching process leaves the turkey poult in a nutrient-deficient state. This situation is aggravated when access to feed is delayed immediately post-hatch (Corless and Sell, 1999), a common occurrence in commercial field conditions. Delayed feed intake post-hatch is often associated with weakened thyroid function (Christensen and Davis, 2001; Funderburk, 2006), altered morphology of the intestinal mucosa (Bayer et al., 1981), reduced digestive capacity and undermined nutrient absorption (Noy and Sklan, 1997; Uni et al., 1999a), resulting in poor growth and livability. Amniotic fluid supplementation of late-term poultry embryos with metabolic cofactors and/or carbohydrates has been shown to augment the villi size and surface area of the small intestine (at least in chickens) and enhance the activity of digestive enzymes (Tako et al., 2004) and nutrient transporters (Foye et al., 2007), often improving their growth post-hatch (Foye et al., 2005). However, the effects of IOF on the ultra-structural maturation of the intestinal mucosa and their potential correlation with plasma T3 levels had not been explored. Thyroid hormones are of particular importance because these are potent stimulators of intestinal maturation (morphological as well as functional) (Black, 1978) and major regulators of metabolic function (Christensen and Davis, 2001).

Here we present evidence from three separate experiments that support our hypothesis that in-ovo feeding accelerates the morphological maturation of the small intestinal mucosa in hatchling poults (Table 3-5). These results are in close agreement with those reported by Tako et al. (2004). These authors observed that 17.5E chicken embryos in-ovo fed a saline solution containing β-
hydroxy-β-methyl-butyrate (HMB), carbohydrates or HMB plus carbohydrates exhibited increased jejunum villus surface area at 19E, 20E, hatch and 3d post-hatch. It their experiments, the margin of difference (%) in villus height and surface area between the IOF and control treatments was reported to decrease with time, which correlates with our observations. It is noteworthy that the control used in our experiments was 0.4mL of a saline (NaCl) solution, which mimics the stress of puncturing the shell and the amniotic membrane. Although NaCl can potentially affect the metabolic function of molecular transporters in the gut epithelium, it has been reported that injection of up to 1mL of 5 g of NaCl/l does not affect (P<0.05) embryo and chick BW, intestinal development, or brush border membrane enzymatic activity (Tako et al., 2004). In our second experiment, we observed that the difference in villus height between control and IOF of metabolic cofactors plus carbohydrates gradually decreases from 25% at 25E to 11% at hatch (Table 4). We also observed that IOF lactose, a non-digestible carbohydrate in poultry because they lack the digestive enzyme lactase, enhances the villi height and villus surface area at hatch and body weight at 3d (Table 5). Given that bacteria appear to colonize the distal portion of the digestive tract in turkey embryos well before hatch (Chapter III), perhaps lactose can provide a substrate for their colonization and, by that, indirectly influence epithelium maturation. However, the mechanism of this response needs further research.

Besides the persistent histomorphometrical advantages of IOF on the maturation of the small intestinal epithelium, there are certain villi ultra-structural modifications observed among the IOF treated poults that indicate an enhanced physical gut epithelial barrier. Scanning (SEM) and transmission (TEM) electron microscopic analysis revealed that individual villi at hatch were more round in shape, and the microvilli appeared uniformly distributed in the IOF poults as compared to the controls (Figures 1c-d and 2a-b). These observations correlate with those of Uni et al., (1998), who reported that immediate access to feed after hatch prevents clumping of microvilli in the small intestinal mucosa in chicks. Clustering of microvilli physically exposes epithelial cell junctions to direct attachment of pathogenic microorganisms that can compromise gut barrier function (Liévin-Le Moal and Servin, 2006; Sell, 1996). In addition, light and TEM micrographs showed higher activity of mucus secretion in IOF turkey poults at day of hatch (Figure 2c-d). These observations were
confirmed by significant (P<0.01) increases in the goblet cell population among IOF-treated poult at hatch (Table 3). Since the intestinal mucosa is protected physically and chemically by a dynamic blanket of mucus, secreted by goblet cells (Atuma et al., 2001; Liévin-Le Moal and Servin, 2006) stimulation of mucus secretion by IOF likely offers a protective advantage to the epithelium against potential pathogenic invasion. Likewise, Uni et al. (2003) demonstrated that early feeding enhances the maturation and secretory activity of goblet cells. Furthermore, in-ovo feeding of carbohydrates to the late-term chicken embryo has been shown to enhance the expression of mucin genes at hatch in comparison with saline-injected controls (Smirnov et al., 2006). These results confirm that IOF not only accelerates the gross morphological maturation of the turkey epithelium at hatch, but also provides ultra-structural advantages, such as better villus integrity and increase mucus secretion at the time of hatch. Such characteristics may improve the physical barrier of the epithelium at a time when a large influx of foreign particles and microbes invade the intestine (Liévin-Le Moal and Servin, 2006).

Although the advantages of IOF on the morphological maturation of the small intestinal epithelium dissipated with time, the IOF treatment effects on genes mediating nutrient metabolism and epithelial cell renewal linger beyond the hatching period. Using microarray analysis, we present evidence that in-ovo feeding the turkey embryo with a solution containing metabolic cofactors up-regulates genes mediating carbohydrate and aminoacid metabolism (maltase-glucoamylase, glycogen synthase kinase, aminopeptidase 1), cell growth (EGFr, ILGF-BP1, thyroid receptors α1 & β1) and innate immune response (IL-8, TLR2-2, Fas-ligand) until 4 days after hatch (Figures 3a-b and 4). The up-regulation of carbohydrate and protein metabolism by in-ovo feeding turkey and chicken embryos have been extensively studied by Foye (2005) and Tako et al., (2004), though the IOF effects on genes mediating cell proliferation and immune response remains unexplored. Foye et al., (2007) reported that IOF of arginine and/or HMB enhanced the activity of intestinal disaccharidases (e.g. sucrase, maltase) and jejunal peptidases (leucine aminopeptidase) in turkey poult. In-ovo feeding 23E turkey embryos a solution containing arginine and HMB enhanced the activity of jejunal sucrase and maltase by 3-fold as compared to non-injected controls, and these effects remained until to 14
days post-hatch (last time-point evaluated). Moreover, Tako et al., (2004) reported that jejunal maltase was about 50% higher in IOF chicks 3 days after hatch than controls. These positive effects on digestive capacity of carbohydrates and proteins explain the persistent up-regulation of liver glycogen storage in IOF-treated poults or chicks (de Oliveira, 2007; Foye et al., 2003; Uni et al., 2005). For instance, providing carbohydrates and HMB to 17.5E chicken embryos increases liver glycogen up to 5-fold (Uni et al., 2005).

Because the ability to use glycogen reserves to drive morphological maturation of the intestinal epithelium has been positively correlated to circulating thyroid hormone levels (Black, 1978; Christensen et al., 1996), we wondered if the IOF effects on morphological maturation of the intestinal epithelium are correlated to a correspondent increase in the active form of thyroid hormones, triiodothyronine (T\(_3\)). Previous research by Christensen at North Carolina State has demonstrated that in modern turkey embryos the plasma T\(_3\) levels are easily affected by incubation and hatching stressors (Christensen and Davis, 2001; Christensen et al., 1996; Funderburk, 2006). Consequently, hatchlings are not able to mobilize energy reserves during the hatching process, even though they have enough glycogen in liver and muscle reserves (Christensen et al., 2003). Here, we show that in-ovo feeding turkey embryos a solution containing metabolic cofactors (e.g. L-tyrosine and vitamin cofactors necessary for the synthesis of thyroid hormones) and carbohydrates increases circulating levels of plasma T\(_3\) around the time when metabolic reserves must be mobilized for the hatching process (Figure 5). These effects are primarily observed at 27E and hatch, which correspond to the time when IOF enhances morphological and ultra-structural maturation of the epithelium. Elegant experiments performed by Black (1978) and Moog (1979) demonstrated the importance of thyroid hormones in the regulation of morphological development of the mucosa in birds and mammals. Black (1978) has shown that injection of an inhibitor of thyroid function (thiourea) in chicken embryos, prior to in vitro culturing of their duodenum, causes severe retardation of villi elongation and reduced epithelial cell height. These effects can be reversed in the presence of thyroxine (T\(_4\)), the major precursor of T\(_3\) (Black, 1978). Moreover, Black and Moog (1977) reported a direct stimulatory effect of T\(_4\) on goblet cell differentiation in the developing embryonic intestine. The up-regulation of T\(_3\) by IOF
treatment explains the previous effects on the morphological and ultra-structural maturation of the epithelium in hatchling poults. It may also explain the enhanced bird feeding behavior of IOF-treated poults that has been reported elsewhere (Bohórquez et al., 2008; de Oliveira, 2007).

The consequences of in-ovo feeding turkey embryos on the ultra-structural maturation of the gut mucosa and T₃ activity, result in better post-hatch growth performance and feed efficiency (Figure 6). The body weights of 11d poults in-ovo fed at 23E with metabolic cofactors were 5% higher (240.3 vs. 228.7g, P<0.05) than controls. In addition, the IOF-treated poults showed better cumulative feed conversion at 4d and 11d post-hatch when compared to saline injected controls. These results agree with Tako et al. (2004) who observed that chicken embryos in-ovo fed saline solutions containing HMB with or without carbohydrates increased the body weight by about 6% at 10d post-hatch as compared to controls. Likewise, Foye (2005) has observed that poults in-ovo fed saline solutions containing HMB and arginine had up to 11% heavier body weights at 14d than controls.

Based on the supporting evidence presented above, we conclude that turkey embryos in-ovo fed at 23 days of incubation with a solution containing metabolic cofactors with or without carbohydrates or the non-digestible carbohydrate lactose¹⁶, enhances the ultra-structural maturation of the small intestinal epithelium as well as plasma T₃ activity. The consequences of these in-ovo feeding treatments are heavier body weights and improved feed efficiency during the brooding period.

ACKNOWLEDGMENTS

This work was supported by the U.S. Poultry & Egg Association (Tucker, GA). The author wishes to thank the following people for their technical assistance: Valerie Knowlton (NCSU Microbiology Dept. center for electron microscopy), Sandra Horton (NCSU College of Veterinary histology lab), Dr. Eva Johannes (NCSU Botany Dept. Cellular and Molecular Imaging Facility), Dr. Jean de Oliveira and Dr. Shelly Druyan (Microarray assays and data analysis), Mrs. Vickie Hedgpeth (Thyroid radioimmunoassay).

¹⁶ Although we speculate that the mechanism mediating this response is indirectly through the enhancement of commensal bacterial colonization in the embryonic gut (Chapter III), the details of these responses are not entirely understood.
### 4.6 TABLES AND FIGURES

**Table 1.** Ingredient composition and nutrient analysis of diet fed to turkey poults upon hatch.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Inclusion (%)</th>
<th>Nutrient</th>
<th>Calculated Analysis</th>
<th>Chemical Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Dry Matter, %</td>
</tr>
<tr>
<td>Corn</td>
<td>40.32</td>
<td>Kcal ME/kg</td>
<td>3000</td>
<td>89.41</td>
</tr>
<tr>
<td>Soybean Meal (48%)</td>
<td>45.80</td>
<td>C. Protein, %</td>
<td>28.5</td>
<td>27.36</td>
</tr>
<tr>
<td>L-Lysine (HCl)</td>
<td>0.22</td>
<td>Lysine, %</td>
<td>1.80</td>
<td>7.48</td>
</tr>
<tr>
<td>DL-Methionine</td>
<td>0.40</td>
<td>Methionine, %</td>
<td>0.82</td>
<td>6.23</td>
</tr>
<tr>
<td>Poultry meal (60%)</td>
<td>6.00</td>
<td>Met + Cys, %</td>
<td>1.25</td>
<td>0.17</td>
</tr>
<tr>
<td>Poultry Fat</td>
<td>2.00</td>
<td>Added Copper, ppm</td>
<td>145</td>
<td>20</td>
</tr>
<tr>
<td>L-Threonine</td>
<td>0.06</td>
<td>Threonine, %</td>
<td>1.15</td>
<td>0.39</td>
</tr>
<tr>
<td>Limestone</td>
<td>0.85</td>
<td>Calcium, %</td>
<td>1.45</td>
<td>1.73</td>
</tr>
<tr>
<td>Dical Phosphate (18.5%)</td>
<td>3.23</td>
<td>nPP %</td>
<td>0.85</td>
<td>1.45</td>
</tr>
<tr>
<td>Salt</td>
<td>0.27</td>
<td>Sodium, %</td>
<td>0.18</td>
<td>0.17</td>
</tr>
<tr>
<td>Choline Chloride (60%)</td>
<td>0.21</td>
<td>Na+K-Cl, Meq/kg</td>
<td>258</td>
<td>0.83</td>
</tr>
<tr>
<td>Vitamin Premix(^1)</td>
<td>0.10</td>
<td>Added Zinc, ppm</td>
<td>150</td>
<td>189</td>
</tr>
<tr>
<td>Mineral Premix(^2)</td>
<td>0.25</td>
<td>Added Manganese, ppm</td>
<td>150</td>
<td>289</td>
</tr>
<tr>
<td>NaSeO3 premix(^3)</td>
<td>0.15</td>
<td>Added Selenium, ppm</td>
<td>0.03</td>
<td>761</td>
</tr>
<tr>
<td>Sand</td>
<td>0.10</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1 Supplied the following per kilogram of feed: vitamin A, 13,200 IU; cholecalciferol, 4,000 IU; niacin, 110mg; pantothenic acid, 22mg; riboflavin, 13.2mg; pyridoxine, 7.9mg; menadione, 4mg; folic acid, 2.2mg; thiamin, 4mg; biotin, 0.253mg; vitamin B12, 0.04mg; ethoxyquin, 100mg; selenium, 0.30mg. The vitamin E premix provided the necessary amount of vitamin E as DL-α-tocopheryl acetate.

2 Supplied the following per kilogram of feed: 120mg Zn as ZnSO₄·H₂O; 120mg Mn as MnSO₄·H₂O; 80mg Fe as FeSO₄·H₂O; 10mg Cu as CuSO₄; 2.5mg I as Ca(IO₃)₂; 1.0mg Co as CoSO₄.

3 Selenium premix supplied 3ppm Se as sodium selenate.
Table 2. Composition of *in-ovo* feeding solutions.

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
<th>Experiment 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca-Lactate (90%), g/L</td>
<td>20.00</td>
<td>20.00</td>
<td>-</td>
</tr>
<tr>
<td>Sodium Chloride, g/L</td>
<td>4.00</td>
<td>4.00</td>
<td>-</td>
</tr>
<tr>
<td>Ca-3 Hydroxy 3 methyl-butryrate, g/L</td>
<td>2.00</td>
<td>2.00</td>
<td>-</td>
</tr>
<tr>
<td>Zinc Gluconate, g/L</td>
<td>1.00</td>
<td>1.00</td>
<td>-</td>
</tr>
<tr>
<td>Nicotinic acid, g/L</td>
<td>0.10</td>
<td>0.10</td>
<td>-</td>
</tr>
<tr>
<td>Biotin, g/L</td>
<td>0.10</td>
<td>0.10</td>
<td>-</td>
</tr>
<tr>
<td>Pyrodoxy-hydrochloride, g/L</td>
<td>0.20</td>
<td>0.20</td>
<td>-</td>
</tr>
<tr>
<td>Folic acid, g/L</td>
<td>0.02</td>
<td>0.02</td>
<td>-</td>
</tr>
<tr>
<td>Gentamicin sulfate, ppm</td>
<td>34.00</td>
<td>34.00</td>
<td>50.00</td>
</tr>
<tr>
<td>Amphoterycin B (250ug/mL), mL</td>
<td>10.00</td>
<td>10.00</td>
<td>-</td>
</tr>
<tr>
<td>L-Tyrosine, g/L</td>
<td>1.50</td>
<td>1.50</td>
<td>-</td>
</tr>
<tr>
<td>Maltodextrin DE4-7, g/L</td>
<td>-</td>
<td>80.00</td>
<td>-</td>
</tr>
<tr>
<td>Lactose hydrate, g/L</td>
<td>-</td>
<td>-</td>
<td>90.00</td>
</tr>
<tr>
<td>Parabens, ppm</td>
<td>-</td>
<td>-</td>
<td>50.00</td>
</tr>
</tbody>
</table>

*Final pH of solution = 5.6. Adjusted with 3mL of sodium citrate (10% in water).*

*Solution was filtered using a 0.2µm pore size filter.*
Table 3. Effects of *in-ovo* feeding on jejunum villi histomorphometrics of turkey poults at hatch, 4 days and 11 days of age (Experiment 1).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Villus height, µm</th>
<th>Crypt depth, µm</th>
<th>Villus surface area, µm²</th>
<th>Goblet cells, per 100 µm²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hatch 4d 11d</td>
<td>Hatch 4d 11d</td>
<td>Hatch 4d 11d</td>
<td>Hatch 4d 11d</td>
</tr>
<tr>
<td>Control</td>
<td>598b 790 1080</td>
<td>101 155 187</td>
<td>57,297b</td>
<td>6.45b</td>
</tr>
<tr>
<td><em>In-ovo fed</em></td>
<td>681a 729 1028</td>
<td>108 149 190</td>
<td>66,280a</td>
<td>11.48a</td>
</tr>
<tr>
<td>P-value</td>
<td>0.061 0.202 0.487</td>
<td>0.168 0.536 0.662</td>
<td>0.077 0.587</td>
<td>0.663 &lt;.001</td>
</tr>
<tr>
<td>SEM(df=23)</td>
<td>20.5 22.0 36.1</td>
<td>2.20 4.68 3.81</td>
<td>2303 4664</td>
<td>670 0.579 0.719</td>
</tr>
</tbody>
</table>

*Apparent villus surface area = [(villus tip width+villus base width)/2]² height. Means represent the average of 12 replicates (poults). Ten villi were measured and averaged per poult.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Hatch 3 days</th>
<th>Villus height, µm</th>
<th>Crypt depth, µm</th>
<th>Villus:Crypt, µm</th>
<th>Villus surface area, µm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>63.68</td>
<td>120b</td>
<td>46</td>
<td>2.90b</td>
<td>3963b</td>
</tr>
<tr>
<td><em>In-ovo fed</em></td>
<td>62.88</td>
<td>142a</td>
<td>41</td>
<td>3.45a</td>
<td>4845a</td>
</tr>
<tr>
<td>Lactose</td>
<td>65.72a</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P-value</td>
<td>0.690</td>
<td>&lt;0.001</td>
<td>0.457</td>
<td>&lt;0.001</td>
<td>0.002</td>
</tr>
<tr>
<td>SEM</td>
<td>1.038d</td>
<td>2.057d</td>
<td>0.781</td>
<td>0.060</td>
<td>118.36</td>
</tr>
</tbody>
</table>

Means represent the average of 75 poults. *Means represent the average of 10 replicates (poults). Ten villi were measured per poult.* Differ significantly at P≤0.001. *Non-injected controls. *In-ovo fed with 1.5mL of *in-ovo* feeding solution (Table 5-experiment 3) at 22E. *Pooled standard error of the mean with 19 degrees of freedom. n=75. *Pooled standard error of the mean with 19 degrees of freedom. n=10.

Table 4. Effects of *in-ovo* feeding on jejunum villi histomorphometrics of turkey embryos at 25E, 27E and day of hatch (Experiment 2).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Villus height, µm</th>
<th>Crypt depth, µm</th>
<th>Villus surface area, µm²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>25E 27E Hatch</td>
<td>25E 27E Hatch</td>
<td>Hatch</td>
</tr>
<tr>
<td>Control</td>
<td>238b 392b 516b</td>
<td>56 91b 109</td>
<td>15,468b</td>
</tr>
<tr>
<td><em>In-ovo fed</em></td>
<td>296a 462a 572a</td>
<td>58 99a 109</td>
<td>20,270a</td>
</tr>
<tr>
<td>P-value</td>
<td>0.056 0.002 0.014</td>
<td>0.597 0.102 0.967</td>
<td>0.060 0.053 0.021</td>
</tr>
<tr>
<td>SEM(df=11)</td>
<td>14.4 10.0 10.2</td>
<td>2.07 2.26 2.86</td>
<td>1211 1341 1414</td>
</tr>
</tbody>
</table>

Means represent the average of six replicates (poults). At least ten villi were measured and averaged per poult. *Differ significantly at P≤0.05. **Differ significantly at P≤0.01. *Saline injected (0.8mL). *In-ovo fed with 0.8mL of *in-ovo* feeding solution (Table 5-experiment 2) at 23E. *Pooled standard error of the mean with 11 degrees of freedom. n=6.

Table 5. Effects of *in-ovo* feeding lactose on body weight and ileum villi histomorphometrics of turkey poults at hatch.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Body Weight, g</th>
<th>Villus height, µm</th>
<th>Crypt depth, µm</th>
<th>Villus:Crypt, µm</th>
<th>Villus surface area, µm²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hatch 3 days</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>63.68</td>
<td>120b</td>
<td>46</td>
<td>2.90b</td>
<td>3963b</td>
</tr>
<tr>
<td><em>In-ovo fed</em></td>
<td>62.88</td>
<td>142a</td>
<td>41</td>
<td>3.45a</td>
<td>4845a</td>
</tr>
<tr>
<td>Lactose</td>
<td>65.72a</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P-value</td>
<td>0.690</td>
<td>&lt;0.001</td>
<td>0.457</td>
<td>&lt;0.001</td>
<td>0.002</td>
</tr>
<tr>
<td>SEM</td>
<td>1.038d</td>
<td>2.057d</td>
<td>0.781</td>
<td>0.060</td>
<td>118.36</td>
</tr>
</tbody>
</table>

Means represent the average of 75 poults. *Means represent the average of 10 replicates (poults). Ten villi were measured per poult.* Differ significantly at P≤0.001. *Non-injected controls. *In-ovo fed with 1.5mL of *in-ovo* feeding solution (Table 5-experiment 3) at 22E. *Pooled standard error of the mean with 154 degrees of freedom. n=75. *Pooled standard error of the mean with 19 degrees of freedom. n=10.
Figure 1. Histomorphometrical and ultra-structural characteristics of small intestinal mucosa of in-ovo fed poults compared to controls at hatch. (a) Alcian blue stained histological section at day of hatch from the proximal jejunum of 23E saline-injected (control) turkey embryos (experiment 1). (b) Alcian blue stained histological section at day of hatch from the proximal jejunum of 23E in-ovo fed turkey embryos (experiment 1). Note the amount of mucus secretion in the lumen of in-ovo fed compared to controls (Figure 1a). (c) Scanning electron micrograph at day of hatch from the proximal jejunum of 23E saline-injected (control) turkey embryos (experiment 1). (d) Scanning electron micrograph at day of hatch from the proximal jejunum of 23E in-ovo fed turkey embryos (experiment 1). Note the size of villi and the tip roundness of individual villi of in-ovo fed compared to controls (Figure 1c).
Figure 2. Ultra-structural characteristics of small intestinal villi of in-oovo fed poult compared to controls at hatch. (a) Scanning electron micrograph at day of hatch from individual villi of the proximal jejunum of 23E saline-injected (control) turkey embryos (experiment 1). Note clumping of microvilli in the apical end exposes hexagonal shape of enterocytes. (b) Scanning electron micrograph at day of hatch from individual villi of the proximal jejunum of 23E in-oovo fed turkey embryos (experiment 1). Note uniformity of microvilli on the apical end as well as the presence of mucus clusters compared to controls. (c) Transmission electron micrograph of goblet cells in the proximal jejunum of 23E saline-injected (control) turkey embryos (experiment 1). (d) Transmission electron micrograph of goblet cells in the proximal jejunum of 23E in-oovo fed turkey embryos (experiment 1).
Figure 3. Microarray analysis of small intestinal gene expression of 4-day old in-ovo fed turkey poults compared to controls. (a) Volcano plot of microarray analysis in the proximal jejunum of 4 day-old turkey poults. Notice the differences in gene expression between saline-injected controls and 23E in-ovo fed turkey poults. (b) Microarray heat map showing gene expression differences between the jejunum of saline-injected controls and that of 23E in-ovo fed turkey poults. Inset shows the experimental design of microarray analysis.
Table 6. Fold change and P-values of gene expression profiles in the jejunum of 4-day old turkey poults that were *in-ovo* fed at 23 days of incubation.

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Gene Name</th>
<th>Ascession No</th>
<th>Fold change&lt;sup&gt;0&lt;/sup&gt;</th>
<th>P value (-log&lt;sub&gt;10&lt;/sub&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TYR</td>
<td>Tyrosinase</td>
<td>TC227576</td>
<td>0.62</td>
<td>15.39</td>
</tr>
<tr>
<td>HOXA3</td>
<td>Homeobox A3</td>
<td>NM_204548</td>
<td>0.79</td>
<td>4.19</td>
</tr>
<tr>
<td>CDK1</td>
<td>Cyclin-dependent kinase 1</td>
<td>TC230512</td>
<td>0.86</td>
<td>9.84</td>
</tr>
<tr>
<td>CKM</td>
<td>Creatine kinase B chain (B-CK)</td>
<td>TC186914</td>
<td>0.87</td>
<td>5.62</td>
</tr>
<tr>
<td>14_3_3</td>
<td>14-3-3 protein zeta</td>
<td>TC229596</td>
<td>0.89</td>
<td>5.00</td>
</tr>
<tr>
<td>SM22</td>
<td>Smooth muscle cell specific protein</td>
<td>M83105</td>
<td>0.90</td>
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<td>F_actin</td>
<td>Alpha-actinin-1</td>
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<td>NPPA</td>
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<td>LOC395765 / NM_204925</td>
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<td>MYL9</td>
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<tr>
<td>MDH2</td>
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<td>TC192153</td>
<td>0.92</td>
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<td>HMOX1</td>
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<td>NM_205344</td>
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<td>4.32</td>
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<tr>
<td>HAND1</td>
<td>Heart and neural crest derivatives expressed 1</td>
<td>NM_204965</td>
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<td>4.01</td>
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<td>ACAAA</td>
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<td>ALDOC</td>
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<td>TC207134</td>
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<td>NM_205479</td>
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<td>ELN</td>
<td>Tropoelastin</td>
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<td>Decorin</td>
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<td>E2F</td>
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<td>TC229068</td>
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<td>IL10</td>
<td>Interleukin-10</td>
<td>TC197579</td>
<td>1.05</td>
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<td>NOS2A</td>
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<td>FasL</td>
<td>Fas ligand (TNF superfamily, member 6)</td>
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<td>4.39</td>
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<td>PLB</td>
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<td>TLR2 -2</td>
<td>Toll-like receptor 2 type 2 precursor</td>
<td>TC218153</td>
<td>1.08</td>
<td>4.81</td>
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<td>NM_204120</td>
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<td>IL8</td>
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<td>EDN1</td>
<td>Preproendothelin-1</td>
<td>TC224053</td>
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<td>5.19</td>
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<td>CHP2</td>
<td>Gallinacin-1 alpha precursor (Gal-1 alpha) (Antimicrobial peptide CHP2)</td>
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<td>IFN</td>
<td>Interferon gamma precursor</td>
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<tr>
<td>GSK3B</td>
<td>Glycogen synthase kinase-3 beta</td>
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**Table 6.** Continuation.

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<th>Gene Symbol</th>
<th>Gene Name</th>
<th>Ascession No</th>
<th>Fold change&lt;sup&gt;Ω&lt;/sup&gt;</th>
<th>P value (-log&lt;sub&gt;10&lt;/sub&gt;)</th>
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<tr>
<td>HIF1A</td>
<td>Hypoxia-inducible factor 1 alpha subunit</td>
<td>NM_204297</td>
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<td>ZC3H15</td>
<td>Erythropoietin 4 immediate early response</td>
<td>NM_001006510</td>
<td>1.12</td>
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<td>XPNPEP1</td>
<td>Membrane-bound aminopeptidase P (X-prolyl aminopeptidase P) 2</td>
<td>TC212978</td>
<td>1.12</td>
<td>4.90</td>
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<td>Interleukin-13</td>
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<td>Toll-like receptor 4</td>
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<td>IGFBP1</td>
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<td>NM_001001294</td>
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<td>Epidermal growth factor receptor</td>
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<td>TBX20</td>
<td>T-box 20</td>
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<td>IRX4</td>
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<td>Actin alpha 2 smooth muscle aorta</td>
<td>NM_001031229</td>
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<td>MGAM</td>
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<td>ALDOB</td>
<td>fructose-bisphosphate aldolase B</td>
<td>TC187124</td>
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<td>HK1</td>
<td>Hexokinase 1</td>
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*ΩDenotes the expression of genes the in-ovo fed treatment compared to control group.
Figure 4. Metacore™ metabolic map showing potential pathways affected in the small intestinal epithelium of 23E in-ovo fed turkey poults at 4 days post-hatch.
Table 7. Key for gene map presented in Figure 9.

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Gene Description</th>
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<tbody>
<tr>
<td>AMYP</td>
<td>Pancreatic alpha-amylase 1</td>
</tr>
<tr>
<td>SLC5A1</td>
<td>Sodium/Glucose cotransporter 1</td>
</tr>
<tr>
<td>PEPT1</td>
<td>Oligopeptide transporter, small intestinal isoform</td>
</tr>
<tr>
<td>Aminopeptidase P1</td>
<td>Xaa-Pro aminopeptidase 1</td>
</tr>
<tr>
<td>P2X1</td>
<td>P2X purinoreceptor</td>
</tr>
<tr>
<td>HXK1</td>
<td>Hexokinase 1</td>
</tr>
<tr>
<td>LDHD</td>
<td>D-lactate dehydrogenase</td>
</tr>
<tr>
<td>LDHA</td>
<td>L-lactate dehydrogenase</td>
</tr>
<tr>
<td>TR-Alpha</td>
<td>Thyroid hormone receptor alpha</td>
</tr>
<tr>
<td>TR-Beta1</td>
<td>Thyroid hormone receptor beta-1</td>
</tr>
<tr>
<td>GCGR</td>
<td>Glucagon receptor precursor</td>
</tr>
<tr>
<td>GHR</td>
<td>Growth hormone receptor precursor</td>
</tr>
<tr>
<td>LPL</td>
<td>Lipoprotein lipase precursor</td>
</tr>
<tr>
<td>Insulin Receptor</td>
<td>Insulin receptor precursor</td>
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</table>
Figure 5. Plasma triiodothyronine (T₃) levels in 23E in-ovo fed turkey poults from 25E to 12d, compared to saline-injected controls (Experiment 2).
**Figure 6.** Growth performance and feed efficiency of 23E *in-ovo* fed turkey poults from hatch to 11d, compared to controls.
4.7 REFERENCES


Bohórquez, D., A. Santos, and P. Ferket. Year. In-ovo feeding and dietary \( \beta \)-hydroxy-\( \beta \)-methylbutyrate effects on poult quality, growth performance and ileum microanatomy of turkey poults from 1 to 11 days of age. Proc. Int Poult Sci For, Atlanta, GA.


CHAPTER V

THE EFFECTS OF IN-OVO FEEDING AND DIETARY SUPPLEMENTATION OF YEAST EXTRACT NUCLEOTIDES ON THE MORPHOLOGICAL MATURATION OF THE SMALL INTESTINAL MUCOSA OF THE PERINATAL TURKEY POULT
5.1 ABSTRACT

Perinatal development of the intestine is critical for the post-hatch survival and growth of poults. In-ovo feeding enhances the morphological maturation of the small intestinal mucosa in the hatching turkey poult. However, this apparent advantage diminishes after hatch, perhaps due to limits in de novo and dietary nucleotides that support the rapid mitotic proliferation intestinal epithelial cells during the neonatal period. Thus, dietary supplementation of yeast-extract nucleotides may help maintain the post-hatch maturation of the small intestinal mucosa of in-ovo fed (IOF) turkey poults. To test this hypothesis, 600 Nicholas turkey embryos were injected into the amnion with 0.8mL of a saline or IOF solution at 23 days of incubation (E). Hatched poults from each group were subjected to basal diets supplemented with 0% or 3% Nupro®, resulting in 4 post-hatch treatments: (1) Saline-injected + 0% Nupro®, (CTRL); (2) In-ovo fed + 0% Nupro® (IOF); (3) Saline-injected + 3% Nupro® (NP); (4) In-ovo fed + 3% Nupro® (IOF-NP). Each treatment was replicated by 6 cages with 10 poults per cage. Body weights (BW), cumulative feed:gain ratio (cFCR), feed intake (cFI), mortality rates and small intestinal weights (SIW) were evaluated at hatch, 4d and 12d. Jejunum tissue samples from 6 poults per treatment were collected for qRT-PCR analysis of egfr, igfbp-1 and muc2 at 25E, 27E, hatch, 4d and 12d. Jejunum histomorphometrics were assessed in 6 poults per treatment at hatch, 4d and 12d. At hatch, IOF treatment had greater (P<0.05) SIW (3.54 vs. 2.92g/100g BW), villus height (572 vs 504µm) and villus surface area (58502 vs. 49663µm²) than CTRL. At 4d, only NP treatment significantly increased crypt depths (166 vs 140µm, P<0.05) compared to CTRL. There were no IOF or NP treatment effects on jejunum histomorphology at 12d. However, at this time, IOF+NP significantly (P≤0.05) increase villus height (1082 vs 910µm) and villus surface area (147819 vs 112725µm²) compared to CTRL. Analysis of egfr, igfbp-1 and muc2 expression revealed that IOF up-regulates these genes prior to hatch as compared to CTRL. At 12d, egfr and igfbp-1 were up-regulated (P≤0.1) in the IOF+NP treatment compared to CTRL. In addition, NP significantly (P<0.05) increased (~5%) cFI at 4d and 12d. We conclude that dietary supplementation Nupro® to in-ovo fed turkey poults enhances the morphological maturation of the small intestinal mucosa after hatch, perhaps because dietary nucleotides stimulate post-hatch appetite of turkey poults.
5.2 INTRODUCTION

In poultry, the genetic potential for growth, feed efficiency, and disease resistance depends to a great extent upon the morphological and functional maturity of the gastrointestinal tract (GIT) at hatch to allow for a smooth transition to an exogenous supply of nutrients post-hatch (Dibner et al., 1996; Noy and Sklan, 1998; Uni et al., 1999). In turn, the maturation of the intestine relies primarily on the presence of nutrients in its lumen prior to and after hatch (Corless and Sell, 1999; Noy and Sklan, 1999; Shah and Sanderson, 2000). Prior to hatch, swallowing of the amniotic fluid by the embryo is arguably the single most important signal for morphological and functional preparation of the intestinal mucosa for post-hatch feed intake (Bloomfield et al., 2002; Burlingame et al., 2003; Chopra and Crandall, 1975; Cleveland et al., 1991). Mammalian models in which fetal swallowing is prevented by esophageal ligation have shown that amniotic fluid consumption is critical for mucosal development (Trahair and Harding, 1995), internalization of the intestines into the abdominal cavity (i.e. gastroschisis) (Bloomfield et al., 2002), and for the onset of dipsogenic (i.e. thirst) and orexic (i.e. appetite) mechanisms (Bradley and Mistretta, 1973). Thus, amniotic fluid manipulation has the potential to program fetal thirst and appetite after birth (El-Haddad et al., 2004). In poultry, this procedure is known as in-ovo feeding (Uni and Ferket, 2003) and is defined as the administration of nutrients into the amnion of the late-term avian embryo (Uni and Ferket, 2004), primarily to accelerate enteric development prior to hatch and smooth the transition to dietary nutrients immediately after hatch (Uni and Ferket, 2001; Uni and Ferket, 2004).

In-ovo feeding (IOF) of late-term chicken and turkey embryos has shown promising results in terms of increasing the energy reserves available for the hatching process and stimulating the morphological maturation of the digestive tract prior to hatch (Uni and Ferket, 2004). For instance, Smirnov et al. (2006) have shown that injecting a solution containing carbohydrates into the amnion of chicken embryos at 17.5 days of incubation (E) has a trophic effect on the small intestine mucosa around the time of hatch. In fact, in-ovo feeding carbohydrates or β-hydroxy-β-methyl-butyrate to chicken embryos has been associated with >30% increase in villus surface area and about a 50% increase in the activity of digestive enzymes at hatch (Tako et al., 2004). In turkeys, in-ovo feeding
arginine and β-hydroxy-β-methyl-butyrate increases intestinal absorption of carbohydrates and amino acids at hatch (Foye et al., 2005). Moreover, de Oliveira (2007) showed that genes mediating mucosal and nutrient utilization in the small intestine are up-regulated around the time of hatch in IOF-treated turkey embryos, favoring improved poult quality scores and skeletal development post-hatch.

In-ovo feeding also enhances the development of the mucosal brush border and barrier function. In-ovo feeding carbohydrates significantly increases goblet cell numbers and mucins secretion in the small intestine of hatching chicks (Smirnov et al., 2006). Mucins glycoproteins make up an integral part of the mucus blanket that protects the intestinal epithelium and mediates nutrient uptake (Liévin-Le Moal and Servin, 2006). Moreover, in-ovo feeding turkey embryos at 23E enhances the goblet cell numbers and the morphological and ultra-structural development of the small intestinal mucosa during the perinatal period (Chapter IV). These results have been correlated with the up-regulation of several genes involved in carbohydrate and protein metabolism and cell proliferation, including those encoding for epidermal growth factor receptor (EGFR) and insulin-like growth factor binding protein-1 (IGFBP-1). These 2 proteins are important mediators of cell division and proliferation during early development of the intestinal epithelium (Adamson and Rees, 1981; Lee et al., 1997). These reports consistently illustrate the morphological maturation and digestive capacity of IOF hatchlings is functionally similar to that of a conventionally fed 2 day-old chick (Tako et al., 2004; Uni and Ferket, 2004). However, these advantages apparently dissipate with time after hatch, perhaps because the accelerated enteric development of IOF-treated poults reaches a plateau soon after hatch, a point that is achieved by the controls with time. Despite this possibility, it is noteworthy that rapid enteric development, particularly in poultry (de Oliveira et al., 2009), requires the presence of highly digestible nutrients at hatch that can sustain the accelerated proliferation of epithelial cells.

It has been well documented that rapidly dividing cells (e.g. intestinal epithelial cells) have a voracious demand for nutrients and metabolic cofactors, in particular for nucleotides, the building blocks of DNA and RNA (He et al., 1993; Tanaka et al., 1996). Nucleotides are chemical compounds that consist of three molecular fragments: sugar, heterocyclic base and phosphate group (Horton et
al., 2002). These compounds not only are essential for nucleic acid synthesis and replication, but also mediate signaling of peptide hormones responsible for growth, and provide energy (e.g. ATP) for enzymatic processes and muscle work (Saenger, 1984). Although nucleotides can be synthesized by cells de novo, recycling or using pre-formed dietary nucleotides is far more energetically efficient (Grimble, 1994; Saenger, 1984). Mammalian research has shown the importance of dietary nucleotide supplementation in the regulation of metabolism, modulation of the immune response, and normal enteric maturation (Carver and Allan Walker, 1995; Pickering et al., 1998; Uauy et al., 1994). In piglets, dietary nucleotide supplementation can ameliorate the erosion of the mucosa observed immediately after weaning and reduce the occurrence of diarrhea (Martinez-Puig et al., 2007). Furthermore, dietary supplementation with yeast-extract nucleotides enhances the morphological development of the small intestine of turkey poults one week after hatch, and it increases their body weights up to 3 weeks of age (Bohórquez et al., 2008). Therefore, we hypothesized that post-hatch dietary supplementation of in-ovo fed turkey poults with dietary nucleotides stimulates the morphological development of the small intestinal mucosa after hatch. For this purpose we used a commercial yeast-extract product containing up to 6% of nucleotides called Nupro®. To test our hypothesis, we injected into the amnion of 23E turkey embryos a solution containing metabolic cofactors and carbohydrates (Table 1) or saline (control). At hatch, we subjected turkey poults from each group to a diet containing 0 or 3% Nupro®. We then used quantitative Real Time-PCR (qRT-PCR) to evaluate the treatment effects on the expression of genes involved in mucosal maturation (egfr, igfbp-1 and muc2) from 25E to 12 days post-hatch. We chose these genes based on a previous microarray analysis presented in Chapter IV. We also evaluated the treatment effects on the histomorphometrical characteristics of the jejunum and the growth performance of turkey poults from hatch to 12 days of age.

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1 Alltech Inc. Lexington, KY
5.3 MATERIALS AND METHODS

Bird husbandry and tissue collection

Fertile Nicholas turkey eggs (600) were obtained from a commercial hatchery and incubated at 37.5°C and 52% relative humidity. At 20E, all eggs were candled and equally distributed by weight into two groups. At 23E, 250 eggs per group were injected with either 0.8mL of a solution containing nutritional cofactors of metabolism and carbohydrates (IOF) (Table 1) or 0.8mL of a saline-solution (CTRL). The saline control was a prepared as 4g of NaCl per litter of solution. The solutions were manually injected into the amnion using a 23g needle (injection depth 318mm). Manual IOF injection procedures have been previously described in detail by Foye (2005) and de Oliveira (2007). At hatch, 120 turkey poults (males and females) from each group (IOF and CTRL) were randomly distributed among 24 alternative design battery² cages (10 poults per cage). Poults were then fed basal diets supplemented with 0% or 3% Nupro®, resulting in 4 post-hatch treatments: (1) Saline-injected + 0% Nupro®, (CTRL); (2) In-ovo fed + 0% Nupro® (IOF); (3) Saline-injected + 3% Nupro® (NP); and (4) in-ovo fed + 3% Nupro® (IOF-NP). All birds were given ad libitum access to feed and water. The composition of the dietary treatments and Nupro® product are presented in Table 2 and 3.

Growth performance and intestinal weights. Individual bird body weights (BW), bird feed intake (FI), cumulative feed:gain ratio (cFCR) and mortality were determined at hatch, 4d, and 12d.

Small intestinal weights and histology samples. Six turkey poults per treatment were euthanized by cervical dislocation at hatch, 4d, and 12d. Small intestinal weights from the gizzard/duodenum sphincter to the ileo-cecal junction were recorded at each time point. The pancreas was removed and the intestinal lumen was flushed with saline buffer prior to intestinal weight determination. A 1cm tissue segment was removed from the proximal jejunum and immediately fixed in 10% neutral buffered formalin for at least 72 hours prior to histology slides preparation.

Tissue collection for total RNA extraction. Six turkey embryos or poults per treatment were euthanized by cervical dislocation and intestinal samples were collected at 25E, 27E, hatch, 4d, and 12d. A 2cm segment of the mid-jejunum was excised from each embryo or poult, flushed with saline buffer, and fixed in 10% neutral buffered formalin for at least 72 hours prior to histology slides preparation.

² Alternative design Inc. Siloam Springs, AR
buffer, and placed individually in a vial containing 5mL of RNA/ater. Samples were stored at -80°C before RNA extraction.

**Histomorphometrical analysis**

**Slides preparation.** Histological samples from all three experiments were processed according to the following protocol. Three sections (~3 mm) were obtained from each fixed segment, placed into tissue cassettes and submerged into 10% neutral buffered formalin. Cassettes were then sent to the North Carolina State University College of Veterinary Medicine histology lab for preparation of slides and hematoxylin and eosin staining.

**Image acquisition and histomorphometrical analysis.** Light microscopy photographs were taken of the transverse sections and analyzed using UTHSCSA Image Tool software. The following measurements were performed without knowledge of the treatment identity on at least 10 villi per sampled bird: villus height, villus apical width at the villus tip, villus basal width at the crypt-villus junction, crypt depth, and muscularis depth. Apparent villus surface area was estimated by the mathematical formula: 

\[ \frac{\text{((villus tip+villus base)/2)} \times \text{villus height}} \]

**Data analysis.** Ten villi measurements were averaged within each sampled poult (6 poults per treatment) and this number served as the experimental unit. Villi data were analyzed by a one-way ANOVA procedure of JMP software and means that differed significantly among treatments were separated by the Tukey’s test procedure at P≤0.05. The same procedure was used to analyze the small intestinal weights relative to body weight.

**Total RNA extraction and quantitative RT-PCR analysis**

**RNA extraction and quality control.** Total RNA was extracted from 100mg of jejunum tissue per sample (6 samples per treatment) using the TRIzol extraction method according to the protocol previously described by de Oliveira (2007). Extracted RNA pellets were resuspended in 100µL of nuclease-free water and its concentration was determined using a NanoDrop spectrophotometer.

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3 Applied biosystems / Ambion. Austin, TX  
4 Department of Dental Diagnostic Science at The University of Texas Health Science Center, San Antonio, Texas  
5 SAS Inc. Cary, NC  
6 Invitrogen corporation, Carlsbad, CA  
7 Thermo Scientific – NanoDrop products. Wilmington, DE
Then, 20µg of total RNA from each sample were DNase treated with TURBOTM DNase according to the manufacturer’s protocol. The integrity of RNA was verified by electrophoresis on 1.2% agarose gel and samples were stored at -80°C prior to reverse transcription into cDNA.

**Synthesis of cDNA.** One microgram of DNase-treated RNA per sample (6 samples per treatment) were reverse transcribed into cDNA using iScript Select cDNA Synthesis Kit. All procedures were performed according to the manufacturer’s recommendation and the PCR conditions were: 65 min at 42°C, followed by 5 min at 85°C to heat-inactivate the reverse transcriptase. Finally, cDNA concentration was determined using a NanoDrop spectrophotometer prior to storage at -20°C.

**Primer design and Quantitative RT-PCR analysis.** Primers were designed using the Primer Express v2.0 software and ordered from IDT. Primers characteristics and sequences are presented in Table 4. Quantitative RT-PCR was performed using an iQ SYBR Green Supermix kit and the protocol was performed according to the manufacturer’s recommendations. Each sample was assayed in triplicate of 20µL reactions containing: 1µL of (1:20) cDNA, 1µL of primers (20µM of reverse and 20µM forward), 10µL of iQ SYBR Green Supermix and 8µL of nuclease-free water. In addition, a standard curve was built from four serial dilutions (1:5, 1:25, 1:125, 1:625) of pooled cDNA from all samples (assayed in triplicates) to calculate the efficiency of the reaction. The qRT-PCR assay was performed using a Bio-Rad iCycleriQ Real Time PCR and the cycling conditions are described in Table 5.

**Data Analysis.** Calculated threshold (Ct) values were obtained using the iCycleriQ software and used to evaluate fold change of the gene of interest (GOI) with respect to the housekeeping gene (HKG, 18S rRNA). Expression of GOI relative to HKG was analyzed using the ΔΔCt method described by Pfaff (2001). Statistical significance of treatment changes in expression was determined by a one-way ANOVA analysis of ΔCt (GOI-HKG) values among treatments (Yuan et al., 2006). Means that differed significantly were separated by a Student’s test at a P<0.05 using JMP.

**Statistical Analysis of Growth Performance**
Body weights, cumulative feed:gain ratio, cumulative feed intake and percentage mortality rates were
analyzed using the general linear models procedure for analysis of variance (ANOVA) of JMP<sup>5</sup>. Mortality data were transformed to arc sine of the square root percentage data distribution before statistical analysis. The Model used was \( Y_{ijklm} = \mu + B_i + F_j + N_k + F_jN_k + \varepsilon_{ijkl} \), where, \( B_i \) = Block location in battery cages, \( F_j = \text{in-ovo feeding effect} \), \( N_k = \text{Nupro® effect} \), \( F_jN_k = \text{interaction of in-ovo feeding X Nupro®} \), and \( \varepsilon_{ijkl} = \text{Error term} \). The means of poults within each cage served as the experimental units for statistical analysis. Variables having a significant F-test were compared using the Tukey’s test function of JMP<sup>5</sup>, and the treatment effects were considered to be significant at \( P<0.05 \).

**Animal ethics**

Turkey embryos and poults were managed according to normal husbandry practices and the experiments were conducted according to the experimental protocol approved by the Institutional Animal Care and Use Committee (IACUC) of North Carolina State University.

### 5.4 RESULTS

*Treatment effects on small intestinal weights and morphological maturation of the jejunal mucosa from hatch to 12d (Table 6).* At hatch, the IOF treatment increased small intestinal weights (3.54 vs. 2.92g/100g of BW, \( P \leq 0.01 \)), villus height (571.9 vs. 504.2\( \mu \)m, \( P \leq 0.01 \)) and villus surface area (58502 vs. 49662\( \mu \)m<sup>2</sup>, \( P \leq 0.01 \)) as compared to CTRL. At 4d, the small intestinal weight was greater in the NP than CTRL group (11.12 vs. 9.32g/100g of BW, \( P \leq 0.05 \)). Likewise, the crypt depth in NP treatment group was greater than CTRL at 4d (166.3 vs. 139.8\( \mu \)m, \( P \leq 0.05 \)). There was no other significant treatment effects on the histomorphometrical variables evaluated at 4d. Moreover, there were no treatment effects on intestinal weight and crypt depth at 12d. However as compared to CTRL, the IOF+NP treatment had increased villi height (1081.7 vs. 910.3\( \mu \)m, \( P \leq 0.05 \)) and villus surface area (147819 vs. 112725\( \mu \)m<sup>2</sup>, \( P \leq 0.05 \)). *In-ovo* feeding increases intestinal mass per 100g of BW and absorptive capacity at hatch as indicated by increased villi height, and surface area. This advantage can be sustained by dietary inclusion of a nucleotide source, such as Nupro®, which stimulates crypt maturation at least until 4d post-hatch.
**Treatment effects on the jejunum expression of egfr, igfbp-1 and muc2 from 23E to 12d (Figure 1)**. There were not significant treatment effects on muc2 expression from 27E to 12d, yet IOF significantly (P≤0.05) increased the expression of muc2 at 25E. Treatment effects on igfbp-1 expression were not significant from 25E to 4d; however at 12d, IOF+NP treatment increased (P≤0.1) igfbp-1 expression by about 125% as compared to CTRL. Treatment effects on egfr expression appeared to be more consistent throughout the evaluation period. At 27E, IOF treatment significantly (P≤0.05) increased egfr expression as compared to CTRL, but at hatch IOF significantly (P≤0.05) reduced egfr expression as compared to CTRL. Although there were no significant treatment effects on egfr expression at 4d, the NP and IOF+NP treatments increased (P≤0.1) the egfr expression by 12d, compared to CTRL. Though not statistically significant, the trends in the expression of these three genes are affected by the prenatal and neonatal treatments differently. Apparently, IOF up-regulated the expression of these genes from the time of injection until hatching; but after hatch, IOF down-regulated the expression of these genes unless the diet was supplemented with Nupro®. These data suggest that even though IOF will accelerate maturation prior to hatch, its effects can be sustained after hatch by dietary supplementation of a nucleotide source in the diet.

**Treatment effects on growth performance of turkey poults from hatch to 12d (Table 8)**. There were no significant (P≤0.05) treatment effects on body weights or cumulative feed:gain ratio at hatch 4d and 12d. However in comparison to controls, Nupro® alone significantly improved cumulative feed intake at 4d (28.1 vs. 26.4g, P=0.046) and 12d (257 vs. 246g, P=0.023). Evidently, dietary supplementation with a nucleotide source, such as Nupro®, has a consistent effect on feed intake post-hatch, which may stimulate enteric development.

### 5.5 DISCUSSION

It is now well accepted that a functional digestive tract at hatch is critical for hatchlings to adapt to an exogenous supply of nutrients and for poultry to achieve the genetic potential for growth and feed efficiency (Dibner et al., 1998; Noy et al., 2001; Uni and Ferket, 2004). Further morphological and functional development of the digestive tract depends primarily on the luminal presence of nutrients
that stimulate digestive and absorptive capacity of the epithelium (Noy et al., 2001; Shah and Sanderson, 2000). Consequently, early feeding strategies have the potential to program the growth performance of poultry. \textit{In-ovo} feeding (IOF) or the administration of nutrients into the late-term avian embryo (Uni and Ferket, 2001; Uni and Ferket, 2004) has consistently been shown to accelerate enteric development and digestive function (de Oliveira, 2007; Foye, 2005; Tako et al., 2004). Because rapid enteric development requires an adequate supply of readily available nutrients and cofactors, perhaps the potential of IOF can be enhanced by post-hatch dietary supplementation of enteric modulators such as nucleotides. For that reason, we hypothesized that post-hatch dietary supplementation of IOF turkey poults with dietary nucleotides helps to sustain or even stimulate the morphological development of the small intestinal mucosa after hatch. The source of dietary nucleotides used was a commercially available yeast-extract product, Nupro®, which contains up to 6% of nucleotides.

\textit{In-ovo} feeding has been shown to accelerate the morphological and ultra-structural development of the small intestinal mucosa up to the time of hatch (Chapter IV). In the current study, IOF not only increases villi height and villus surface area by the time of hatch, but it also increases the gross weights of the small intestine at this time (Table 6). Morphological maturation of the small intestinal epithelium increases the surface area exposed to nutrient absorption and provides a physical platform for the onset of digestive enzymes that mediate nutrient absorption and utilization (Noy et al., 2001; Sell et al., 1991; Uni et al., 1999). Similar effects have been observed in several other IOF studies in chicken, turkey and duck hatchlings (Chen et al., 2009; de Oliveira, 2007; Foye, 2005; Smirnov et al., 2006; Tako et al., 2004). Based on our qRT-PCR data, we conclude that \textit{in-ovo} feeding of turkeys at 23E up-regulates \textit{egfr} and \textit{muc2} prior to hatch, which likely mediate the development of mucosal morphology up to hatch (Figure 1). Smirnov et al. (2006) reported that \textit{in-ovo} feeding chicken embryos at 17.5E have higher expression of \textit{muc2} gene, which encodes for the secreted mucin glycoproteins of the protective mucus blanket (Liévin-Le Moal and Servin, 2006). Similarly, IOF has been shown to up-regulate the expression of several other genes involved in carbohydrate and protein metabolism and cell proliferation, including the genes encoding for epidermal growth factor.
receptor (EGFR) and insulin-like growth factor binding protein-1 (IGFBP-1) (Chapter IV). Binding of epidermal growth factor (EGF) to its receptor (e.g. EGFR) can trigger a number of biological responses including increased amino acid transport, increased glucose uptake and cell growth (Adamson and Rees, 1981). Although igfbp-1 functions have not been clearly defined, it is known to regulate the effects IGF-I and II on cell growth, and its expression is stimulated by thyroid hormones and epidermal growth factor (Lee et al., 1997).

Due to rapid turnover, tissues of the GIT require increased levels of nucleotides as precursors for nucleic acid synthesis (He et al., 1993; Tanaka et al., 1996). Thus, dietary supplementation of a nucleotide source immediately after hatch may optimize morphological and functional maturation of the digestive tract (Carver and Allan Walker, 1995). Because IOF accelerates enteric growth, an exogenous supply of nucleotides may favor rapidly dividing cells of the intestinal mucosa. Dietary supplementation of the nucleotide-rich Nupro® has been demonstrated to increase villus surface area and crypt depths in turkey poults one week post-hatch (Bohórquez et al., 2008). In the current study, the IOF-treated poults supplemented with Nupro® had significantly larger crypt depths at 4d, as well as higher villus height and greater villus surface area at 12d than controls (Table 6). These results are in agreement with the treatment effects on egfr and igfbp-1 expression at 12d. At this time, the igfbp-1 and egfr expression in IOF-treated poults supplemented with Nupro® were higher (P≤0.1) than controls. Moreover, regardless of the prenatal in-ovo treatment, dietary supplementation of Nupro® increased the expression of egfr at 12d. Reports from mammalian research have demonstrated the positive effects of an exogenous supply of nucleotides upon the proliferation and differentiation of intestinal epithelial cells (Martinez-Puig et al., 2007; Tanaka et al., 1996; Uauy et al., 1994). In fact, nucleotides are commonly supplemented in commercially available infant formulas, in particular to stimulate enteric development and immune function during the weaning period (Carver et al., 1991; Yu, 2002).

Treatment effects on body weight and feed efficiency were not significant throughout the evaluation period. Contrary to previous reports where only male turkey poults were used (de Oliveira, 2007; Foye, 2005), we used hatchling poults of both sexes. Males and females have distinctive
patterns of growth, which can affect the average growth performance of the flock. Regardless of sex, the feed intake was consistently increased by dietary supplementation of nucleotides. Although the mechanisms by which dietary supplementation of nucleotide-rich yeast extract may enhance feed intake in poultry are poorly understood, nucleotides are known to stimulate appetite in mammals by enhancing food palatability (Carver and Allan Walker, 1995).

In conclusion, dietary supplementation of the nucleotide-rich yeast extract Nupro® to in-ovo fed turkey poults enhances the morphological maturation of the small intestinal epithelium after hatch, as evidenced by an increase in villus surface area and up-regulation of genes involved in epithelial cell growth. The positive effects of dietary supplementation of Nupro® appears associated with post-hatch stimulation of appetite and feed intake in turkey poults.

ACKNOWLEDGMENTS

This work was supported by the U.S. Poultry & Egg Association (Tucker, GA). The author wishes to thank: Sandra Horton (NCSU College of Veterinary histology lab), Dr. Eva Johannes (NCSU Botany Dept. Cellular and Molecular Imaging Facility) and Chris Miller (assistance with primer design and qRT-PCR optimization).
### 5.6 TABLES AND FIGURES

**Table 1.** Composition of *in-ovo* feeding solution.

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Amount per litter of solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca-Lactate (90%), g/L</td>
<td>20.00</td>
</tr>
<tr>
<td>Sodium Chloride, g/L</td>
<td>4.00</td>
</tr>
<tr>
<td>Ca-3 Hydroxy 3 methyl-butyrates, g/L</td>
<td>2.00</td>
</tr>
<tr>
<td>Zinc Gluconate, g/L</td>
<td>1.00</td>
</tr>
<tr>
<td>Nicotinic acid, g/L</td>
<td>0.10</td>
</tr>
<tr>
<td>Biotin, g/L</td>
<td>0.10</td>
</tr>
<tr>
<td>Pyrodoxy-hydrochloride, g/L</td>
<td>0.20</td>
</tr>
<tr>
<td>Folic acid, g/L</td>
<td>0.02</td>
</tr>
<tr>
<td>Gentamicin sulfate, ppm</td>
<td>34.00</td>
</tr>
<tr>
<td>Amphoterycin B (250ug/mL), mL</td>
<td>10.00</td>
</tr>
<tr>
<td>L-Tyrosine, g/L</td>
<td>1.50</td>
</tr>
<tr>
<td>Maltodextrin DE4-7, g/L</td>
<td>80.00</td>
</tr>
</tbody>
</table>

*Final pH of solution = 5.6. Adjusted with 3mL of sodium citrate (10% in water). Solution was filtered using a 0.2µm pore size filter.

**Table 2.** Total amino acid content and chemical analysis of nutrient content of Nupro® yeast-extract nucleotides

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Wet basis</th>
<th>Dry basis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry Matter, %</td>
<td>95.30</td>
<td>51.13</td>
</tr>
<tr>
<td>Crude Protein, %</td>
<td>48.73</td>
<td>51.13</td>
</tr>
<tr>
<td>Calcium, %</td>
<td>0.10</td>
<td>0.10</td>
</tr>
<tr>
<td>Phosphorous, %</td>
<td>0.97</td>
<td>1.02</td>
</tr>
<tr>
<td>Sulfur, %</td>
<td>0.41</td>
<td>0.44</td>
</tr>
<tr>
<td>Magnesium, %</td>
<td>0.17</td>
<td>0.18</td>
</tr>
<tr>
<td>Sodium, %</td>
<td>0.08</td>
<td>0.09</td>
</tr>
<tr>
<td>Potassium, %</td>
<td>0.79</td>
<td>0.83</td>
</tr>
<tr>
<td>Copper, ppm</td>
<td>21</td>
<td>22</td>
</tr>
<tr>
<td>Iron, ppm</td>
<td>194</td>
<td>204</td>
</tr>
<tr>
<td>Manganese, ppm</td>
<td>65</td>
<td>68</td>
</tr>
<tr>
<td>Zinc, ppm</td>
<td>132</td>
<td>139</td>
</tr>
<tr>
<td>Acid Detergent Fiber, %</td>
<td>1.54</td>
<td>1.62</td>
</tr>
<tr>
<td>Aflatoxin, ppb</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Ash, %</td>
<td>6.47</td>
<td>6.79</td>
</tr>
</tbody>
</table>

*Alltech, Inc. Lexington, KY. As reported by the manufacturer. Total nucleic acids are an average of range content (5.2-6.6) reported by manufacturer.
Table 3. Composition and chemical analysis of dietary treatments fed to turkey poults from hatch to 12d.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Control</th>
<th>+ Nupro</th>
<th>Nutrient</th>
<th>Wet Basis</th>
<th>Dry Basis</th>
<th>Wet Basis</th>
<th>Dry Basis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn (yellow)</td>
<td>43.66</td>
<td>43.63</td>
<td>Dry Matter, %</td>
<td>90.32</td>
<td>90.43</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADM Soybean meal</td>
<td>41.94</td>
<td>39.20</td>
<td>Crude Protein, %</td>
<td>28.10</td>
<td>28.61</td>
<td>31.12</td>
<td>31.64</td>
</tr>
<tr>
<td>Poultry meal</td>
<td>5.00</td>
<td>5.00</td>
<td>Acid Detergent Fiber, %</td>
<td>4.43</td>
<td>8.87</td>
<td>4.90</td>
<td>5.38</td>
</tr>
<tr>
<td>Dical phosphate (18.5%)</td>
<td>3.34</td>
<td>3.22</td>
<td>Calcium, %</td>
<td>1.37</td>
<td>1.36</td>
<td>1.52</td>
<td>1.50</td>
</tr>
<tr>
<td>Poultry fat</td>
<td>3.26</td>
<td>3.11</td>
<td>Phosphorous, %</td>
<td>1.06</td>
<td>1.06</td>
<td>1.17</td>
<td>1.17</td>
</tr>
<tr>
<td>Lime stone</td>
<td>0.94</td>
<td>1.00</td>
<td>Sulfur, %</td>
<td>0.34</td>
<td>0.35</td>
<td>0.38</td>
<td>0.39</td>
</tr>
<tr>
<td>DL-Methionine</td>
<td>0.40</td>
<td>0.40</td>
<td>Magnesium, %</td>
<td>0.17</td>
<td>0.16</td>
<td>0.19</td>
<td>0.18</td>
</tr>
<tr>
<td>Lysine</td>
<td>0.34</td>
<td>0.33</td>
<td>Sodium, %</td>
<td>0.12</td>
<td>0.10</td>
<td>0.13</td>
<td>0.11</td>
</tr>
<tr>
<td>Micro salt</td>
<td>0.28</td>
<td>0.27</td>
<td>Potassium, %</td>
<td>0.95</td>
<td>0.89</td>
<td>1.05</td>
<td>0.99</td>
</tr>
<tr>
<td>Choline chloride (60%)</td>
<td>0.22</td>
<td>0.24</td>
<td>Copper, ppm</td>
<td>14.00</td>
<td>17.00</td>
<td>15.00</td>
<td>19.00</td>
</tr>
<tr>
<td>Mineral Premix</td>
<td>0.20</td>
<td>0.20</td>
<td>Iron, ppm</td>
<td>630</td>
<td>647</td>
<td>697</td>
<td>715</td>
</tr>
<tr>
<td>Vitamin Premix</td>
<td>0.15</td>
<td>0.15</td>
<td>Manganese, ppm</td>
<td>182</td>
<td>161</td>
<td>202</td>
<td>178</td>
</tr>
<tr>
<td>NaSeO3 premix</td>
<td>0.15</td>
<td>0.15</td>
<td>Zinc, ppm</td>
<td>141</td>
<td>150</td>
<td>156</td>
<td>166</td>
</tr>
<tr>
<td>L-Threonine</td>
<td>0.11</td>
<td>0.10</td>
<td>Aflatoxin, ppb</td>
<td>4.52</td>
<td>0.00</td>
<td>5.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Nupro</td>
<td>3.00</td>
<td>3.00</td>
<td>Ash, %</td>
<td>7.20</td>
<td>7.38</td>
<td>7.97</td>
<td>8.16</td>
</tr>
</tbody>
</table>

1 Supplied the following per kilogram of feed: 120mg Zn as ZnSO4·H2O; 120mg Mn as MnSO4·H2O; 80mg Fe as FeSO4·H2O; 10mg Cu as CuSO4; 2.5mg I as Ca(IO3)2; 1.0mg Co as CoSO4. 2 Supplied the following per kilogram of feed: vitamin A, 13,200 IU; cholecalciferol, 4,000 IU; niacin, 110mg; pantothenic acid, 22mg; riboflavin, 13.2mg; pyridoxine, 7.9mg; menadione, 4mg; folic acid, 2.2mg; thiamin, 4mg; biotin, 0.253mg; vitamin B12, 0.04mg; ethoxyquin, 100mg; selenium, 0.30mg. The vitamin E premix provided the necessary amount of vitamin E as DL-α-tocopheryl acetate. 3 Selenium premix supplied 3ppm Se as sodium selenate. *Altech Inc., Lexington, KY.
Table 4. Primer characteristics for genes of interest (egfr, igfp-1 and muc2) and house keeping gene (18S rRNA).

<table>
<thead>
<tr>
<th>Gene (Gallus gallus)</th>
<th>Primer*</th>
<th>Accession number</th>
<th>Melting temperature, °C</th>
<th>5’ to 3’ Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mucin 2, oligomeric mucus/gel-forming</td>
<td>MUC2-F</td>
<td>423101</td>
<td>59</td>
<td>AAGCCAGTCTCCACCTTCAGTAA</td>
</tr>
<tr>
<td></td>
<td>MUC2-R</td>
<td>423101</td>
<td>59</td>
<td>TGGTGTTGGAGCAGTGGTT</td>
</tr>
<tr>
<td>Epidermal growth factor receptor</td>
<td>EGFr-F</td>
<td>396494</td>
<td>57</td>
<td>TCATTGAGCGCTACTAGCAACAA</td>
</tr>
<tr>
<td></td>
<td>EGFr-R</td>
<td>396494</td>
<td>57</td>
<td>TGCCCTGCCCCATTTCT</td>
</tr>
<tr>
<td>Insulin-like growth factor binding protein 1</td>
<td>IGFBP1-F</td>
<td>408027</td>
<td>55</td>
<td>CCATAAGCCAGGACAAATCCA</td>
</tr>
<tr>
<td></td>
<td>IGFBP1-R</td>
<td>408027</td>
<td>55</td>
<td>GGTCCCTGTTCCTTTCCATTTT</td>
</tr>
<tr>
<td>18S ribosomal RNA (House keeping)</td>
<td>18S rRNA-F</td>
<td>855269</td>
<td>57</td>
<td>CCGAGAGGGAGCCTGAGAA</td>
</tr>
<tr>
<td></td>
<td>18S rRNA-R</td>
<td>855269</td>
<td>57</td>
<td>CGCCAGCTCGATCCCCAAGA</td>
</tr>
</tbody>
</table>

*Primers for genes of interest were designed using the Primer Express v2.0 software (Applied Biosystems, Foster City, CA). F = forward and R = reverse

Table 5. Conditions of quantitative RT-PCR assay.

<table>
<thead>
<tr>
<th>Steps</th>
<th>Temperature, °C</th>
<th>Time</th>
<th>Number of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>95°</td>
<td>7min</td>
<td>1</td>
</tr>
<tr>
<td>Cycling</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Denature</td>
<td>95°</td>
<td>30sec</td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>51-62°¹</td>
<td>30sec</td>
<td>50</td>
</tr>
<tr>
<td>Extension</td>
<td>72°</td>
<td>30sec</td>
<td></td>
</tr>
<tr>
<td>De-activation</td>
<td>72°</td>
<td>5min</td>
<td>1</td>
</tr>
<tr>
<td>Melt curve</td>
<td>55° + .5° each cycle</td>
<td>10sec</td>
<td>80</td>
</tr>
</tbody>
</table>

¹The actual melting temperature depends on the gene used (Table 3).
Table 6. Effects of *in-ovo* feeding at 23E and post-hatch dietary feeding of Nupro® yeast-extract nucleotides on jejunum villi histomorphometrics of turkey poults from hatch to 12d.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Small intestine weight, g/100g of body weight</th>
<th>Villus height, µm</th>
<th>Crypt depth, µm</th>
<th>Villus surface area, µm²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hatch 4d 12d</td>
<td>Hatch 4d 12d</td>
<td>Hatch 4d 12d</td>
<td>Hatch 4d 12d</td>
</tr>
<tr>
<td><strong>Control</strong></td>
<td>2.92&lt;sup&gt;B&lt;/sup&gt; 9.32&lt;sup&gt;b&lt;/sup&gt; 8.49</td>
<td>504.18&lt;sup&gt;B&lt;/sup&gt; 739.55 910.27&lt;sup&gt;b&lt;/sup&gt;</td>
<td>109.12 139.78&lt;sup&gt;b&lt;/sup&gt; 148.69</td>
<td>49662.7&lt;sup&gt;B&lt;/sup&gt; 83296.5 112725.5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>In-ovo fed&lt;sup&gt;Ω&lt;/sup&gt;</td>
<td>3.54&lt;sup&gt;A&lt;/sup&gt; 9.88&lt;sup&gt;ab&lt;/sup&gt; 8.87</td>
<td>571.89&lt;sup&gt;A&lt;/sup&gt; 709.91 976.32&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>108.88 139.52&lt;sup&gt;b&lt;/sup&gt; 150.75</td>
<td>58501.7&lt;sup&gt;A&lt;/sup&gt; 78663.2 126485.8&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>Nupro&lt;sup&gt;♣&lt;/sup&gt;</td>
<td>- 11.12&lt;sup&gt;a&lt;/sup&gt; 7.52</td>
<td>- 736.71 950.94&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>- 146.09&lt;sup&gt;ab&lt;/sup&gt; 148.13</td>
<td>- 80947.6 114171.0&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>In-ovo fed + Nupro&lt;sup&gt;®&lt;/sup&gt;</td>
<td>- 10.03&lt;sup&gt;ab&lt;/sup&gt; 7.65</td>
<td>- 742.82 1081.70&lt;sup&gt;a&lt;/sup&gt;</td>
<td>- 166.28&lt;sup&gt;a&lt;/sup&gt; 149.95</td>
<td>- 86675.3 147818.7&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>P-value</strong></td>
<td>0.007 0.032 0.067</td>
<td>0.008 0.938 0.052</td>
<td>0.967 0.028 0.990</td>
<td>0.011 0.844 0.022</td>
</tr>
<tr>
<td><strong>SEM(df=23)</strong>&lt;sup&gt;3&lt;/sup&gt;</td>
<td>0.099 0.202 0.185</td>
<td>11.56 20.57 20.80</td>
<td>2.86 2.98 3.08</td>
<td>1598.3 327.6 3883.3</td>
</tr>
</tbody>
</table>

<sup>1</sup>Means represent the average of six replicates (poults) except at hatch. At hatch, means represent the average of 12 poults per treatment. At least ten villi were measured and averaged per poult. <sup>A-B</sup>Differ significantly at P≤0.01. <sup>a-b</sup>Differ significantly at P≤0.5. <sup>♠</sup>Saline injected (0.8mL).<sup>Ω</sup>In-ovo fed with 0.8mL of *in-ovo* feeding solution (Table 5-experiment 2) at 23E. <sup>♣</sup>Altech Inc., Lexington, KY. <sup>3</sup>Pooled standard error of the mean with 23 degrees of freedom. n=6. At hatch, n=12.
Figure 1. Ratio difference between the treatment GOI/HKG vs. the control GOI/HKG in the jejunum of turkey poults *in-ovo* fed at 23E and post-hatch supplemented with or without dietary Nupro® (Alltech Inc. Lexington, KY) up to 12d. GOI = gene of interest. HKG = house keeping gene (18SrRNA).** = *P* ≤ 0.05 and * = *P* ≤ 0.1. *egfr* = epidermal growth factor receptor; *igfbp-1* = insulin like growth factor binding protein – 1; *muc2* = mucin encoding gene 2. Fold change values at each time point represent 6 biological replicates. Ratio change of gene of interest versus house keeping gene was calculated according to the method described by Pfaff (2001).
Table 7. Effects of in-ovo feeding at 23E and post-hatch dietary feeding of yeast-extract nucleotides Nupro® on the growth performance of turkey poults from hatch to 12d.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Body weight, g</th>
<th>Cumulative feed:gain, g:g</th>
<th>Cumulative feed intake, g</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hatch</td>
<td>4d</td>
<td>12d</td>
</tr>
<tr>
<td>Saline-injected 0% Nupro®</td>
<td>69.48</td>
<td>90.01</td>
<td>280.76b</td>
</tr>
<tr>
<td>In-ovo fed 0% Nupro®</td>
<td>68.90</td>
<td>93.01</td>
<td>285.24ab</td>
</tr>
<tr>
<td>Saline-injected 3% Nupro®</td>
<td>69.08</td>
<td>92.16</td>
<td>292.52a</td>
</tr>
<tr>
<td>In-ovo fed 3% Nupro®</td>
<td>69.03</td>
<td>92.15</td>
<td>282.88b</td>
</tr>
</tbody>
</table>

Means of factor effects

In-ovo treatments

Saline-injected | 69.28 | 91.08 | 286.64 | 1.243a | 1.351 | 27.51 | 251.35 |
In-ovo fed | 68.96 | 92.58 | 284.06 | 1.162a | 1.383 | 27.08 | 251.85 |

Dietary treatments

0% Nupro® | 69.19 | 91.51 | 283.00 | 1.203 | 1.355 | 26.45A | 246.54B |
3% Nupro® | 69.05 | 92.15 | 287.70 | 1.202 | 1.379 | 28.14A | 256.65A |

Source of variation

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Pooled SEM (19)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IOF</td>
<td>0.646</td>
</tr>
<tr>
<td>Nupro</td>
<td>0.844</td>
</tr>
<tr>
<td>IOFxNupro</td>
<td>0.692</td>
</tr>
<tr>
<td>Pooled SEM (19)</td>
<td>0.334</td>
</tr>
</tbody>
</table>

1Means represent the average of six replicate cages of 10 poults per treatment, except at hatch. At hatch, means represent the average of 12 cages of 10 poults per treatment. 2Differ significantly at P≤0.05. 3Differ significantly at P≤0.1. *Saline injected (0.8mL). †In-ovo fed with 0.8mL of in-ovo feeding solution (Table 5-experiment 2) at 23E. ‡Alltech Inc. Lexington, KY. §Pooled standard error of the mean with 19 degrees of freedom. n=6.
5.7 REFERENCES


Bohórquez, D., A. Santos, R. Nanney, and P. Ferket. Year. Small intestine development and growth performance of turkey poults is enhanced by dietary supplementation of NUPRO® yeast extract. Proc. Proc. of the XXII World’s Poultry Congress, Brisbane, AUS.


CHAPTER VI

SUMMARY AND CONCLUSIONS
6.1 INTRODUCTION

The adaptation from lipid-rich *in-ovo* nutrient supply to a carbohydrate- and protein-based diet is perhaps the biggest challenge that poultry hatchlings must overcome during their life. This metabolic hurdle occurs at a time when bacteria and exogenous food compounds are introduced to an immature enteric mucosa, which is devoid of adaptive immune surveillance (Cook, 2000; Dibner et al., 1998). Up to 5% of turkey poultts do not survive this critical adaptive challenge during the first 3 to 5 days post-hatch, and many of those that survive continue to struggle with enteric disease, skeletal defects, immuno-incompetence, stunted growth, and poor feed efficiency (Christensen et al., 2003; Noy et al., 2001; Uni and Ferket, 2004). These developmental problems may be ameliorated by genetic manipulation, hatchery and brooding practices, or early nutrition and feeding strategies.

From a nutritional standpoint, early feeding strategies that encourage immediate post-hatch feed intake have been shown to ease this difficult transition by stimulating the morphological maturation and digestive capacity of the intestinal epithelium in poultry hatchlings (Noy et al., 2001; Noy and Sklan, 1999; Uni et al., 1999). Epithelial cells lining the digestive tract are the main interface between ingested and absorbed nutrients; hence, the proliferation and metabolic functions of these cells are directly dependent upon luminal and blood circulation factors (Liévin-Le Moal and Servin, 2006; Shah and Sanderson, 2000; Walker, 2002). This is especially evident when the embryo swallows the amniotic fluid prior to internal pipping, which presents the developing intestinal epithelium with a complex mixture of nutrients, growth factors and other compounds that stimulate enteric development in preparation for hatch (Romanoff, 1960). Consequently, amniotic fluid supplementation of the late-term avian embryo, also known as *in-ovo* feeding\(^1\) (IOF), has emerged as novel early feeding strategy to enhance the enteric maturation of poultry and ease the stress of hatching (Uni and Ferket, 2004).

Moreover, understanding the nutritional needs of rapidly dividing epithelial cells can facilitate experimental design or husbandry approaches. Intestinal epithelial cells have a voracious demand for nutrients and metabolic cofactors, including nucleotides, the building blocks of DNA and RNA (Carver et al., 1991; He et al., 1993; Tanaka et al., 1996). Intestinal epithelial cells cannot synthesize

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enough nucleotides \textit{de novo} to meet their metabolic and developmental demands; instead, salvage mechanisms allow these cells to scavenge essential nucleotides from other sources, including those of dietary origin (Tanaka et al., 1996). There is much evidence from mammalian research about the importance of dietary nucleotide supplementation on the regulation of metabolism, modulation of the immune response, and in gut maturation (Carver and Allan Walker, 1995; Carver et al., 1991; Grimble, 1994; Pickering et al., 1998; Uauy et al., 1994). Moreover, mammalian milk, in particular that of humans, is a rich source of nucleotides for the newborn (Carver and Allan Walker, 1995). Because young hatchlings lack the maternal milk supply, as do mammalian newborns, dietary supplementation of nucleotides may help support the enteric maturation and immune defenses of poultry after hatch.

6.2 RESEARCH SUMMARY

The hypothesis in this dissertation tests that early nutritional strategies, in particular those influencing \textit{in-ovo} nutrition and post-hatch dietary feed intake, enhance the morphological development of the intestinal mucosa in perinatal turkey poults. Since there are no previous reports that delineate the ultra-structural changes in the small intestinal mucosa of the perinatal turkey, it is useful to document the micro-anatomical adaptations that accompany the development of the intestinal mucosa prior to and after hatch, before attempting to introduce modulating treatments. This micro-anatomical survey of the mucosal development was the primary objective of the study presented in chapter II, and some of the observations are discussed below. Longitudinal pre-villus ridges at 15 days of incubation (E) gradually form zigzag patterns that precede the appearance of two parallel lines of villi by 21E. Interestingly, villi topography and size change profoundly between 23E and 25E, perhaps as amniotic fluid is swallowed by the embryo. Villus surface area increases by about 3.5-fold from 23E to 25E, and subsequently continues to increase linearly until reaching a plateau at 8 days post-hatch. Overall villi shape also changes from finger-like projections before hatch to leaf-like projections by 12 days post-hatch. At this time, microbial colonization of the gut is evident, and some organisms, such as segmented filamentous bacteria, likely influence villi microanatomy development. Profound morphological adaptations occur in the gut epithelium during perinatal
development of the turkey embryo or poult, which contribute to the unique specialization and compartmentalization of the small intestine in preparation for an influx of exogenous nutrients.

The ultra-structural characterization of the intestinal epithelium in the perinatal turkey also raised some questions about when bacteria colonization in the avian digestive tract first begins. In chapter III, we hypothesized that microbial colonization of the intestinal epithelium in turkeys occurs in-ovo during the last stages of embryonic development. The presence of microbial colonies in the ceca of turkey embryos was surveyed from 15E up to 27E, using scanning electron microscopy (SEM). In addition, the potential types of bacteria present in the luminal contents of late-term turkey embryos (25E) were characterized using 16S rRNA bacterial profiling and terminal restriction fragment length polymorphism (TRFLP) analysis. SEM analysis of ceca samples revealed micro-colonies of bacteria from 17E through 27E. These micro-colonies appeared to be composed of at least 3 different shapes of bacteria-like microorganisms, and the complexity of these micro-colonies seemed to increase with age. These observations were supported by TRFLP analysis, which confirmed the presence of a few bacterial species in the ceca of 25E embryos, including *Bacillus, Lactobacilli, Pseudomonas*, and several uncultured bacteria. This microscopic and molecular biological evidence confirms that microbial colonization of the gut of turkeys begins during embryonic development, several days prior to hatch.

In Chapter IV, the effects of in-ovo feeding on the morphological maturation of the small intestinal epithelium during the perinatal period were investigated. Although the effects of IOF on the digestive capacity of late-term chicken and turkey embryos have been previously studied, the research reported here was the first to document the influence of IOF on the ultra-structural maturation of the small intestinal epithelium of turkey embryos and poults. Because plasma triiodothyronine (T3) is a potent stimulator of morphological maturation of the intestinal mucosa, the positive effects of IOF on mucosal development was considered to be related to a concomitant rise in plasma T3 levels. IOF consistently enhanced villus height and villus surface area up to hatch in comparison to controls, and these results agreed with the IOF effects on T3 circulating levels. Radioimmunoassay analysis of plasma T3 showed higher T3 activity in IOF-treated embryos at 27E and poults at hatch and 12d as
compared to saline-injected controls. Microarray analysis of gene expression in the jejunum of 4 day-old turkey poults also showed up-regulation of intestinal disaccharidases, epithelial cell growth, thyroid receptors, and innate immune response pathways in IOF poults. Moreover, by 11 days of age, IOF birds had 5% heavier (P<0.05) body weights and 6% (P<0.05) better cumulative feed conversion ratios. *In-ovo* feeding of turkey embryos clearly accelerates the morphological maturation of the jejunum mucosa up to hatch. These effects involve localized up-regulation of genes mediating epithelial cell growth and a systemic increase of plasma T<sub>3</sub>. This study provides evidence that amniotic fluid supplementation of turkey embryos can have a lasting impact on intestinal morphology and function.

Because IOF consistently accelerates maturation of the intestinal epithelium in hatchlings, we considered if these positive developmental responses could be further enhanced or maintained by post-hatch dietary supplementation of nucleotides from yeast extract (Nupro<sup>®</sup>, Alltech Inc.) which stimulate enteric development (Chapter V). Besides assessing the treatment effects on jejunum histo-morphometrics, we also evaluated the treatment effects on the expression of genes involved in mucosal maturation (*egfr*, *igfbp-1* and *muc2*). These genes were chosen based on the previous microarray analysis discussed in chapter IV. At hatch, *in-ovo* fed turkey poults had significantly (P<0.05) higher villus height and villus surface area by ~13 and ~18% respectively, than the controls. At 4d, dietary nucleotide supplementation only significantly (P<0.05) increased crypt depths by ~18%, compared to controls. At 12d, dietary nucleotide supplementation to *in-ovo* fed turkey poults significantly (P≤0.05) increased villus height (~19%) and villus surface area (~31%), compared to controls. We also observed in this study that IOF up-regulates the expression of *egfr*, and *muc2* prior to hatch; though, after hatch, dietary supplementation of nucleotides to *in-ovo* fed turkey poults increased the expression (P≤0.1) of *egfr* and *igf-p-1 at 12d, compared to controls. Moreover, regardless of the prenatal treatment, dietary supplementation of yeast-extract nucleotides during the neonatal period significantly (P≤0.05) increased (~5%) cumulative feed intake up to 12 days of age. Evidently dietary supplementation of yeast extract nucleotides enhanced the post-hatch morphological maturation of the small intestinal epithelium, and this effect was more pronounced in
IOF poults. The positive effects of dietary nucleotides were apparently related to increased appetite of turkey poults.

6.3 CONCLUSIONS

In light of the evidence presented above, the following conclusions have been drawn from this dissertation. (1) Profound ultra-structural adaptations take place in late-term turkey embryos in preparation for life post-hatch. These adaptations coincide with the imbibing of the amniotic fluid by the embryo. (2) Microflora colonization of the avian gut evidently begins well before hatch. (3) Amniotic fluid supplementation consistently stimulates the morphological and ultra-structural maturation of the small intestinal epithelium at hatch. These effects appeared to be mediated by up-regulation of genes involved in epithelial cell proliferation, as well as an increase in plasma triiodothyronine levels. (4) Dietary supplementation of nucleotide-rich yeast-extract enhances the post-hatch morphological maturation of the small intestinal epithelium, and its effects are more pronounced among in-ovo fed turkey poults. The positive effects of nucleotides-rich yeast-extract are apparently associated with increased appetite of starting turkey poults.

6.4 IMPLICATIONS AND FUTURE DIRECTIONS

Early nutrition has a tremendous influence on the adult phenotype of any living organism. In honeybees (Apis mellifera), for example, adult fertile queens and sterile workers originate from genetically identical larvae following differential feeding with royal jelly (Kucharski et al., 2008). Larvae raised to become worker bees are fed small quantities of royal jelly initially and then honey subsequently, whereas those destined to become queen bees are raised bathed in royal jelly. Consequently, queen bees develop into an adult phenotype that has a much larger body frame with fully developed sexual organs and a distinctive social behavior than the worker honeybee phenotype (Foret et al., 2009). In vertebrates, the embryonic imbibing of amniotic fluid during late term pregnancy is a process with analogous effects. Deficiencies in this essential fluid during fetal development are strongly associated with gastrointestinal tract dysfunction. For instance, maternal
diabetes can alter the composition of amniotic fluid and is often correlated with neonatal disorders that involve immature development of the digestive tract, such as necrotizing enterocolitis (Koski and Fergusson, 1992; Neu, 1996). We have shown here that profound ultra-structural adaptations occur in the intestinal mucosa of turkey embryos around the time that the amniotic fluid is swallowed. Such changes are perhaps essential to prepare the digestive tract for post-hatch feed intake and nutrient utilization. Moreover, amniotic fluid supplementation accelerates the morphological maturation of the intestinal epithelium and exerts long-lasting influences on the expression of genes that mediate mucosal maturation and nutrient utilization during early life. Interestingly, in mammals, fetal swallowing of amniotic fluid has been proposed as the trigger for the onset of appetite and thirst (El-Haddad et al., 2004), perhaps through the modulation of gut taste receptors that have recently been described as major mechanisms for food intake regulation (Jang et al., 2007; Jeon et al., 2008).

The ability to regulate feed intake and nutrient partitioning for metabolic processes have been at the forefront of research in metabolic disorders (e.g. diabetes type II, obesity, hypertension, cardiovascular disease). In the US alone, about 47 million people suffer from at least one of these disorders and, what used to be considered a disorder of adults only, now affects about 10% of US children under the age of 10 (Cook et al., 2008; Flegal et al., 2002). Furthermore, metabolic disorders have expanded to include livestock animals. About 11% of US poultry have skeletal abnormalities, and with 8 to 9 billion poultry produced yearly, these problems lead to losses of hundreds of millions of dollars annually due to increased mortality rates and suppressed growth and feed conversion (Sanotra et al., 2001; Uni and Ferket, 2004). However, little research has been done on how prenatal nutrition may modulate the onset of thirst and appetite mechanisms in birds and mammals. Because of inherent difficulties of studying the mammalian embryo (e.g. continuous maternal input and the invasiveness of the procedure) the avian embryo is a more suitable model for understanding early life programming. The studies presented in this dissertation are an example of the potential for early life “programming” through amniotic fluid modulation. Nutrient supplementation of the amniotic fluid consistently accelerates enteric maturation of the perinatal turkey poult, which may alleviate aberrations of early growth.
6.5 REFERENCES


