Effects of Probiotic and Prebiotic Supplementation in Turkey Poults on Intestinal Morphology and *MUC2* Gene Expression

THESIS

Presented in Partial Fulfillment of the Requirements for the Degree Master of Science in the Graduate School of The Ohio State University

By

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Graduate Program in Animal Sciences

The Ohio State University

2014

Master's Examination

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ABSTRACT

Ohio has a 158 million dollar turkey industry. Gut growth and development occurs first before the turkey can realize its full muscle producing potential. Alternative and adjunctive approaches to decreasing the use of feed grade antibiotics are becoming an important area of research due to increasing consumer and legislative concerns with antibiotic resistance. Probiotics or supplemental dietary commensal microbes is one such potential approach as they can colonize the intestine, particularly in young animals with a relatively naïve intestine microbiome. Intestinal mucosa is made up of mucin glycoproteins that play a key role in preventing the attachment and colonization of pathogenic bacteria. These proteins are made up of a protein backbone that is coded for by the *MUC* gene family. At hatch, the turkey intestine is relatively aseptic and therefore vulnerable to bacterial colonization from both commensal and pathogenic microbes. In this study, we determined the expression of *MUC2*, which codes for a secretory gel-forming mucin that is predominantly found in the small and large intestine, from immediately post-hatch through day 11 of age in poults fed a conventional starter diet, the starter diet supplemented with two commercial probiotics (A and B), or the starter diet supplemented with a commercial prebiotic. This was done by comparing the *MUC2* transcription levels to the transcription level of a housekeeping gene. Multiple potential housekeeping genes were investigated and one, *RPS13*, was found to be stably expressed across all ages and treatments in the turkey poult. The effects of the supplemented diets on intestinal development were also analyzed. While *MUC2* transcription increased with

age, there were no significant effects due to diet. The intestinal parameters of villus height, area and crypt depth were all increased with supplementation of probiotic B and the prebiotic.

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Loeffler, Stephanie L.,M. Lilburn, M.Wick. 2013 Mucin 2 gene expression in the young poult. Poultry Science Association Annual Conference. San Diego, CA.

Loeffler, Stephanie L., M.Lilburn, M.Wick. 2014. The effect of probiotic or prebiotic supplementation on gut morphology in young turkey poults. Poultry Science Association Annual Conference. Corpus Christi, TX.

Fields of Study

Major Field: Animal Sciences

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LITERATURE REVIEW

The epithelial cells lining the villus of the gastrointestinal tract are covered by a mucus layer which provides protection, lubrication and aids in the transport of luminal contents. The mucus layer consists largely of water interspersed with immunoglobulins, electrolytes, enzymes, sloughed cells and glycoproteins called mucins (Satchithanandam et al., 1990). Mucins are composed of a peptide backbone, coded by the MUC gene family, which contains alternating glycosylated (major domain) and non-glycosylated domains which are sometimes referred to as the minor domain (Deplancke and Gaskins, 2013). The major domain is rich in serine, threonine and proline and consists of densely glycosylated O-linked oligosaccharide side chains. The minor domain is non-glycosylated, is rich in cysteine and located at the C and N terminal regions of the protein (Forder et al., 2007).

Goblet cells are present throughout the small and large intestine and are responsible for the production and secretion of mucins (Tellez et al., 2010). These cells arise via mitosis from stem cells at the base of the crypt or from poorly differentiated cells in the lower crypt. The goblet cells migrate from the crypt toward the villus tip and are subsequently sloughed off into the lumen, a process that takes 2 to 3 days (Uni et al., 2003). Secretion from goblet cells occurs by two distinct processes, baseline secretion, or simple exocytosis, and compound exocytosis (Deplancke and Gaskins, 2013). In baseline secretion, mucin glycoproteins are assembled and stored in granules before they are

secreted from the apical surface (Uni, 2006). If exposed to a mucin secretogogue, goblet cells experience compound exocytosis, which is an accelerated secretory event leading to the acute release of stored granules. Various biological agents can act as mucin secretagogues including hormones, cytokines, lipids and neuropepetides (Deplancke and Gaskins, 2013).

There are three categories or classifications of mucins: secretory gel-forming (*MUC2, MUC5AC, MUC5B, MUC6*), secretory soluble (*MUC7*), and membrane-bound (*MUC1, MUC3, MUC4* and *MUC12*). Secretory gel-forming mucins are large in size, have a high carbohydrate content and exhibit both viscous and elastic characteristics (Uni, 2006). These mucins contain at least one repeating domain which is rich in proline, threonine and serine as well as a cysteine rich domain. The predominant gel-forming mucin in the small and large intestine is *MUC2*. Membrane-bound mucins, or receptor mucins, are involved in signal transduction, oncogenic processes and gel formation (Jiang et al., 2013).

Mucins are further classified into neutral and acidic subtypes, with the latter being further differentiated by their sulfated or non-sulfated side chains. Neutral mucins appear to be the major subtype within gastric mucosa while acidic mucins are present throughout the intestinal epithelium and are predominant within the large intestine (Deplancke and Gaskins, 2013). The oligosaccharide side chains of mucins are often terminated with sialic acid (non-sulfated mucins) or sulfated sugars. The terminus of the side chain accounts for the polyanionic and visco-elastic properties of mucins (Hino et al., 2012). The composition of gut mucin largely depends on MUC gene expression (protein

backbone) and epithelial glycosyltransferase activity which is required for the transfer of monosaccharides to the protein backbone (Tsirtsikos et al., 2012).

The expression of different mucins as defined by their unique protein backbones and different glycosylation patterns can vary within tissues. For example, *MUC2* has been observed to be widely expressed in the small intestine and the colon whereas *MUC5AC* is mainly expressed in the stomach. This is the case in poultry where *MUC5AC* is more highly expressed in the proventriculus compared with the small intestine (Forder et al., 2007, Smirnov et al., 2004). Mucin gene expression is regulated by cytokines, bacterial products and growth factors. Mucin biosynthesis is influenced by factors that affect protein synthesis in general, the differentiation of precursor cells into mature goblet cells, the rate of epithelial cell migration from the proliferating zones within the crypt, and any disruption in the glycosylation of the oligosaccharide side chains (Smirnov et al., 2004).

Intestinal development in poultry is different from what is observed in mammals (Geyra et al., 2001). The gastrointestinal tract in the hatchling is functionally immature, despite going through significant morphological, cellular and molecular changes towards the end of incubation (Uni, 2006). During the first week post-hatch, the chick small intestine grows rapidly with significant increases in villus height and crypt depth as well as an increased capacity to digest and absorb carbohydrates (Smirnov et al., 2006). At hatch, the crypt has few cells and invagination is not complete but by 48 hours post-hatch, invagination was observed to be complete in all three intestinal segments (Geyra et al., 2001). Crypt development is a crucial step in intestinal maturation because stem cells proliferate in the crypt and as they migrate up the villus, they differentiate into

enterocytes and mucin producing goblet cells. Intestinal mass increases by a magnitude of seven to ten while length increases by a magnitude of two to four times within the first twelve days posthatch in poults, suggesting that the mucosal structures also are developing rapidly during that time. A similar growth pattern has been observed in chicks (Uni et al., 1999).

Mucin producing cells can be observed in the chick small intestine beginning at embryonic day 17 and by day 18 of embryogenesis, 13% of all epithelial cells are goblet cells but they only produce acidic mucin. Immediately after hatch through day 7 post-hatch, however, there is an equal proportion of goblet cells producing acidic and neutral mucins (Uni et al., 2003). At hatch, goblet cells made up 23% and 26% of the epithelial cells in the jejunum and ileum, respectively and this proportion remained constant during the first 7 days of age (Uni et al., 2003).

Forder et al. (2007) reported that at day 1 post-hatch in chicks, goblet cells within the jejunum produced only sulfated mucins but by day 4, non-sulfated (sialic acid containing) mucins appeared. With the yolk sac becoming depleted of maternal antibody reserves during the first few days post-hatch, the stimulation of goblet cells to alter their mucin glycosylation pattern may help the hatchling fight bacterial challenges. Acidic mucins at early developmental stages may act as an innate barrier against bacterial translocation in that sulfated mucins appear to be less susceptible to degradation by bacterial glycosidases (Uni et al., 2003). At day 4 post-hatch, there is an upregulation of mRNA expression of proteins associated with the immune system, such as antimicrobial peptides and pro-inflammatory cytokines. Cytokines are known to stimulate mucin production and goblet cell proliferation as well as alter mucin glycosylation (Forder et al.,

2007). The synthesis and secretion of different mucin species is not equal. Sulfated mucins are characteristic of immature goblet cells and as they migrate up the villus the mucins produced become increasingly sialated (Tellez et al., 2010). The changes that are observed in mucin composition by day 4 post-hatch could be partially due to the increased development of the immune system (Forder et al., 2007).

The presence, or lack thereof, of feed in the gastrointestinal tract of a chick plays an important role in mucin synthesis and composition. Smirnov et al. (2004) reported that depriving 28 day old chicks of food and water for 72 hours increased expression of mucin mRNA in the duodenum and jejunum as well as increasing the glycoprotein concentration in both sections. The thickness of the mucous layer was similar throughout the small intestine, but was decreased in those birds that had been starved. The thickness of the mucous layer is determined by the rate of mucin secretion and degradation. There was an observed enlargement of goblet cells in the starved chicks, which is indicative of an accumulation of mucin. Since both mucin mRNA levels and mucin glycoprotein concentration were increased in starved birds, the decreased thickness may be due to a disruption in the secretion of the mucin glycoproteins.

Smirnov et al. (2006) reported that in ovo feeding (IOF) of carbohydrates increased mucin mRNA expression at hatch compared to controls who had not received IOF. The presence of food in the alimentary canal is crucial to normal mucosal function and triggers the protein kinase C (PKC) pathway. In the intestine, glucose transport through the SGLT1 receptor activates the PKC-dependent pathway. The PKC pathway has been shown to increase *MUC2* and *MUC5AC* in cultured colonocytes, so it is possible that the enhanced expression of mucin in IOF embryos is due to the activation of

PKC-dependent pathways by glucose (Hong et al., 1999). The composition of mucin was altered as well within ovo carbohydrate feeding, as there was an increased proportion of acidic mucins in mucin producing goblet cells. This may be due to the fact that goblet cells can utilize glucose for conversion to nucleotide-sugars followed by the incorporation into the carbohydrate portion of the mucin glycoproteins (Neutra and Leblond, 1966).

Intestinal microbial populations alter mucin properties, either directly or indirectly. Bacteria may directly affect goblet cell functions through the local release of bioactive factors. On the other hand, goblet cells may be affected and altered in response to host derived factors produced by activated epithelial or lamina propria cells after their contact with intestinal bacteria. Mucin oligosaccharide side chains can act as a source of carbohydrate, peptides and exogenous nutrients for many bacterial species. Those bacteria that are capable of colonizing the mucous layer of the small intestine can avoid rapid expulsion and would therefore have an advantage over those species that cannot colonize the mucous layer. Commensal bacterial colonization represents a symbiotic relationship with the host and it is not surprising that mucus secretion is typically enhanced in response to intestinal microbes (Deplancke and Gaskins, 2001).

Studies in germ free animals illustrate the fact that bacterial populations can and do affect mucin production and composition. Goblet cell numbers in germfree rodents are decreased and smaller in size compared to conventionally raised mice. This can lead to a mucus layer in conventionally raised rodents that is twice as thick as in germfree rodents, indicating greater mucin production (Kandori et al., 1996). Water holding capacity of cecal mucins is also altered in germfree rodents as illustrated by cecal enlargement. This

is thought to reflect the absence of mucin degrading bacteria rather than an increase in mucin production as cecal mucins are rapidly degraded and morphology normalizes when commensal bacteria are introduced (Gordon et al., 1972; Hoskins and Boulding, 1981; Gustafsson and Carlstedt-Duke, 1984).

The composition of mucin is also altered under germfree conditions. The ratio of neutral to acidic mucins in the colon is higher in germfree rodents and sulfomucins seem to increase at the expense of sialylated mucins. Sharma and Schumacher (1995) reported fewer sialylated mucins in the small intestine of germfree rats when compared to conventionally raised rats. Sialomucin containing goblet cells were also greater in number in the large intestine of rats raised under germfree conditions and inoculated with microbes derived from human fecal material (human- microbiota-associated) when compared with germfree rats. Additionally, sulfomucin-containing goblet cells were greater in the human-microbiota-associated rats than in conventional rats but were reduced in number in the large intestine. The microflora population of the chick small intestine primarily consists of facultative anaerobes such as *Streptococcus, Staphylococcus, Lactobacillus and E. coli,* but in the duodenum and the ileum there are also large numbers of anaerobes, accounting for 9-39% of the total microbial population (Smirnov et al., 2005) .

Smirnov et al. (2005) reported that mucin mRNA was increased in the jejunum with the addition of a probiotic, a viable, nonpathogenic microorganism that is able to reach the intestines in sufficient numbers to be of benefit to the host, and that mucin glycoprotein concentration was also increased. The probiotic increased the proportion of *Lactobacillus* species in the ileum by147%. An antibiotic growth promoter was

supplemented as a separate treatment and this increased the levels of *Bifidobacterium*. The increased proportion of *Bifidobacterium* could explain the decreased levels of mucin glycoprotein in the duodenum without a corresponding decrease in mucin mRNA. This suggests that there was an increased rate of microbial mucin degradation and *Bifidobacterium* possess mucin degrading activity. Mucins are resistant to the proteolytic enzymes in the gastrointestinal tract, so the role of bacteria in mucin degradation is crucial.

CHAPTER 1: VALIDATION OF HOUSEKEEPING GENES

ABSTRACT

Housekeeping genes are used in quantifying the transcription levels of a gene of interest. However, due to the fact that a suitable housekeeping gene must be stably expressed across all ages and treatments for a given species, finding one is a challenge. In this study, we evaluated four potential housekeeping gene primer sets for use in the turkey poult to quantify the expression of the *MUC2* gene. The *RPS13* and *TBP* primer sets were determined to be suitable for use in the turkey poult.

INTRODUCTION

Quantifying gene expression requires normalizing the gene of interest against at least two housekeeping genes. A housekeeping gene should be expressed at relatively stable levels across all ages and experimental treatments. The evaluation of multiple housekeeping genes is prudent, as some are more stable under different treatment conditions than others. There are few gene sequences available in the turkey, so finding and designing housekeeping genes requires literature searches for genes used in related species and using those species' sequences to design primer sets, by degenerative primer design based on either a multiple sequence alignment or by designing a primer set from a single species' sequence. In order to accurately determine relative gene expression multiple potential housekeeping genes need to be evaluated for stable expression across

treatments. The goal of this study was to validate at least two housekeeping genes in the turkey

MATERIALS AND METHODS

A. Primer design

A *MUC2* gene primer set was designed based on the report of Jiang et al. (2013). A primer set for *RPS13* (Ribosomal Protein S13), which encodes for a ribosomal protein that is a component of the 40S subunit, was designed based on the duck sequence (XM_005015078.1) and was obtained from Dr. Kichoon Lee (The Ohio State University).

B. Degenerate primer design

Housekeeping genes are used to normalize the expression of a target gene and it is becoming increasingly apparent that more than one housekeeping gene should be used for a given set of reactions. *HPRT1* and *HMBS* are two housekeeping genes that have been validated for chicken samples in our lab so we chose them for this study. Since the sequences for the turkey are not available for the *HPRT1* and *HMBS* genes, four to five closely related sequences were found using the NCBI database for *HPRT1* and those sequences were subsequently run through Clustal W, a multiple alignment program. Once the sequences were aligned, homologous sections of greater than 15 base pairs (bp) were targeted and primers designed from those sections. There was a one base pair mismatch in the homologous section used for the forward primer, with the chicken having a T and the other four species having a C. Two degenerate primers were designed, one with a T

and the other with the C at that position. The forward primer that worked had a cytosine in that position. Primers for *HMBS* were designed based on the chicken sequence (XM_417846.4) using NCBI Primer Blast.

TATA binding protein *TBP* codes for a transcription factor that binds to the TATA box. *TBP* was used as a housekeeping gene for the chicken by Forder et al. (2012). The sequence for TBP is not available in the turkey so primers were designed using the chicken sequence (NM_205103). The primer sequences and amplicon sizes for all genes proposed for this study are show in Table 1.

Table 1: Primer sequences and amplicon sizes for all genes.

Gene	Direction	Sequence	Amplicon size
MUC2	F	ATTGTGGTAACACCAACATTCATC	134bp
	R	CTTTATAATGTCAGCACCAACTTCTC	
HPRT1	F	GAAGATATAATTGACACTGG	145bp
	R	CAAATCCAACAAAGTCTGG	
HMBS	F	ATTCAGACTGACAGCGTGGTT	499bp
	R	CCCAGCCCATTCTCTTCAGT	
TBP	F	ATGAATGCGACCGTGTCACT	479bp
	R	AGGCTGAATGTTGCCAAGGA	
RPS13	F	CAAGAAAGCTGTTGCTGTTCG	169bp
	R	GGCAGAAGCTGTCGATGATT	

C. Sampling

Turkey poults were randomly selected on day 0, 4 and 10 posthatch for intestinal sampling. Five poults per day were euthanized via cervical dislocation and the lower small intestine from Meckle's Diverticulum to the ileo-cecal junction was removed. The intestinal segment was flushed with distilled water and immediately placed into a sterile 15mL conical tube and submerged in liquid N. Each sample was stored at -80^{o}C until RNA isolation.

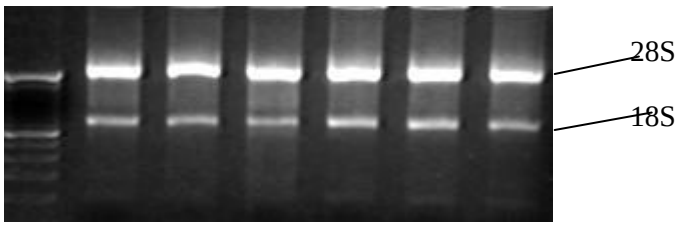

Figure 1: 1.5% agarose gel electrophoresis of RNA isolated from day of hatch intestinal samples in lanes 2-7.

D. RNA Isolation

RNA isolation was performed using a Norgen ® Animal Tissue Isolation Kit. Samples were taken from -80^{o} C freezer and immediately submerged in liquid N. They were pulverized in a frozen mortar with a frozen pestle, all while staying submerged in liquid N. Pieces weighing approximately 10mg were placed into a sterile cryovial containing 300uL of the Lysis Buffer, weighed and the process was repeated until 30mg of sample was obtained. The pooled sample was further pulverized with a homogenizer

for 30 seconds to a minute. The homogenized sample was transferred to a sterile microcentrifuge tube and 600uL of RNase free water was added along with 20uL of reconstituted Proteinase K. The sample was incubated at 55^{o}C in a water bath for 15 minutes, vortexing every 5 minutes. After incubation was complete, the sample was placed in a microcentrifuge for 1 minute at 14,000 x g to pellet any cell debris. The supernatant was removed and transferred to a new microcentifuge tube.

An aliquot of 450ul of 95% ethanol was added to the supernatant and the column provided with the kit was assembled. An aliquot of 650uL of the ethanol-supernatant mixture was added to the top of the column and spun down for 1 minute at 14,000 x g and the flow through, consisting of any organic matter and remaining cell debris, was discarded. This step was repeated one to three times until all the ethanol-supernatant mixture had passed through the column.

An enzyme incubation buffer (100ul) was then added to the column, along with 15uL of DNase 1 and spun down for 1 minute at 14,000 x g. The entire flow through which consisted of the DNase and the enzyme incubation buffer (115 uL) was pipetted back onto the column and incubated at room temperature for 15 minutes. A Wash Solution (400 uL) was added to the column and the column was spun down at 14,000 x g for one minute. The flow through, which consisted of any DNA and DNase enzyme was discarded, an additional 400uL of Wash Solution was added to the column and spun down again at 14,000 x g for one minute. The flow through was discarded and the column was spun for two minutes at 14,000 x g to thoroughly dry the column.

The column was placed into a clean elution tube (provided with the kit) and 50uL of the Elution Buffer (RNase free water) was added to the column and then spun down at 200 x g for two minutes followed by a one minute spin at 14,000 x g. This process was repeated once more for a second elution in a separate tube. The RNA samples were stored at -80^{o}C until cDNA synthesis.

RNA samples were applied to a 1.5% agarose gel for electrophoresis to check the integrity of the 28s and 18s bands (**Figure 1**). Nanodrop measurements were also taken to determine the concentration as well as 260/230 and 260/280 ratios. RNA absorbs at 260nm while proteins absorb at 280nm and guanidine, which is commonly used in column based isolation kits, absorbs at 230nm. A low 260/280 and/or 260/230 ratio indicates contamination.

E. cDNA Synthesis and RT-PCR

The synthesis of cDNA was done using Invitrogen ®M-MLV ReverseTranscriptase and anchored OligodT (OligodTA). Each RNA sample was diluted to 0.2ug/uL and 5ul of diluted RNA was added to a PCR tube. A 1uL aliquot of OligodTA, 1uL of 100uM dNTP and 5ul of RNAse free water was added to each tube as well. The tubes were then incubated at 65^{o}C for 5 minutes and then chilled on ice for 30 seconds. An aliquot of the 5X 1st Strand Buffer (4 uL) and 2uL of 0.1M DTT were added to each tube, along with 1uL of RNase free water to obtain a volume of 19uL. Each tube was then incubated at 37^{o}C for two minutes and 1uL of the M-MLV RT was added to each tube and mixed by pipetting up and down 4 to 5 times. The tubes were then

incubated at 37°C for 50 minutes, followed by 15 minutes at 70°C. Each sample was stored at 4°C until RT-PCR was performed.

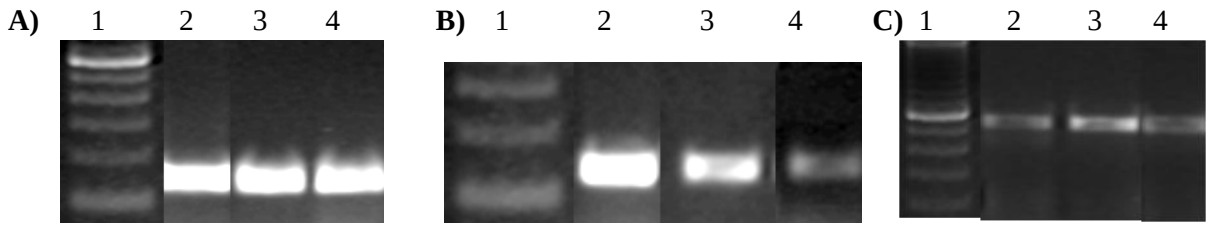

Figure 2: 2.0% agarose gels showing the RT-PCR products of *RPS13* (A), *MUC2* (B), and *TBP* (C) primer sets. Lane 2 represents day 10 posthatch poult intestinal cDNA, with lane 3 being day 4 posthatch and lane 4 being day of hatch, or embryonic day 28.

RT-PCR was performed using each primer set and cDNA samples from each age using GeneMate Taq polymerase. A master mix consisting of 10X NH_4 buffer, 50mM M_gCl_2, 100mM dNTPs, Taq polymerase, RNase free water was used for each primer pair. A microliter of each 100uM primer (forward and reverse) was added to the master mix. There was a denaturing phase at 95°C for 5 minutes, 35 cycles of 95°C for 30s followed by 59°C for 30s and 72°C for 1 minute, then an elongation phase for 10 minutes at 72°C. All samples were stored at 4°C. Each RT-PCR product was subsequently run on a 2.0% agarose gel via gel electrophoresis at 120V to determine primer success.

RESULTS

When RT-PCR was done on cDNA samples from embryonic day 28 and post hatch days 4 and 10, all primer sets produced a single amplicon of the expected size. In **Figure 2,** the amplicons produced using *RPS13* (**Figure 2.A**.), *TBP* (**Figure 2.B**.) and *MUC2* (**Figure 2.C**.) are shown with the cDNA samples from the three different ages.

The *HMBS* and *HPRT1*primer sets also produced an amplicon of the expected size when run in a PCR reaction with cDNA from samples at all three ages. The results from these two primer sets were not consistent, however, as more RT-PCR reactions were run on more samples. Multiple bands were observed when agarose gel electrophoresis was done with the *HPRT1* primers. Approximately 60% of the samples on a given day would produce an amplicon of 499bp when run in a PCR reaction with *HMBS*. Due to the variation observed with these two genes, we opted not to use them as our housekeeping genes.

CHAPTER 2: INTESTINAL MORPHOLOGY and *MUC2* EXPRESSION

ABSTRACT

Two separate experiments were carried out to investigate the effects of age and dietary treatments on *MUC2* expression and intestinal morphology. The first experiment, referred to as the ontogeny study looked at the effect of age and the second experiment, referred to as the probiotic study, looked at the effect of four different dietary treatments. Turkey poults were sampled on day of hatch and days 4 and 10 post-hatch in the ontogeny study. In the probiotic study, birds were assigned to one of four diets: Control, Probiotic A, mannan oligosaccharide (MOS) or Probiotic B and also sampled on days 4, 9 and 11 post-thatch. In both studies, we hypothesized that as the birds increased in age, histological morphology measurements of villus height, area and crypt depth will increase together with an increase in *MUC2* gene expression. In Experiment 2, the hypothesis tested was that supplementation with probiotics and the MOS would increase *MUC2* expression and histological morphology measurements. From days 4 to 10 post-hatch, villus height and villus area were increased in Experiment 1 while in Experiment 2, supplementing Probiotic B increased villus height and villus area. Crypt depth was increased with Probiotic B and MOS supplementation, while Probiotic A supplementation led to a decrease in crypt depth. There was a numerical increase in *MUC2* expression with age in Experiment 1 ($P < .16$). In the probiotic study there was a significant increase with age but no differences due to dietary treatment

INTRODUCTION

Optimizing early growth and development of the intestine in young poults is crucial to optimizing the growth of a commercial turkey. The gastrointestinal tract in the hatchling poult is functionally immature, despite going through significant morphological, cellular and molecular changes toward the end of incubation (Uni, 2006). During the first week post-hatch, the small intestine in poults grows rapidly with significant increases in villus height and crypt depth as well as an increased capacity to digest and absorb carbohydrates (Uni et al., 1999, Applegate et al., 2005; Smirnov et al., 2006). At hatch, the crypt has few cells and invagination is not complete but by 48 hours post-hatch, invagination is observed to be complete in all three intestinal segments (Geyra et al., 2001). Crypt development is a crucial step in intestinal maturation because stem cells proliferate in the crypt and as they migrate up the villus, they differentiate into enterocytes and mucin producing goblet cells. This suggests that the mucosal structures are also developing rapidly during that time.

The epithelium of the fully developed intestine is lined with a layer of mucus that is made up of mucin glycoproteins, water, various macromolecules, resident microorganisms, and sloughed cells. The thickness of the mucus layer varies along the length of the intestine and is a protective barrier against colonization by harmful bacteria because a pathogen must first pass through the mucus layer to reach the underlying epithelial cells (Smirnov et al., 2005). The glycoprotein portion of the mucus layer, mucin, is secreted by goblet cells in the villi of the intestinal epithelium and is thought to be protective because the glycoprotein provides beneficial bacteria with binding sites thus preventing the colonization of harmful bacteria (Forder et al., 2007). This implicates

MUC2 expression in the development of a healthy gut, as it is the primary mucin expressed in the small intestine.

One factor that affects mucin protein expression is the mucin-bacterial interaction. Not only is mucin a site for adhesion and competition among bacteria, but it can also serve as an energy source to some microbes (Dohrman et al., 1998). To date, there are no reports in the literature describing the ontogeny of mucin gene expression in turkey poults. While the effects of probiotics on mucin proteins have been investigated previously, there are no reports on this relationship during the initial, critical stages of intestinal maturation. It has been shown that bacterial populations can be altered by the diet and that intestinal bacteria interact with mucin (Deplanke and Gaskins, 2001; Smirnov et al., 2005; Tellez et al., 2010) Therefore, it is reasonable to postulate that a microbiome rich in these populations may have a positive impact on the development of a healthy gut and the growth of a young poults.

The objective of this study was to quantify mucin gene expression during the maturation process of the small intestine and determine the extent to which mucin expression and intestinal morphology can be altered by supplementing probiotics and mannan oligosaccharides. We hypothesize that probiotic and mannan oligosaccharide supplementation will increase *MUC2* expression, as well as overall intestinal development as determined by increased villus height, villus area and crypt depth.

MATERIALS AND METHODS

A. Birds and Diets

In the ontogeny study, fertile commercial turkey eggs were obtained from Cooper

Farms, Oakwood, OH and incubated at the OARDC Poultry Research Farm. Once hatched, poults were fed a conventional starter diet formulated to meet or exceed the NRC (1994) nutrient recommendations for turkeys.In the probiotic study, fertile turkey eggs (n=200) were incubated and at hatch, 160 poults were randomly assigned to one of four dietary treatments. A control diet was formulated according to the NRC (1994) nutrient recommendations and this served as the base diet for each of two experimental

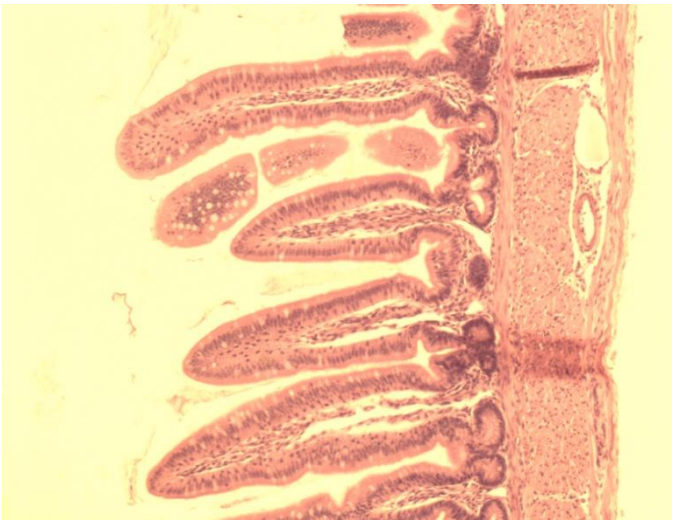

Figure 3: Day 10 posthatch turkey poult lower intestine segment from the ontogeny study stained with HE at 10X magnification.

diets (Probiotic A; Probiotic B) that each contained a different commercial probiotic incorporated at the recommended commercial dosage. A third experimental diet contained a commercial mannan oligosaccharide (MOS). All birds for both studies were housed in starter batteries maintained at the Turkey Research Farm located at the Ohio Agricultural Research and Developmental Center, Wooster, OH.

B. Intestinal Sampling

All birds were handled in accordance with procedures approved by the Institution Animal Care and Use Committee (Animal Care protocol; 2013A00000075). On embryonic day 28 (day of hatch) in the ontogeny study, 5 hatchlings were euthanized by cervical dislocation and used for yolk free body weight and intestinal weight determination. Five additional hatchlings were euthanized and used for body weight and intestinal RNA isolation. The entire small intestine was extracted, placed in a sterile cryogenic vial and immediately placed in liquid N_2. On days 4 and 10 post-hatch, five poults were euthanized each day and used for body weight and intestinal segment weight determination. These birds were also be used for sample collection for histology. The lower small intestine, defined as beginning at Meckle's Diverticulum to the ileo-cecal junction, was flushed with Prefer fixative (Anatech Ltd., Battle Creek, MI). A 1 inch segment was cut from the flushed lower small intestine, tied off at one end with yarn, filled with a fixative (Prefer), tied off at the other end, and then placed in a 50 mL conical vial filled with Prefer and the vial was labeled according to day and treatment. An additional five poults per day were euthanized and the intestine flushed with distilled water. Once flushed, the duodenum and the lower small intestine were placed separately into 15 mL conical tubes and then snap frozen in liquid N_2. Samples in liquid N_2 were stored in -80^{o}C until total RNA isolation could be performed.

On embryonic day 28 (day of hatch) of the probiotic study, 10 hatchlings were also sampled. Five hatchlings were euthanized and used for yolk-free body weight and intestinal weight determination. The remaining five hatchlings were euthanized and used for intestinal RNA isolation as described previously.

An additional 10 poults per dietary treatment were randomly sampled on days 4, 9, and 11 days post-hatch. Five poults per treatment per day were euthanized and used for body weight and intestinal segment weight determination. On days 9 and 11, these birds were also used for histological sample collection, as described for the ontogeny study. The other five poults per treatment per day were euthanized and used for intestinal RNA sampling, as described above.

C. RNA Isolation

Total RNA isolation was performed using a Norgen® Animal Tissue RNA Isolation Kit, as described previously from the lower small intestine, beginning at Meckle's diverticulum and ending at the ileo-cecal junction, from each poult. A Nanodrop assay was performed on each sample to determine RNA concentration and 260/230 and 260/280 ratios. A 1.5% agarose gel electrophoresis was performed to determine RNA integrity and to check for any degradation. Each sample was stored at -80^{o}C until RT-PCR was initiated.

D. RT-PCR

cDNA was synthesized as described previously with the following modifications. Both the OligodTA primer and random hexamers were used. PCR was run on each cDNA sample using *MUC2*, *RPS13* and *TBP* primer sets in separate tubes. To account for human pipetting error, three replicates of each primer set per sample were run as well. Products were run via electrophoresis on 2.0% agarose gels and volumes were determined using TotalLab Quant.

E. Histology Measurements

The lower small intestinal segments, beginning at Meckle's diverticulum and ending at the ileo-cecal junction, fixed in Prefer were sent to the Goss Laboratory which is a core histology lab within the College of Veterinary Medicine at The Ohio State University. Each sample was embedded in paraffin and 4 micron sections were stained with hematoxylin and eosin stain (HE).

Each stained section was photographed using a BX51 Olympus microscope at 10X magnification (**Figure 3**). Villi height, total villus area and crypt depth were measured using Image J software (NIH Bethesda, MD). At least 9 replicate intestinal measurements were taken from each bird. The "free hand selection" tool within Image J was used to measure villus area. The outline of each villus was traced from where the villus meets the crypt on one side to where it meets the crypt on the other side. To measure villus length the “Straight Line Selections” tool was used. A line from the tip of the villus to the beginning of the crypt was drawn and measured. Some villi were too crooked or to measure accurately with a straight line. In that case, the “Spline Fit” tool was used, which resulted in a series of straight lines between points to represent the curve of the villus. A line would form between the lines and continue at an angle to the next place you click, until the endpoint. Two to three points worked well for most villi. Crypt depth was also measured using the "Straight Line Selections" tool, drawing a straight line from the point where the villus meets the crypt to the point where the crypt meets the epithelium.

STATISTICAL ANALYSIS

Histological measurements in the ontogeny study were statistically analyzed via a Student's T-Test and via analysis of variance using the PROC GLM procedure of SAS in the probiotic study ($P<0.05$). Housekeeping gene validation comparisons were done using a Student's T-Test. Body weights and intestinal segment weights were analyzed using the PROC GLM procedure of SAS ($P<0.05$).

RESULTS

A. Ontogeny Study: Body Weights and Intestinal Weights

Table 2: Average body, duodenum and lower small intestine weights by day.

Day	Body Weight (g)	Duodenum (g)	Lower Small Intestine (g)
4	94.062[a]	2.64[a]	4.228[a]
10	197.89[b]	3.6[b]	6.636[b]
P-Value	<0.001	0.0006	0.039
Pooled SEM	6.76	0.176	0.424

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

Average body weight, duodenal and lower small intestinal weights increased significantly from day 4 post-hatch to day 10 post-hatch (**Table 2**).

B. Ontogeny Study: Histology

Average villus length and villus area increased significantly from day 4 to day 10 post-hatch. There were no differences in crypt depth (**Table 3**).

Table 3: Differences in intestinal morphology in the lower small intestine of poults from the ontogeny study.

Age (d)	Villus Area(μm^2)	Villus Length (µm)	Crypt Depth (µm)
4	125276[a]	736[a]	206
10	216275[b]	1024[b]	213
Pooled SEM	79324	335	78

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

C. Ontogeny Study: Housekeeping Gene Validation

Houskeeping genes *RPS13* and *TBP* did not change from day 4 to day 10 post-hatch or from day of hatch (E28) to day 10 post-hatch. The P values for daily comparisons of each gene is presented in **Table 4**. However, *TBP* increased from day of hatch to day 4 post-hatch ($P= 0.007$). A gene is determined to be a suitable housekeeping gene with a $P > 0.05$. Therefore, all *MUC2* expression data is presented as a ratio of *RPS13*.

Table 4: Student's T-Test probability values housekeeping gene expression comparisons at each day of age.

Days Compared	*RPS13*	*TBP*
10 vs. 4	0.663718	0.142459
10 vs. E28	0.352769	0.562901
4 vs. E28	0.098847	0.00703

D. Ontogeny Study: *MUC2* Expression

Average *MUC2* expression increased in a linear fashion from day 0 to day 10 post-hatch but these differences were not significant ($P < 0.16$; **Figure 4).**

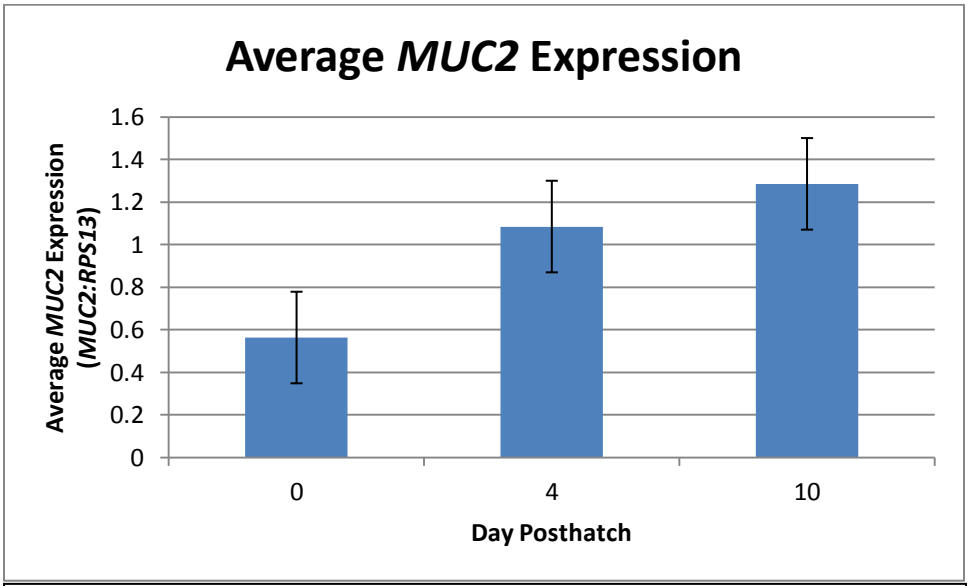

Figure 4: Average *MUC2* expression by day as a ratio of *RPS13* expression. ($P < 0.16$).

E. Probiotic Study: Histology

There was a significant increase in villus length on Day 11 versus Day 9 but no age effects on villus area or crypt depth. Average villus length, villus area, and crypt depth were significantly increased in the poults fed MOS and Probiotic B compared with Control poults on Day 9 but the Control and MOS poults were not different on Day 11 (Day X Diet interaction, $P < .110$). There were no differences between the Control and Probiotic A poults in villus length or villus area but crypt depth was significantly reduced in poults fed Probiotic A. There was an increase in villus length, villus area, and crypt depth in Control poults on Day 11 and this resulted in a significant Day X Diet interaction for crypt depth ($P < .007$; **Table 5**).

Table 5: The effect of dietary probiotics or a MOS prebiotic on small intestinal morphology in poults at 9 or 11 days post-hatch.

	Villus Area (μm^2)	Villus Length (μm)	Crypt Depth (μm)
Age (Day)			
9	435948	1265[b]	253
11	449555	1382[a]	258
Diet			
Control	416652 [a]	1238 [a]	244 [b]
Probiotic A	409129 [a]	1273 [a]	225 [a]
MOS	450873 [b]	1336 [b]	267 [c]
Probiotic B	494351 [c]	1447 [c]	286 [d]

Continued

Table 5 continued

Pooled SEM	14857		28		5	
	Day		Day		Day	
Day * Diet	9	11	9	11	9	11
Control	389521	443783	1119	1357	224	263
Probiotic A	423252	395007	1230	1315	221	228
MOS	456198	445549	1309	1362	276	263
Probiotic B	474822	513881	1400	1494	295	276
Analysis of Variance	Probability					
Day	0.382		<0.0001		0.511	
Diet	0.001		<0.0001		<0.0001	
Day x Diet	0.144		0.110		0.007	

[a,b,c,d] Means with different superscripts differ significantly ($P<0.05$)

F. Probiotic Study: Housekeeping Gene Validation

On day 11 post-hatch, *TBP* expression differed significantly between the Control and Probiotic B diets and the MOS and Probiotic B diets. *RPS13* expression did not change. On day 9 post-hatch, *TBP* differed significantly between the Probiotic A and MOS diets as well as the Probiotic A and Probiotic B diets. *RPS13* expression did not change. On day 4 post-hatch, neither *TBP* expression nor *RPS13* expression changed significantly among treatments but due to the variability in *TBP* expression among the four treatments on days 9 and 11, *MUC2* expression data will be presented as a ratio of *RPS13* (**Table 6**)

Table 6: Student's T-Test comparisons of housekeeping gene expression between dietary treatments on each sample day.

Day	Diets Compared	*Probability*	
		RPS13	*TBP*
4	Control vs. Probiotic A	0.3447	0.5137
	Control vs. MOS	0.848	0.1479
	Control vs. Probiotic B	0.7997	0.4644
	Probitoic A vs. MOS	0.2659	0.4879
	Probiotic A vs. Probiotic B	0.4658	0.919
	MOS vs. Probiotic B	0.6566	0.3699
9	Control vs. Probiotic A	0.6764	0.9556
	Control vs. MOS	0.6347	0.0571
	Control vs. Probiotic B	0.4546	0.0651
	Probitoic A vs. MOS	0.7819	0.0396
	Probiotic A vs. Probiotic B	0.2141	0.0413
	MOS vs. Probiotic B	0.2797	0.3488
11	Control vs. Probiotic A	0.84991	0.0638
	Control vs. MOS	0.328	0.9834
	Control vs. Probiotic B	0.5157	0.0175
	Probitoic A vs. MOS	0.54357	0.2576
	Probiotic A vs. Probiotic B	0.5158	0.2322
	MOS vs. Probiotic B	0.24722	0.0103

G. Probiotic Study: *MUC2* Expression

MUC2 expression significantly increased from day of hatch to day 4 post-hatch ($P<0.02$) but there were no further increases between days 4 and 9 **(Figure 5)**. There were no significant dietary treatment differences on days 4, 9 and 11 and no day x dietary treatment interactions **(Table 7)**.

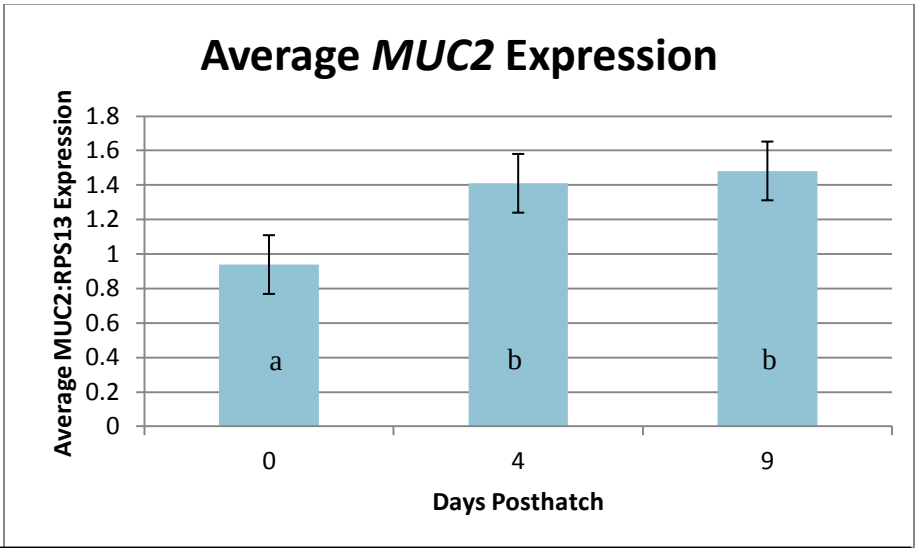

Figure 5: Average *MUC2* expression by day. [a,b] Means with different letters differ significantly ($P<0.05$).

Table 7: Average *MUC2* expression by dietary treatment.

Treatment	Average *MUC2* Expression (*MUC2:RPS13*)
Control	1.15
Probiotic A	1.259
MOS	1.363
Probiotic B	1.198
Pooled SEM	.045
Analysis of Variance	Probability
Day	<0.0001
Diet	0.557
Day x Diet	0.9078

DISCUSSION

In the ontogeny study, villus length and villus area increased significantly ($P<0.005$) from day 4 to day 10 post-hatch, which is consistent with the literature relative to intestinal development in turkey poults immediately post-hatch **(Table 2)**. Uni et al. (1999) found that villus length and area increased rapidly in the duodenum and jejunum, with smaller increases in the ileum. Crypt depth in the ontogeny study did not increase

with age, which is also consistent with previous studies. Crypt depth changes are smaller with age and initially faster in the duodenum (Uni et al., 1999)

Probiotics are defined as a live microbial feed supplement which beneficially affects the host animal (Fuller, 1989). To confer benefit to the host, probiotics accomplish this in multiple ways: increasing mucin expression, producing antimicrobial factors, competitive epithelial adherence and potentially increasing total and pathogen specific IgA levels. Contrary to popular belief, a probiotic does not necessarily need to colonize the intestinal tract to exert effects. Those bacteria that do not colonize, therefore, need to exert effects in a transient manner by influencing the existing microbiota as they pass through the gastrointestinal tract (Ohland and MacNaughton, 2010). There was an increase in villus length between day 9 and 11 post-hatch but no significant differences in villus area in the probiotic study. Supplementation with Probiotic B increased both villus length and area as well as crypt depth **(Table 5)**.

MUC2 expression was not altered by supplementing either probiotic **(Table 7)** but given that our experiment was only conducted through day 11 and that both probiotics were *Bacillus* spores, it is possible that Probiotic A did not have sufficient time to colonize the intestine or transiently effect intestinal morphology and subsequent *MUC2* gene transcription.

Each diet was sent out for analysis and the control diet was found to have 3.4 X 10^4 CFU/g of total *Bacillus* species, but no *Bacillus licheniformis*, the active probiotic species comprising Probiotic B. Probiotic A is comprised of *Bacillus subtillus* and both treatment diets were analyzed and confirmed to contain their respective bacterial species.

There is evidence to support the theory that multistrain and/or multispecies probiotic supplementation is more effective than a single strain. Weight loss is a common effect of *Salmonella* infection. Van Es and Timmerman (2002) found that rats challenged with *Salmonella* Enteritidis and supplemented with a probiotic containing more than two strains of *Lactobacillus* had the least body weight loss when compared with rats supplemented with a single *Lactobacillus* strain . Given that there were other strains of Bacillus in the Probiotic B diet, the same enhanced multistrain effect seen in Van Es and Timmerman (2002) could have facilitated the positive intestinal histology data on Day 11 observed in the current study. One possible mechanism for increased benefits seen in multistrain probiotics is that they may be able to more effectively create a probiotic niche which enhances colonization of commensal bacteria (Timmerman et al., 2004). *Bacillus licheniformis* and *Bacillus subtillus* are native to the poultry gut (Barbosa et al., 2005). They are both aerobes so they use the oxygen in the small intestine to create an anaerobic environment for species such as *Lactobacillus* and *Bifidobacterium*. The latter two bacteria are lactic acid producers and increasing their numbers creates a more acidic intestinal environment, which inhibits opportunistic pathogens from colonizing and proliferating in the small intestine (Song et al., 2014).

Deng et al. (2012) found that supplementing laying hens with *Bacillus licheniformis* under heat stress conditions increased villus height in the ileum and cecum at 6 days after the treatment was initiated when compared with intestinal samples from control hens fed a conventional layer diet. When the group supplemented with *Bacillus licheniformis* was compared with control hens fed a conventional layer diet under normal temperature conditions, there was no difference in villus height. This suggests that

Bacillus licheniformis supplementation might be most effective under stressed environmental conditions. In the current study, supplementing turkey poults immediately post-hatch with *Bacillus licheniformis* (Probiotic B) increased villus length, villus area, and crypt depth. This suggests that probiotic supplementation of young poults under normal conditions could lead to positive changes within the intestine that are not the result of overcoming a stressful environment.

Probiotic A supplementation resulted in decreased crypt depth and there was a Day X Diet interaction **(Table 5)**. The results could be due simply to the wide variation in intestinal morphology seen in young poults or the fact that poults fed the control diet had an increase in crypt depth between days 9 and 11 while poults fed the other diets remained unchanged. The overall lack of a positive effect due to Probiotic A when compared with Probiotic B emphasizes the differences that exist with the commercial products that utilize the same species of bacteria, albeit different strains. This underscores the variability and confusion that exists in commercial practice relative to the use of these potentially beneficial microbes.

Prebiotics are defined as indigestible feed ingredients that beneficially affect the host by selectively stimulating the proliferation and activity of one or a few bacteria (Sohail et al., 2012). Mannan oligosaccharides are prebiotics that are polysaccharide-protein complexes derived from yeast. They are known to create favorable conditions for *Lactobacillus* but they also act as competitive binding sites for pathogens with type 1 fimbriae that recognize D-mannose receptors. *Salmonella* and *E.coli* are two gram negative pathogenic bacteria that will bind to supplemented mannan oligosaccharide, causing them to pass through the intestinal tract without attaching thereby decreasing

their colonization efficiency. In the current study, supplementation with a MOS prebiotic increased crypt depth at both ages, which is consistent with the observations by Solis de los Santos et al., (2007) in poults. These authors used a different MOS product than what was used in the current experiment. In the current probiotic study, supplementation with MOS increased crypt depth ($P < .012$),villus length ($P < .018$) and villus area ($P < .103$) when compared to the control poults. The increase in crypt depth could be beneficial to the host as it may represent an increase in the number of proliferating stem cells which could increase the number of mucin producing goblet cells. Increased crypt depth may also be associated with inflammation so there is no definitive conclusion as to the mechanism(s) underlying this observation.

Iji et al. (2001) reported that supplementing chickens with the same prebiotic used in the current study led to an increase in villus height, similar to what observed in the current experiment although there was a Day X Diet interaction. Of interest is the observation that the poults were supplemented with 4lb./ton or 0.2% (11 days) while the chickens in the Iji et al. (2001) study were supplemented at a 0.5% level (21 days) . When the Solis de los Santos (2007; poults), Iji et al. (2001; chicks) and current data with poults are combined, it certainly supports the hypothesize that MOS products have a positive effect on early intestinal development in poultry.

Even though *MUC2* transcription did not differ significantly between treatments, poults supplemented with MOS had the numerically highest *MUC2* expression levels. If we had continued to sample poults through a later age, we may have observed increasingly significant effects with the MOS treatment.

CONCLUSION

We were successful in designing houskeeping gene primer sets for *TBP* and *RPS13* and validated *RPS13* as a suitable housekeeping gene in the turkey poult. A validated housekeeping gene was crucial in order to quantify *MUC2* expression. *MUC2* transcription increased with age from day of hatch through day 10 posthatch with significant increases from day of hatch to day 4 posthatch, but was not affected by probiotic or prebiotic supplementation.

Intestinal development was measured in terms of three parameters: villus height, area and crypt depth. Villus height and area increased significantly with age, though crypt depth did not change. Villus height, area and crypt depth were all increased with Probiotic B supplementation. Crypt depth was also significantly increased with MOS supplementation. This suggests that probiotic and prebiotic supplementation may be beneficial to the intestinal development in the immediate posthatch turkey poult.

Further research is required to determine the full scope of effects of probiotic and prebiotic supplementation in young and developing turkey poults.

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