EFFECTS OF EARLY NUTRITION ON THE WNT AND BONE MORPHOGENIC PROTEIN PATHWAYS IN THE DEVELOPING INTESTINE

BY

ALLYSON L BARES

THESIS

Submitted in partial fulfillment of the requirements for the degree of Master of Science in Nutritional Sciences in the Graduate College of the University of Illinois at Urbana-Champaign, 2010

Urbana, Illinois

Master’s Committee:

Associate Professor Kelly S. Swanson, Chair
Professor Sharon M. Donovan, Director of Research
Assistant Professor Hong Chen
ABSTRACT

Infant formula is consumed by the majority of infants in the United States for at least part of the first year of life. Infant formula lacks many of the bioactive compounds that are naturally occurring in breast milk. Because of this, there has been an increased interest by the companies that manufacture infant formula to include additives that would potentially allow formula to more closely mimic breast milk activity. One such ingredient currently being added to infant formula is prebiotics.

Prebiotics are non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth of specific healthful bacteria in the colon. It is speculated that prebiotics replicate the activity of breast milk oligosaccharides, which through the production of butyrate by intestinal microbiota, may interact with the Wnt/BMP pathways. The Wnt/BMP pathways regulate intestinal stem cells, which determine the growth, development and maintenance of the intestine. Therefore, the objective of this study was to explore the effects that the addition of prebiotics to formula have on the regulation of the Wnt/BMP pathways when fed to neonatal piglets, a model commonly used in the study of infant nutrition. Piglets (n=5) were randomized into sow-reared (SR), fed control formula (F), or fed formula with added prebiotics (F+P). Fructooligosaccharides (FOS) (2 g/L) and polydextrose (PDX) (2 g/L) were chosen as the prebiotics for this study, because this combination had been less studied than other combinations. Ileum and ascending colon were collected at 7 and 14 days-of-age. Dry matter content, pH, and short chain fatty acid (SCFA) content was measured. The mRNA expression of β-catenin, sFRP3, sFRP4, frizzled 6, DKK1 (Wnt pathway), gremlin (BMP pathway), TNF-α, HNF-4α and osteopontin (OPN) was measured by RT-
qPCR. Piglets fed the F+P diet had greater acetate concentration and lower pH in the ileum at day 14 and in the colon at day 7 and day 14 than F piglets. Butyrate concentrations were highest in SR with F+P not differing from F in ileum at day 14 and colon at day 7 and day 14. Effects of age were seen in all genes, with the exception of OPN, sFRP-3 and sFRP-4. On day 7, no effect of diet was observed in the ileum, however, mRNA expression of DKK1 and frizzled 6 were greater in F+P than SR (p≤0.05). On day 14, gremlin expression was lower and OPN was greater in the ileum of SR piglets compared to F and F+P. Also on day 14, HNF-4α mRNA expression was greater in both ileum and colon of F+P piglets and sFRP3 mRNA expression was greater in the colon than F or SR. In summary, differences were observed between gene expression of F+P and SR piglet intestines, but the supplementation of 2 g/L scFOS and 2 g/L PDX to formula did not shift expression of genes in the Wnt/BMP pathways to be more similar to SR than F.

As the Wnt/BMP pathway is known to exist in a gradient along the crypt-villus axis, with Wnt expression dominating in the crypt region and BMP expression dominating in the villi, it was possible that pooling whole tissue reduced our ability to detect treatment effects that would be concentrated in either region. A method was therefore developed to remove intestinal epithelial cells along the villus-to-crypt axis. Twenty-five-day-old F and SR piglets were euthanized and ileal tissue was collected and placed in a dissociation buffer in a shaking water bath. Exfoliated cells were removed at increasing time points from 5 to 100 minutes in order to remove cells along the villus-to-crypt axis. After the final incubation, remaining mucosal tissue was removed using a sterile glass microscope slide and pooled with the final exfoliated cell isolation. After
each cell collection, a section of tissue was fixed in formalin for histomorphological examination. Expression of genes in the Wnt/BMP pathways, along with crypt marker genes (CDK5 and v-myb), were measured in both whole ileal tissue, pooled epithelial cells, and separate epithelial cell isolations from the same piglet. The expression of β-catenin, HNF-4α, TNF-α, TGF-β and the crypt marker v-myb matched the expected villus-to-crypt pattern in cells collected after 10 (incubation 1), 30 (incubation 2) and 60 (incubation 3) minutes. However, expression of expression in cells collected after 100 minutes (incubation 4) was variable, which may be due to the fact that crypt cells were not efficiently removed and the presence of unwanted non-epithelial tissue. Gremlin, OPN, DKK1, sFRP3 and sFRP4 expression was not statistically different along the villus-to-crypt axis. Frizzled 6 and CDK5 did not express as we had predicted, with expression highest towards the villi. In summary, the epithelial cell collection method used was not entirely successful. While much of the gene data suggests that cells were removed along the villus-to-crypt axis through the first three incubations, the last incubation, which involved scraping the tissue, removed non-epithelial components of the mucosa, while leaving the crypts intact.

In conclusion, the addition of 2 g/L PDX and 2 g/L scFOS did not cause gene expression of the Wnt/BMP pathways to mirror either F or SR expression. New isolation methods to extract cells along the crypt-villus axis should be considered, including the use of a laser capture microdissection. While this combination of prebiotics did not yield the intended effects, future research should be done on other combinations, such as the inclusion of galactooligosaccharides (GOS), which is commonly added to food products including infant formula.
ACKNOWLEDGMENTS

First, I would like to thank my family and friends. I would like to acknowledge my parents for all their support and love, and my brother for telling me to never do something because it is marketable, but only because it makes getting out of bed in the morning easier. I want to thank Anthony for all his patience and constant encouragement. I would like to acknowledge all the members of the Donovan lab for their friendship and, particularly, Marcia Siegel. Without her, I am convinced we would only accomplish half of what we currently do. I want to thank Emily Radlowski, Mei Wang and Dimitri Kashtanov. I am forever grateful to them for allowing me to take samples from the studies they worked so hard on. I would also like to thank Laura Bauer, in Dr. George Fahey's laboratory, for analysis of the short chain fatty acids. Thanks also to my advisor, Dr. Donovan, and Dr. Chen and Dr. Swanson who sat on my committee, for their time and suggestions throughout my time here. I would like to acknowledge Mead Johnson Nutrition and Bristol Myers-Squibb for providing funding for the research. And a big thank you to all the students within the Division of Nutritional Sciences for your friendship and encouragement, both in and out of the lab, and for making my two years in Champaign-Urbana memorable.
TABLE OF CONTENTS

List of Abbreviations ........................................................................................................ viii

Chapter 1: Literature Review .............................................................................................. 1

Regulation of Intestinal Development .............................................................................. 1

Microbial Colonization .................................................................................................... 2

Factors impacting the Development of the Microbiota ............................................... 4

Bacterial Fermentation .................................................................................................. 5

Epigenetics and Intestinal Development ....................................................................... 5

Wnt/BMP Pathway ........................................................................................................ 8

Wnt Pathway ................................................................................................................ 8

BMP Pathway .............................................................................................................. 10

Human Milk ................................................................................................................ 10

Health Benefits ............................................................................................................. 10

Breastfeeding Statistics ............................................................................................... 11

Human Milk Oligosaccharides ...................................................................................... 11

Prebiotic Additives to Infant Formula .......................................................................... 14

Table .............................................................................................................................. 17

Figures .......................................................................................................................... 18

Chapter 2: Summary and Research Goals ....................................................................... 23

Figure ........................................................................................................................... 26

Chapter 3: Impact of Prebiotic Addition to Infant Formula on the Expression of Genes

Associated with Wnt and BMP Pathways of Neonatal Piglets ..................................... 27

Introduction ................................................................................................................ 27
LIST OF ABBREVIATIONS

AC       Ascending Colon
AF       Amniotic Fluid
BMP      Bone Morphogenic Pathway
CDC      Center for Disease Control and Prevention
ChIP     Chromatin immunoprecipitation
DC       Descending Colon
DKK1     Dickkopf homologue 1
DM       Dry Matter
Duod     Duodenum
ELISA    Enzyme-Linked Immunosorbent Assay
F        Formula Fed
F+P      Formula plus Prebiotic Fed
FOS      Fructooligosaccharide
GF       Germ-Free
GI       Gastrointestinal
GOS      Galactooligosaccharide
GLUT-2   Facilitated glucose transporter-2
HATs     Histone Acetyl Transferases
HDACI    Histone Deacetylase Inhibitor
HDACs    Histone Deacetylases
HMO      Human Milk Oligosaccharide
HNF-4α   Hepatocyte Nuclear Factor Four Alpha
ISC      Intestinal Stem Cells
NEC      Necrotizing Enterocolitis
PDX      Polydextrose
SCFA     Short-Chain Fatty Acids
sFRP     Secreted Frizzled Receptor Protein
Smads    TGB-β superfamily of signaling proteins defined by the homology
         between MAD and sma-2,3,4
SR       Sow Reared
TGF-β    Transforming Growth Factor Beta
TNF-α    Tumor Necrosis Factor Alpha
TPN      Total Parenteral Nutrition
Regulation of Intestinal Development

The development of the intestine relies on a whole host of factors, including genetic and environmental factors. Before birth, development of the gastrointestinal (GI) tract is primarily regulated by genetics. The small intestine develops early in gestation and is quite developed by the end of the first trimester. The development of the gut begins at three weeks gestation, when the primitive gut tube is formed. Soon afterwards, enterocytes are formed and the villus formation begins around 9 weeks gestation (Montgomery, 1999). All epithelial cell types are present by the end of the first trimester (Drozdowski et al, 2010). Amniotic fluid (AF) provides the first stimulus for the developing fetal intestine. The human infant near term swallows more than 750 mL/d of AF, which contains of host of nutrients, growth and immunologic factors (Canani, 2008). Intestinal atrophy observed in fetal sheep following ligation of the esophagus demonstrates the importance of luminal stimulation by AF in promoting prenatal intestinal development (Sangild et al, 2002).

After birth, feeding is critical to development and drives the maturation of the intestine and its microbial ecosystem (Canani et al, 2008). While previously nourished predominantly by the umbilical cord, the infant must now rely on the intestine for uptake of nutrients, ion balance, and interactions with intestinal microbiota. Human milk or formula provides stimulation for intestinal development after birth. When infants are
placed on total parenteral nutrition (TPN) where they receive no intestinal stimulus, the
GI tract undergoes mucosal atrophy, reduced intestinal blood flow, impaired immune
function and bacterial translocation and overgrowth (Canani et al, 2008; Niinikoski et al,
2004). These data illustrate the importance feeding has on the development of the
neonatal intestine.

While digestion and absorption of nutrients is an important function of the
intestine, it is not its only function. The intestine is one of the largest immune organs in
the body. It also plays major endocrine and exocrine roles, making the development of
the intestine critical to the well-being of the infant (Neu, 2006).

Microbial Colonization

Microbial colonization by the commensal bacteria is critical to the health and
development of the intestine. It is estimated that there are $10^{14}$ microbes living in the
human intestinal tract. From this, it has been calculated that there are 10-times more
microbial cells living in the intestine than make up the whole human body. The number
of microbes increases proximally-to-distally, with fewer than $10^4$ microbes per gram
contents in the stomach to $10^8$ in the ileum, and $10^{12}$ in the colon, which is the most
heavily colonized region (Leser, 2009).

Infants are born with a sterile gut and colonization begins immediately upon birth.
The gut is generally first colonized by facultative bacteria. After one week, anaerobic
genera establish themselves in the gut. With time, these anaerobic bacteria become the
dominant bacteria (Vael and Desager, 2009). The dominant groups found in the infant are
Proteobacteria, Bacteroidetes, Firmicutes, Actinobacteria, and Verrucomicrobia (Palmer et al, 2007). By 6-months-of-age, the human fecal microbiota is dominated by Firmicutes and Bacteroidetes. By one-year-of-age, the gut microbiota resembles that of an adult. Around this time, the microbiota community becomes highly stable and unique to that individual. At least part of the composition of the intestinal microbiota is determined by host genetics, however, a number of environmental factors, including living arrangements, are determinants as well (Leser, 2009).

The importance of microbiota in maintaining gut structure and function has been examined using germ-free (GF) animals, which contain no microbiota. The GF animals exhibit fewer morphological changes in their gastrointestinal (GI)-tract as compared to conventionally-raised animals. Epithelial cell turnover rate in germ-free (GF) animals is approximately half that of conventionally-raised (colonized) animals, which is supported by reduced rates of epithelial cell proliferation and migration in GF animals. Furthermore, GF animals have fewer goblet cells and enteroendocrine cells than colonized animals, suggesting differences in stem cell commitment to these lineages. Other differences include changes in fluid handling, metabolism, immune response and behavior (Leser and Molbak, 2009).

The gastrointestinal tract is the largest immune organ in the body. The gut-associated lymphoid tissue contains about 60% of all lymphocytes in the body (Anderson et al, 2009). The intestinal microbiota is intimately involved in immune system regulation. The body must be able to ‘tolerate’ harmless, commensal microbes present within the gut, while at the same time being prepared to mount an immune response to pathogenic bacteria. Considerable cross-talk occurs between the postnatal gut and the
microbiota, which is believed to be involved in the process by which the body determines if a bacteria is pathogenic or not (Leser and Molbak, 2009). Inappropriate responses by the body are believed to be a cause of atopic diseases seen in many infants (Vael and Desager, 2009).

**Factors Impacting the Development of the Microbiota:**

Infants are exposed to bacteria from their mother and the surrounding environment. This helps to explain the marked inter-individual variability observed between infants. Babies born by caesarean section have a delayed colonization of *Escherichia coli*, *Bifidobacterium* and *Bacteroides*, indicating that these are normally introduced to the infant by the mother during vaginal birth (Vael and Desager, 2009). Route of feeding also affects the microbial composition within the infant gut. For example, studies have shown that there are fewer *Clostridium* and more *Enterococcus* and *Staphylococcus* in breast-fed infants when compared to formula-fed infants, whereas, formula-fed infants tend to have higher amounts of *Bacteroides* and *Enterobacteriaceae* than breast-fed infants (Adlerberth and Wold, 2009).

Bifidobacteria are known to have many health benefits when they colonize the human gut. These benefits include protection from infectious microbes, production of water soluble vitamins, assistance in digestion and absorption of carbohydrates and calcium, and potential reduction in the risk for colorectal cancer (Anderson et al, 2009). Although, breast-fed infants have traditionally been reported to have greater density of *Lactobacillus* and bifidobacteria, more recent studies using molecular approaches (16s rDNA) have not shown consistent differences in these species between breast-fed and
formula-fed infants (Adlerberth and Wold, 2009). Some prebiotics have been reported to increase the amount of bifidobacteria in the gut (Vael and Desager, 2009).

Bacterial Fermentation:

The microbiota present in the gut use carbohydrates as a fuel source, fermenting them into gases (CO₂, H₂, CH₄) and short-chain fatty acids (SCFA). The most common SCFA produced are acetate, propionate and butyrate. Butyrate is particularly important to the colon as it is the preferred energy source of the colonocytes. Butyrate is believed to help regulate proliferation and apoptosis in the colon by potentially controlling gene expression. In addition, SCFA can be transported into the circulation to provide an energy source; in humans, SCFA account for 6-9% of the total energy harvested (Leser and Molbak, 2009).

Epigenetics and Intestinal Development

Epidemiological data indicates that there are different periods of development where nutrition can influence adult susceptibility to chronic diseases. Animal models have demonstrated that even subtle nutritional differences at specific points in the development of the animal can cause changes in gene expression and metabolism that persist through the organism’s life (Waterland, 1999). Monozygotic twin pairs show differences in developing many GI diseases, such as celiac disease, ulcers, Crohn’s disease and ulcerative colitis. This indicates the possible importance of epigenetic process in the gut (Waterland, 2006).
Epigenetics, which means “beyond the genome”, defines the concept that genetic information is not only contained in a linear sequence of DNA, but also the 3-dimensional structure of the chromatin. The human genome is huge, and packing it down small enough to fit into the cell nucleus requires highly-ordered packaging, which is achieved through epigenetics. Certain chromosomal regions are tightly condensed, whereas others are maintained in an open configuration (Waterland, 2006). These epigenetic modifications alter the expression of genes by making them either more accessible to transcription machinery, or making them hard to reach. Epigenetic modifications are not found in equal number throughout the genome, but are concentrated among regions that regulate growth, metabolism and differentiation (Barros and Offenbacher, 2009).

Epigenetics help to explain how different tissues can carry out different tasks, all while containing the same genetic code. For example, a liver cell will never differentiate into a brain cell, because the epigenetic mechanisms that control gene expression are normally efficiently maintained through subsequent cell replication (Waterland, 2006). Although many of these epigenetic modifications are potentially reversible, once established they appear to persist over many cell generations, potentially throughout the life of the organism (Vaissiere et al, 2007; Barros and Offenbacher, 2009).

Epigenetic modifications are influenced by a range of factors, including stress, infection, toxins, and diet. Any one of these induces changes in the epigenetic markers, which then modify the chromatin structure and the subsequent gene expression of the cell. These changes begin in utero and infancy and can occur throughout the lifetime of the organism (Barros and Offenbacher, 2009). It has been proposed that sensitivity to
diet and the environment vary due to genetic and epigenetic differences that exist between the individuals (Lund and Zaina, 2007).

One of the most well-studied type of epigenetic modification is *DNA methylation*, which occurs on the cytosine in CpG dinucleotides. Methylation causes the chromatin to pack more tightly and shuts down transcription. Thus, DNA methylation is involved with silencing of transposons, imprinted genes and the inactive X chromosome of women (Bird, 2002). The other main group of epigenetic modifications involves *post translational modifications of histones*. These include changes in phosphorylation, methylation, acetylation and ubiquitinylation (Barros and Offenbacher, 2009). Acetylation is among the most studied of these. Histone acetylation has evolved to open chromatin structure and allow for transcription of genes to occur. Histone acetylation is a reversible modification of specific residues on histone tails. It is controlled by histone acetyl transferases (HATs) that add the acetyl group, and histone deacetylases (HDACs) that remove them (Vaissiere et al, 2008). The actions of these enzymes facilitate gene transcription or transcriptional repression, respectively (Bartova et al, 2008).

One well-known histone deacetylase inhibitor (HDACI) is butyrate (Bartova et al, 2008). By inhibiting HDAC, butyrate allows for acetylation to occur. Butyrate, a SCFA produced by fermentation of the colonic flora, is hypothesized to play an important role in intestinal growth and development (Kien et al, 2008). Exactly how butyrate interacts with the gut is still under investigation. *In vivo* and *in vitro* experiments with butyrate have yielded conflicting results, finding increased and decreased proliferation, respectively (Blottiere et al, 2003; Kien et al, 2008). Because of its ability to interfere with HDACs, butyrate, through interactions with histones, has the potential to influence
the development of the gut, specifically the activity of proliferation and apoptosis through modulations of the Wnt and bone morphogenic protein (BMP) pathways.

**Wnt/BMP Pathway**

**Wnt Pathway**

In 1987, the *Drosophila* gene *Wingless* and the proto-oncogene *Int-1* were found to share a common origin; the term “Wnt” arose from the combination of these gene names (Pinto and Cleavers, 2005). Wnt proteins are secreted glycoproteins that are soluble and cysteine-rich. In mammals, there are about 20 of these proteins (Nakamura et al, 2007). Since the late 1980’s, numerous studies have been conducted in humans, *Drosophila*, zebrafish and nematodes to determine how the Wnt pathway is involved in normal development, homeostasis and carcinogenesis in the intestine and colon (Pinto and Cleavers, 2005).

The Wnt pathway plays an important role in the regulation of intestinal stem cells (ISC), which are cells located in the crypts of the intestine and are responsible for creating all types of mature intestinal cells. These include enterocytes, which absorb nutrients, goblet cells, which secrete mucus, Paneth cells, which release antimicrobial peptides, and enteroendocrine cells, which release hormones. Intestinal stem cells must continually renew the pluripotent stem cell progenitors, as well as, create these differentiated cells (Scoville et al, 2008) (Figure 1.1). Even under ordinary conditions, the equilibrium between proliferation and differentiation is extraordinarily important. It must be balanced in such a way as to maintain the correct size, shape and function of the intestinal villi. These ISC must also be able to respond to injuries in order to replace
damaged and non-functioning cells (Nakamura et al, 2007). The Wnt pathway plays a critical role in the renewal of the intestinal lining and it is involved in maintaining ISC cell cycle control and differentiation, as well as, controlling the migration and localization of epithelial cells along the crypt-villi axis. Improper regulation of the Wnt pathway is involved in the development of many colorectal cancers (Radtke and Clevers, 2005).

Wnt canonical signaling, which is found in the intestine, involves β-catenin. In the absence of Wnt signals, β-catenin is degraded through the β-catenin destruction complex. When this destruction complex is destabilized, β-catenin binds to the transcription factors LEF/TCF, which then drives transcription of genes within the Wnt pathway (Scoville et al, 2008).

Wnt ligands act to initiate Wnt signals through the “Wnt receptor complex”, which is comprised of a Frizzled receptor (FZD1 through FZD8) and a low-density lipoprotein co-receptor (LRP5 or LPR6) (Figure 1.2). Activation of this complex results in the inactivation of the β-catenin destruction complex. Wnt3, Wnt6 and Wnt9B are believed to be the ligands responsible for the canonical Wnt signaling in the intestine, because they are the only Wnt proteins found in the crypt cells (Scoville 2008). Frizzled 5, 6, 7 and LRP5 and 6 are also expressed in the crypts (Gregorieff et al, 2005) (Table 1.1). Dickkopf homologue 1 (DKK1) is a Wnt inhibitor, and over-expression of DKK1 results in the loss of crypts and decreased villi number and size in mice, which helps to highlight the importance of Wnt signaling in controlling intestinal cell proliferation and keeping stem cells in an undifferentiated state (Pinto et al, 2003).
**BMP Pathway**

The BMP pathway is also involved in the maintenance and control of the intestine. The conditional ablation of BMP proteins in the intestine in mice showed that BMP serves to oppose crypt formation and ISC renewal (Haramis et al, 2004). This suggests that BMP is involved in restricting the space for crypts by controlling ISC number and works against Wnt (Scoville et al, 2008). BMP acts on receptors that activate Smad proteins, which then begin transcription of genes involved in controlled cell death. The Smad proteins are homologs of both the Drosophila protein, mothers against decapentaplegic (MAD) and the *Caenorhabditis elegans* protein *sma*. The name is a combination of the two. Smads are proteins that modulate the activity of transforming growth factor beta ligands (Whitman, 1998). The BMP pathway is down-regulated primarily by gremlin and notch. It is believed that BMP antagonists coordinate with Wnt signals to allow for ISC renewal and proliferation. Accordingly, gradients of Wnt and BMP activity are observed in the intestine (Figure 1.3). Wnt activity is most concentrated at the crypt, where ISC are located. Wnt activity decreases along the crypt-villus axis at the same time that BMP activity is increasing (Scoville et al, 2008).

**Human Milk**

*Health Benefits*

Human breast milk evolved along with humans and is superior to all other food for infants. Exclusive breastfeeding is considered the ‘gold standard’ against which all other infant diets are measured. There are a variety of health benefits that arise from exclusive
breastfeeding, which affects not only the child, but also the mother and the community at-large (Garman et al, 2005).

Breastfeeding decreases the incidence of many diseases in the infant, including respiratory tract infection, meningitis, necrotizing enterocolitis (NEC), otitis media, urinary tract infection, and diarrhea (Garman et al, 2005). Some studies have even suggested that breastfeeding may reduce the incidence of sudden infant death, as well as diseases later in life, such as both type I and type II diabetes mellitus, cancer, asthma, hypercholesterolemia, and obesity and overweight (Garman et al, 2005). Breastfeeding has been reported to affect the development of the intestinal tract, with breastfed infants having smaller crypts and villi than formula-fed infants (Cummins and Thompson, 2002).

**Breastfeeding Statistics**

Despite the many clear benefits of breastfeeding, many mothers still prefer to formula feed. In 2009, the CDC reported that 73.9% of babies born in the United States were given breast milk at some time during the first year of life, however most of these babies were breastfed for a relatively short duration (CDC, 2009). At 6-months-of-age, only 43.4% of infants were reported as receiving some amount of breast milk and at 12-months, it dropped to 22.7% (CDC, 2009). Even fewer babies were reported to be breastfed exclusively. At 3-months-of-age only 33.1% of infants were exclusively breastfed, which dropped to 13.6% at 6-months-of-age (CDC, 2009). Overall, these data show that, while breastfeeding initiation rates have improved steadily over the last two decades, exclusive breastfeeding has remained flat (Garman et al, 2005).
Human Milk Oligosaccharides

Milk evolved to nourish infants. Being the sole food source exerted a strong selective pressure on the genetics of lactation leading to components that promote the health of infants (German et al, 2008). Milk contains all the necessary ingredients for growth and development, as well as several nonessential factors. These compounds act to encourage growth, discourage microbes and bind toxins, as well as to support the development of the immune system and gut microbial community. Although these are nonessential components, they do provide a real advantage to the infant. These components include human milk oligosaccharides (HMOs) (German et al, 2008).

Concentrations and Chemical Structure: The high concentrations and structural diversity of HMO's are unique to humans. Human milk contains 7-12 g/L, making them a major component of human milk, coming after only lipids and lactose. The milk of other species contain oligosaccharide concentrations that are 10- to 100-times lower than that found in human milk (Boehm and Stahl, 2007). Cow’s milk, which is used to make most infant formulas, has less than 1 g/L of oligosaccharides (Coppa et al, 2006).

The HMOs are comprised of D-glucose, D-galactose, N-acetylglucosamine, L-fucose, and sialic acid, with lactose forming the reducing end. There can be anywhere between 3 and 15 repeating carbohydrate units. Approximately 200 different oligosaccharides have been identified in human breast milk (Bode, 2009). The HMO differ not only in size, but also in charge. Both neutral and anionic species are present, however, the majority of HMO are neutral (German et al, 2008). The type of
oligosaccharides produced in the mammary gland is believed to be influenced by maternal genetics, including the mother’s Lewis blood group (Macfarlane et al, 2007).

**Physiological Functions:** The complexity of HMO is reflected in their potential for many different physiological functions within the neonate (German et al, 2008). HMOs were originally thought to be prebiotic in nature. Because infants are born with a sterile gut and have a naïve innate and adaptive immune system, they are very susceptible to gastrointestinal disease. By providing a carbon source for non-pathogenic bacteria, HMOs may help protect against gastrointestinal diseases. All bacteria that are present in the breastfed infant’s intestine are then forced to compete against one another with oligosaccharides as the major carbon source. When HMOs were purified and provided as the sole carbon source for microbes, specific strains were favored, suggesting that these oligosaccharides act to select specific bacteria. Only *Bifidobacteria longum* biovar *infantis* was able to successfully grow on HMO alone (German et al, 2008) (Figure 1.4).

This finding is in line with the other observations. Human milk has been reported to have a “bifidogenic effect” (Coppa et al, 2006). The intestinal bacteria content of breastfed infants has been reported as being comprised of up to 90% by bifidobacteria and lactobacilli, whereas in formula-fed infants these two genera made up only 40-60% of intestinal microbiota (Harmsen et al, 2000).

Despite the prebiotic effects of HMOs, about 90% are found intact in the infant’s feces, which imply that they are relatively resistant to digestion as well as fermentation. This suggests that there is another function of HMOs, as it seems unlikely that such concentrations would be superfluous after the mother spent energy to make them. It has
been proposed that HMOs may provide an anti-adhesive effect to protect against pathogenic microorganisms, which often adhere directly to the host’s epithelial surface. The adhesion factors present in microbes are often glycan-binding proteins. Some HMOs also contain these same glycan binding determinants, which suggest that they may block pathogen adhesion and help protect infants from disease (Bode 2009) (Figure 1.5).

Some of the protective effects of breast milk, perhaps because of HMOs, is seen in clinical trials involving NEC, which is the most common disorder affecting the gut of premature infants. More than 10% of all premature infants develop NEC, with a mortality rate ranging from 10% to 100%, depending on the severity of the disease (Holman et al, 1997). There are many risk factors associated with NEC, including formula feeding. Studies have shown that NEC incidence is six-times higher in premature babies fed formula than those fed breast milk (Sisk et al, 2007).

**Prebiotic Additives to Infant Formula**

Despite the potential for HMOs to protect infants from disease, it is unlikely that they will be added to infant formula anytime in the near future. The methods used to isolate HMOs are expensive and time consuming, making them insufficient to produce the large quantities that would be needed for the addition of HMOs to formula (German, 2008). Currently, biosynthetic pathways of HMOs are poorly understood. Understanding how HMOs are synthesized could provide possible substitutes to HMOs for formula (Bode 2009). As an alternative to HMOs, synthetic prebiotics have been added to infant formulas. However, given the complexity of HMO, synthetic prebiotics are not
anticipated as being anywhere near as successful as HMOs at preventing disease (Bode 2009).

A prebiotic is defined as a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon (Gibson 1995; Roberfroid, 2007). Prebiotics have been argued to be more practical than probiotics (live microorganisms), because probiotics have to compete against the already established microbial communities that live in the gut. Prebiotics can modify gut function by targeting the beneficial bacteria that are already present in the gut (Guarner 2005; Roberfroid, 2007).

Among the most widely used prebiotics are galactooligosaccharides (GOS) and fructooligosaccharides (FOS). GOS is popular in the food industry as well, and is commonly used as a sweetener. It is stable at high temperatures in acidic environments and, like FOS, has a low caloric energy at 1.7 kcal/g. GOS can increase mouth-feel and texture and can act as a bulking agent. FOS is very popular in industry and can be found in more than 500 food products (Guarner 2005). The caloric energy for FOS is quite low at 1.5 kcal/g.

For these reasons, both FOS and GOS are used in a variety of commercial products, including dairy products, sauces, soups, cereals, beverages, animal feeds, and infant formulas (Macfarlane et al, 2007). Prebiotics have been used in infant formulas in Japan for more than 20 years with a safe history of use. They have been introduced into European and American formulas more recently (Macfarlane et al, 2007). Prebiotic supplementation in formula is well tolerated and results in higher fecal bifidobacteria
counts, reduced growth of pathogenic bacteria, accelerated GI transit time, softer stools similar to breastfed infants and did not negatively affect weight gain (Srinivasjois et al, 2009).

Prebiotics have a range of benefits, including immune system modulation. Studies have found that GOS and FOS can lead to increased production of butyrate, which can stimulate apoptosis and may help to protect against colorectal cancer in adults (Rowland 1998). These prebiotics are used to stimulate the growth of beneficial bacteria, particularly bifidobacteria and lactobacilli. Low counts of bifidobacteria have been found in the faeces of infants with allergies, and so adding prebiotics to formula could potentially reduce allergic responses in children (Yoshida et al, 2004). The amount of prebiotics must be limited, as high levels can cause osmotic diarrhea. It should also be noted that while prebiotics are used to stimulate growth of beneficial bacteria, they are not that specific and other organisms that are less desirable are capable of fermenting prebiotics as well (Macfarlane et al, 2007).
Table 1.1. *Major proteins involved in Wnt/BMP pathways and how they affect cell division and apoptosis.*

<table>
<thead>
<tr>
<th>Gene</th>
<th>Pathway</th>
<th>Pathway Regulation*</th>
<th>Location</th>
<th>Overall Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wnt 3, 6, 9b</td>
<td>Wnt</td>
<td>↑</td>
<td>throughout cell division</td>
<td>cell division</td>
</tr>
<tr>
<td>β-catenin</td>
<td>Wnt</td>
<td>↑</td>
<td>4 plus cells up</td>
<td>cell division</td>
</tr>
<tr>
<td>Frizzled 5-7</td>
<td>Wnt</td>
<td>↑</td>
<td>crypt</td>
<td>cell division</td>
</tr>
<tr>
<td>secreted frizzled receptor protein</td>
<td>Wnt</td>
<td>↓</td>
<td>4 plus cells up</td>
<td>no division</td>
</tr>
<tr>
<td>lipoprotein-related protein coreceptor</td>
<td>Wnt</td>
<td>↓</td>
<td>crypt</td>
<td>no division</td>
</tr>
<tr>
<td>Dickkopf homologue 1</td>
<td>Wnt</td>
<td>↓</td>
<td>crypt</td>
<td>no division</td>
</tr>
<tr>
<td>Gremlin</td>
<td>BMP</td>
<td>↓</td>
<td>crypt</td>
<td>continued growth</td>
</tr>
<tr>
<td>BMP receptor</td>
<td>BMP</td>
<td>↑</td>
<td>4 plus cells up</td>
<td>apoptosis</td>
</tr>
<tr>
<td>SMADs</td>
<td>BMP</td>
<td>↑</td>
<td>4 plus cells up</td>
<td>apoptosis</td>
</tr>
</tbody>
</table>

*↑, up-regulated; ↓, down-regulated
Figure 1.1. *Representation of pluripotent potential of intestinal stem cells.* Intestinal stem cells, which are located near the base of the crypt, are able to differentiate into several types of cells, as well as, maintaining the stem cell line. From: Radtke and Clevers, *Science* 2005: 307; 1904-9.
Figure 1.2. *Wnt and BMP pathways.* Each pathway involves the interactions of several proteins. From: Scoville et al. *Gastroenterology* 2008; 134:849-64.
**Figure 1.3.** Distribution of Wnt and BMP along the crypt-to-villus axis. Wnt activity is most concentrated at the crypt where ISCs are located. Wnt activity decreases up the villi at the same time that BMP activity is increasing. From: Scoville et al. *Gastroenterology* 2008; 134:849-64.
Figure 1.4. Growth curves of different bacteria fed on HMOs, inulin and lactose. Only *B. infantis* grew successfully on HMOs. From: German et al. *Nestle Workshop* 2008; 62: 205-18.
Figure 1.5. Representation of interactions between pathogenic bacteria, the intestinal glycocalyx and HMO. Panel A: Bacteria are able to adhere to the epithelial cells through glycan binding proteins. Panel B: HMOs may act as a binding site for bacteria and protect the infant from infection. From: Bode L. Nutrition Reviews 2009; 67; S183-91.
Interactions between genes and nutrients have been well noted. Humans and animals respond in many different ways to their diet because of the difference of their genetics, epigenetics and metabolic status (Go et al, 2005). This is a complex bi-directional interaction. Nutrients can influence the development of a phenotype, but also a response to a specific nutrient can be determined by the individual’s genes (Paoloni-Giacobino et al, 2003).

Nutrition during infancy may cause epigenetic imprinting of the gut, both in disease states and in normal development (Waterland, 2006). It has been demonstrated that intestinal proliferation and morphology differ between formula- and breast-fed infants. For example, formula-fed infants have deeper crypts, longer villi and a higher mitotic count than breast-fed infants (Thompson et al, 1998). Recently, differences in gene expression in exfoliated epithelial cells between formula- and breast-fed infants were reported by our lab using noninvasive stool-based detection (Chapkin et al, 2010). This implies that diet during infancy may be extraordinarily important to the development and future health of the gut.

Differences in diet during the neonatal period can impact intestinal development in several ways. Nutrients can have direct effects or indirect effects through modulation of the microbiota. As noted above, breast-fed infants have fewer *Clostridium* and more *Staphylococcus* in their intestine than do formula-fed infants (Adlerberth and Wold, 2006).
Differences in the microbiota can lead to direct host-microbe interactions or can mediate intestinal development through their metabolic products (e.g. SCFA).

The major pathways that have been implicated in the development of the fetal intestine are the Wnt and BMP signaling (Scoville et al, 2008). These pathways could be influenced by epigenetic changes. Butyrate is produced by some bacteria found in the gut and butyrate is capable of modulating epigenetic changes (Leser and Molbak, 2009). The microbiota differences exist between formula-fed and breast-fed infants (Vael and Desager, 2009), which could imply differences in butyrate production and gene regulation. Furthermore, the addition of prebiotics could affect the microbial makeup of the gut and the regulation of these pathways.

Thus, the goal of this thesis research was to determine how early neonatal nutrition impacts the expression of key genes involved in the regulation of the Wnt/BMP pathway. The overall hypothesis of this research was that the addition of prebiotics to formula would align mRNA expression of genes in the Wnt and BMP pathways in the formula-fed piglet intestine more closely with that seen in sow reared piglets. Chapter 3 compared intestinal growth and gene expression in whole intestinal tissue collected from 7- and 14-day-old piglets fed sow milk, formula or formula supplemented with prebiotics. Chapter 4 compared gene expression in whole tissue compared to exfoliated cells collected along the crypt-to-villus axis in 23-day-old piglets fed sow milk or formula. Due to the differential expression of Wnt and BMP genes along the crypt-to-villus axis, we hypothesized that exfoliated cells would show enrichment of our target genes.
This research was conducted using the piglet model, as it is an exceptional preclinical model, due to its many similarities in metabolism, nutrient requirements, anatomy and physiology with the human infant (Wykes et al, 1993). A comparison of the developmental ontogeny of intestinal development in human infants compared to piglets and rodents is summarized in Figure 2.1 (Sangild, 2006). In humans, the GI tract starts developing as a fetus and reaches full maturity around 1 year-of-age (52 weeks postnatal). Piglets have major developmental periods both before and after birth. Developmental clusters occur around birth and weaning and maturity is reached at 3 months of age (~12 weeks postnatal). In rodents, such as the rat, most development happens around weaning (~5 weeks postnatal). Thus, the rodent gastrointestinal tract is very underdeveloped at birth, which is quite different from humans (Sangild, 2006). One major difference between the development of the human infant and the piglet is the acquisition of the immune system. Human infants receive passive immunity from placental transfer of immunoglobulins (primarily IgG) through the placenta before birth and then from the consumption of colostrum (primarily sIgA) immediately after birth. Piglets do not receive any immunoglobulins through the placenta and are therefore dependent on milk consumption, particularly colostrum, for immunoglobulins (primarily in the form of IgG (Sangild, 2006). Thus, artificially-reared piglets must be provided with immunoglobulins through either colostrum or serum or are susceptible to infection.
Figure 2.1. **Comparison of the timing of gastrointestinal development differs between the human, the pig and rat.** The development of the pig more closely resembles that of the human than the rat. From: Sangild PT. *Experimental Biology and Medicine* 2006; 231: 1695-711.
Chapter 3

Impact of Prebiotic Addition to Infant Formula on the Expression of Genes Associated with Wnt and BMP Pathways of Neonatal Piglets

Introduction

Animal models have demonstrated that even slight differences in available nutrients at specific points in development can have an impact on the gene expression that persist throughout the animal’s life (Waterland, 1999). Epidemiological work done on monozygotic twin pairs show differences in the heritability of genetic diseases, indicating diseases of the gut. This highlights the possibility of epigenetic processes taking place in the gut (Waterland, 2006).

Differences in butyrate production by intestinal microbiota have been proposed to influence the histone structure in the gut. Butyrate, as an HDAC inhibitor, has the ability to interact with genes in the intestine, including the Wnt and bone morphogenic protein (BMP) pathways which govern proliferation, differentiation and apoptosis (Scoville et al, 2008). It has been shown that differences in the microbiota content exist between formula-fed and breast-fed infants (Adlerberth and Wold, 2009), which could be due to differences in butyrate production and gene regulation. The addition of prebiotics could, therefore, affect the microbial composition of the gut and the regulation of these pathways.
Oligosaccharides in milk are believed to be, at least in part, prebiotic in nature. They provide a carbon source for non-pathogenic bacteria and may help protect against gastrointestinal diseases (German et al, 2008). Despite the many proposed benefits of HMOs, it is unlikely that they will be added to infant formula, at least anytime in the near future. The methods used to isolate HMOs are expensive and time consuming, making them too expensive for mass market (German, 2008). As an alternative to HMOs, formula companies have added synthetic prebiotics to infant formulae (Bode, 2009). Studies have found that prebiotics can lead to increased production of butyrate (Rowland, 1998). Therefore, the objective of this study was to explore the effects that the addition of prebiotics to formula have on the regulation of the Wnt/BMP pathways when fed to neonatal piglets, a common model for the use of studying human pediatric nutrition. Fructooligosaccharides (FOS) (2 g/L) and polydextrose (PDX) (2 g/L) were chosen as the prebiotic for this study because the combination was less studied than other combinations. They were added to formula (F+P) and tested against animals given conventional formula (F) and piglets that were reared with a sow (SR).
Materials and Methods

Animal Study and Diets

All animal protocols and procedures were approved by the University of Illinois Institutional Animal Care and Use Committee. Late gestation pregnant sows were obtained from the University of Illinois Swine Research Center and transported to the animal facilities at the Edward R. Madigan Laboratory. Sows were monitored for farrowing and immediately upon delivery, piglets were removed from the sow to avoid ingestion of colostrum. Piglets (n=15) were randomized into three treatment groups: sow-reared (SR), formula-fed (F) or fed-formula containing short chain fructooligosaccharides (scFOS, Nutriflora, GTC nutrition, Westchester, IL) (2 g/L) and polydextrose (PDX, Litesse, Danisco, Madison, WI) (2 g/L) (F+P). Sow-reared piglets were placed back with the sow within 30 min of delivery and received colostrum. Artificially-reared piglets were administered adult pig serum by orogastric gavage in order to provide antibodies to the piglets (Sangild et al., 2002). Piglets were given 4ml/kg body weight immediately after birth, 5 ml/kg body weight at 8-12 h postnatal and 10ml/kg body weight at 22 h postnatal age. Piglets in the F and F+P groups received 360ml/kg/day pig milk replacer with low-level antibiotics (Advance Liqui-Wean, MSC Specialty Nutrition, Dundee, IL) divided into five equal feedings. Prebiotics were added to formula immediately before feedings. Formula intake, piglet body weight and stool consistency were assessed each morning using an arbitrary scale of 1-4, where 1=solid; 2=semisolid; 3=loose and 4=watery (Correa-Matos et al, 2003).

29
Sample Collection

Samples were collected on study day 7 and 14. Piglets were sedated by an intramuscular injection of 7 mg/kg body weight Telazol (Fort Dodge Animal Health, Fort Dodge, IA). Piglets were then euthanized by intracardiac injection of 72 mg/kg body weight of sodium pentobarbital (Fatal Plus, Vortech Pharmaceuticals, Dearborn, MI). The jugular vein was then cut and the animal exsanguinated. The small intestine was quickly removed, its total length determined, and then divided into three segments based on length. The duodenum consisted of the first 10% of the total length, the jejunum was that between 10% and 85% and the last 15% was the ileum. Ascending colon was also measured and collected. For SCFA analysis, content collected from ileum, cecum, AC and DC were placed into glass bottles containing 2N HCl and stored at –20°C until analyses were conducted. Each segment was weighed, flushed with ice cold phosphate buffered saline (PBS) (MP Biomedicals, Solon, OH). Small segments (1-2 cm) were cut and fixed in formalin and the remaining tissue flash frozen in liquid nitrogen and placed into -80°C freezer for later analysis.

Dry matter, pH and SCFA concentrations of luminal contents

Dry matter, pH and SCFA concentrations were measured on luminal contents from ileum, cecum, AC and DC. Dry matter was measured on the samples according to AOAC method (2005). The pH of the contents was measured using a Beckman pH meter and electrode (Beckman Instruments, Inc., Fullerton, CA). Concentrations of SCFAs (acetate, propionate, butyrate, isobutyrate, valerate and isovalerate) were determined as
described by Erwin and colleagues (1961) by using a Hewlett-Packard 5890A series II gas chromatograph (Palo Alto, CA) and a glass column (180 cm x 4 mm id) packed with 10% SP-1200/1%H₃PO₄ on 80/100+ mesh chromosorb WAW (Supelco Inc, Bellefonte, PA).

Isolation of mRNA and Quantitative Real-Time PCR

Messenger RNA was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA) following the manufacturer’s protocol. Total RNA was quantified using a NanoDrop 1000 (Thermo Scientific, Rockford, IL), and RNA integrity was analyzed with the Bionalyzer 2100 system (Agilent Technologies, Santa Clara, CA). cDNA was synthesized with a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA). For gene expression quantification, first-strand cDNA was amplified by real-time PCR using Power SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA) with the primer sets described in Table 3.1 at concentration of 250 nmol/μl of each primer. Primers were either obtained from the published literature or were designed using Primer Express 2.0 software (Applied Biosystems, Carlsbad, CA) with Pubmed sequences. Real-time PCR was performed using an ABI 7900HT real-time PCR system (Applied Biosystems, Carlsbad, CA) and quantification of gene expression was performed by relative standard curve method with data expressed as fold-change relative to the SR group. β-actin expression was chosen as an endogenous loading control.
**Statistical Analyses**

A two-way ANOVA was performed using a General Linear Model (GLM) with a post hoc LSD test using Fisher’s Least Significant Difference test to evaluate the effect of diet (SR, F and F+P), day (7 and 14) and interaction of diet and day on short-chain fatty acids, stool dry matter and pH, and gene expression. A one-way ANOVA was used to evaluate the effect diet had on the weight of piglets and their small intestinal and colon weight and length. Statistics were performed using SAS Version 9.2 (SAS Institute, Cary, NC). Statistical significance was established to be $p \leq 0.05$. Values are expressed as mean ± SD.
Results

Body Weight

Piglets in all treatment groups grew similarly through the first 10 days of life. On days 11-14, piglets in the SR and F groups were statistically larger than F+P animals (Figure 3.1). While they all started off at similar weights, by day 11 F+P animals were statistically smaller and they remained smaller until the completion of the study at day 14. The formula given to F+P animals was the same given to F animals, only with added prebiotics, so it is unlikely that the differences in weight can be contributed to the formula. It is also unlikely that the difference in weight can be contributed to the time it took for piglets to learn to drink from the nipple as F+P were not different from F until well after the first few days of life when training took place. Stool consistency, measured using a scale of 1 to 4 (26) averaged 1.07 ± 0.04 and did not differ by age or dietary treatment.

Intestinal Weight and Length

Small intestinal weight and length in absolute (data not shown) or normalized per kg body weight (Figure 3.2) did not differ between the treatment groups at either day 7 or day 14. Colon weight was not determined. Absolute ascending colon length (cm) at day 7 (Figure 3.3a) showed no difference, however, when the absolute length of the ascending colon was normalized by piglet body weight (cm/kg), F+P had statistically larger colons than either F or SR piglets (Figure 3.3b). At day 14, F+P piglets had longer colons than SR piglets, with F piglets being intermediate between F+P and SR (Figure
3.4a). However, when colon length was normalized by BW, F+P piglets were greater than both SR and F piglets (Figure 3.4b).

**Stool Dry Matter and pH**

Within the ileum, percent dry matter (%DM) differed by day and dietary treatment (Table 3.2). The %DM increased between day 7 and 14. On day 7, the %DM was lower in SR than F or F+P, but no dietary effects on %DM were observed on day 14 (Table 3.2). In the ascending colon, %DM of SR piglets was lower in both F+P and SR compared to F piglets, with F+P piglets being intermediate between the two (Table 3.3).

In terms of pH of luminal contents, an influence of age was found only in ileum with pH at d7 (7.52 ± 0.31) being higher than the pH at d14 (7.27 ± 0.30, p <0.05). Among the dietary treatments, pH was similar between 7-day-old SR and F piglets in the ileum (Table 3.2) and ascending colon (Table 3.3). The addition of prebiotics to formula lowered (p <0.05) the pH in ascending colon (Table 3.3), but not in ileum (Table 3.2). At d14, ileal pH was similar in F and SR, but lower in piglets fed F+P (Table 3.2). In the ascending colon, all three groups differed in pH of luminal contents with SR > F > F+P (Table 3.3).

**SCFA concentrations**

Overall, the predominant SCFA was acetate, followed by propionate and butyrate. The impact of age on SCFA concentrations was observed for all SCFA in the ileum (Table 3.2) and for propionate in ascending colon (Table 3.3), with concentrations
increasing between day 7 and day 14. SCFA concentrations were similar among the diet groups in ileum at day 7 (Table 3.2). At day 14, acetate concentrations in the ileum were highest in F+P piglets, and both F+P and SR piglets had higher acetate concentrations than F (p<0.05). By day 14, no differences in acetate concentrations were observed among the dietary treatment groups in the ascending colon. In the ileum of 7-day-old piglets, propionate and butyrate were higher in SR piglets compared to F or F+P piglets (p<0.05; Table 3.2). By day 14, propionate concentrations in ascending colon contents were similar in SR and F+P, and both were higher than F alone, showing that supplementation of PDX and scFOS to formula increased propionate concentration in the ascending colon of F+P piglets to a level of SR piglets at d14 (p<0.05; Table 2.3). Lastly, on day 14, butyrate concentrations in ileal (Table 2.2) and ascending colon (Table 2.3) contents of F+P piglets were intermediate between F and S, and this difference was statistically significant at day 7.

Gene Expression

The expression of all genes in the ileum and colon of 7- and 14-day-old piglets in the three treatment groups is summarized in Tables 3.4 and 3.5, respectively. More differences were seen within the colon than the ileum.

Gremlin: Expression of gremlin in the ileum and ascending colon is summarized in Figures 3.5a and 3.5b, respectively. Gremlin expression differed by region, being expressed at 5-times higher (p≤0.05) levels in the ileum than the ascending colon (Tables 3.4 and 3.5), differed by age, being expressed at higher (p≤0.05) levels at d14 than d7 in
both ileum and colon, and differed by diet within the ileum. At d14, gremlin expression was lower (p<0.05) in the SR than F or F+P groups.

**β-catenin:** Expression of β-catenin in the ileum and ascending colon is summarized in Figures 3.6a and 3.6b, respectively. β-catenin expression differed by region, being expressed at ~2-times higher levels (p≤0.05) in the ileum than ascending colon (Tables 3.4 and 3.5) and differed by age, being expressed at higher levels (p≤0.05) at d14 than d7 in both ileum and colon. However, no effect of dietary treatment on β-catenin expression was observed.

**sFRP-3:** Expression of sFRP-3 in the ileum and ascending colon is summarized in Figures 3.7a and 3.7b, respectively. sFRP-3 expression differed by region, being expressed at ~2-times higher levels (p≤0.05) in the ascending colon than ileum at d14 (Tables 3.4 and 3.5). Expression levels were unaffected by age or diet in the ileum, whereas an effect of diet was observed on d14 in AC, with piglets in the F+P group expressing higher levels of sFRP-3 than F or SR.

**sFRP-4:** Expression of sFRP-4 in the ileum and ascending colon is summarized in Figures 3.8a and 3.8b, respectively. sFRP-4 expression differed by region, being expressed at ~10-times higher levels (p≤0.05) in the ascending colon than ileum (Tables 3.4 and 3.5). Expression levels were unaffected by age or diet.

**Frizzled 6:** Expression of frizzled 6 in the ileum and ascending colon is summarized in Figures 3.9a and 3.9b, respectively. Frizzled 6 expression differed by region, being expressed at ~20-times higher levels (p≤0.05) in the ascending colon than ileum (Tables 3.4 and 3.5). Expression levels differed by age, being expressed at higher
(p≤0.05) levels at d7 than d14 in the colon, and differed by diet within the ascending colon. At d7, frizzled 6 expression was greater (p<0.05) in the SR than F or F+P groups.

**DKK1**: Expression of DKK1 in the ileum and ascending colon is summarized in Figures 3.10a and 3.10b, respectively. DKK1 expression differed by region, being expressed at ~20-times higher levels (p≤0.05) in the ascending colon than the ileum (Tables 3.4 and 3.5). Expression levels differed by age, being expressed at higher (p≤0.05) levels at d7 than d14 in the colon, and differed by diet within the ascending colon. At d7, DKK1 expression was lower (p<0.05) in the F+P than SR or F+P groups.

**Osteopontin**: Expression of OPN in the ileum and ascending colon is summarized in Figures 3.11a and 3.11b, respectively. Osteopontin expression differed by region, being expressed at ~12-times higher levels (p≤0.05) in the ascending ileum than the colon (Tables 3.4 and 3.5). Expression levels did not differ by age, but did differ by diet within the ileum. At d14, OPN expression was greater (p<0.05) in the SR than F+P group. Neither SR nor S+P differed from F.

**HNF-4α**: Expression of HNF-4α in the ileum and ascending colon is summarized in Figures 3.12a and 3.12b, respectively. HNF-4α expression did not differ by region. Expression levels differed by age, being expressed at higher (p≤0.05) levels at d14 than d7 in both the ileum and the colon, and differed by diet within both the ascending colon and the ileum. At d14, HNF-4α expression was greater (p<0.05) in the F+P than F or SR groups.

**TNF-α**: Expression of TNF-α in the ileum and ascending colon is summarized in Figures 3.13a and 3.13b, respectively. TNF-α expression did not differ by region.
Expression levels differed by age, being expressed at higher (p≤0.05) levels at d14 than d7 in the ileum, and differed by diet within the ascending colon. At d14, TNF-α expression was greater (p<0.05) in the F+P than the SR group. Neither F+P or SR differed from F.
Discussion

Prebiotics are defined as selectively fermented ingredients that allow specific changes, both in the composition and/or activity in the gastrointestinal microflora that confers benefits upon host well-being and health (Roberfroid 2007). The addition of prebiotics to infant formula can increase beneficial bacteria in the digestive system to levels similar to those in the breastfed baby (Ben et al, 2004). In addition, prebiotics can help soften stools to be more like those of the breastfed infant (Costalos et al, 2008; Ben et al, 2004; Moro et al, 2002). Recent clinical studies also suggest that prebiotics may have the potential to reduce the risk of common childhood infections (Arslanoglu et al, 2007) and the incidence of atopic dermatitis (Moro et al, 2006). For these reasons, prebiotics are becoming standard additions to infant formulas world-wide.

The microbiota present in the gut use prebiotics as a fuel source, fermenting them into gases (CO₂, H₂, CH₄) and SCFAs. The most common SCFA produced are acetate, propionate and butyrate, which is consistent with the ratios observed in the current study. Butyrate is particularly important to the colon as it is the preferred energy source of the colonocytes. In addition, SCFA can be transported into the circulation to provide an energy source; in humans, SCFA account for 6-9% of the total energy harvested (Leser and Molbak, 2009).

Butyrate is believed to help regulate proliferation and apoptosis in the colon by potentially controlling gene expression through transcriptional regulation of glucagon-like peptide (Tappenden et al, 1998), as well as, modulating epigenetic changes (Leser and Molbak, 2009). The major pathways that have been implicated in the development
of the fetal intestine are the Wnt and BMP signaling (Scoville et al, 2008). These pathways could be influenced by epigenetic changes. Therefore, the goal of this study was to determine whether the expression of genes in the Wnt and BMP pathways differed between formula-fed and sow-reared piglets and whether the addition of a fermentable prebiotics would modulate the gene expression. We hypothesized that the production of SCFA by the microbial fermentation of the prebiotics would modulate gene expression in the piglet intestine and more closely reflect that of sow-reared piglets.

Piglets fed the formula containing prebiotics demonstrated a slower rate of body weight gain on days 11-14 of the study. This result was somewhat unexpected as previous work in our laboratory had shown that piglets fed formula containing 7.5 g/L of scFOS or soy polysaccharide grew similarly to piglets fed unsupplemented formula (Correa-Matos et al, 2003). Additionally, both GOS and FOS are generally regarded as safe and are commonly consumed by humans (Macfarlane et al, 2007) and human infants fed prebiotics display similar growth curves as infants who are breast-fed or fed formula without prebiotics (Srinivasjois et al, 2009). Prebiotics have been shown to have both beneficial or detrimental effects on mineral (Bosscher et al, 2003), nitrogen (Smiricky-Tjardes et al., 2003) and energy (Anguita et al, 2006) absorption, depending upon the type of prebiotics and the dose administered. Mineral absorption may be enhanced by changes in luminal pH due to SCFA (Bosscher et al, 2003). Additionally, SCFA may directly enhance glucose absorption via up-regulation of mRNA expression for the facilitated glucose transporter (GLUT-2) (Tappenden et al, 1998). In contrast, some studies have shown that prebiotics may inhibit nitrogen/amino acid and energy absorption. This decreased ability to absorb nitrogen may be due to the nitrogen being
tied up in the matrix of the food or by an increased microflora that would compete for nitrogen. High amounts of fiber can also cause an increase in gut osmolarity which may result in a dilution of digestive enzymes (Smiricky-Tjardes et al, 2003; Anguita et al, 2006). Although we did not directly assess nutrient absorption or transit time in our study, it remains a possibility that the prebiotics reduced growth by detrimentally impacting nutrient bioavailability. Future studies could specifically assess these two parameters.

That being said, piglets fed the F+P did not show signs of formula intolerance. Stool consistency was similar in all treatment groups suggesting that the prebiotic level (2g/L each scFOS and PDX) did not cause a watery stool, whereas previous work in our lab had shown that the addition of 7.5 g/L scFOS to formula resulted in a significantly more watery stool than formula alone (Correa-Matos et al., 2003), which was likely due to the high concentration of scFOS used in the study. Furthermore, the %DM was only significantly different among the dietary treatment groups at day 14 in the ascending colon and levels in piglets fed F+P were intermediate (25.5 ± 3.2%) between formula-fed (30.0 ± 2.6%) and sow-reared (21.5 ± 3.3%), suggesting that consumption of the prebiotic-containing formula did not induce excessive fluid accumulation in the colon.

As SCFA are a fuel source for intestinal epithelial cells, the weights and lengths of the ileum and ascending colon were examined. The weights and lengths of both segments were numerically greater in the F+P versus F or SR piglets, but only reached the level of statistical significance for colon length (cm/kg). On-going analyses in the laboratory are investigating whether villus or crypt morphology or rates of proliferation were affected by the addition of prebiotics.
Consistent with previous studies, the addition of scFOS and PDX (Nishibata et al, 2009 and Guarner 2005) increased the production of SCFA, although the degree of fermentation was affected by age and region. Acetate was the predominant SCFA in both ileum and ascending colon at day7 and d14. The addition of the prebiotics increased acetate production in the ascending colon on day 7 and the ileum and ascending colon on day 14 relative to formula alone. Higher levels of acetate at day 14 are likely responsible for the lower pH luminal pH levels in the ascending colon at days 7 and 14 and ileum on day 14.

Ileal propionate concentrations were similar in all the treatment groups at day 7 and were similar and lower in F and F+P than SR at day 14. In the ascending colon, propionate concentrations were higher in SR than F and F+P at day 7, whereas by day 14 propionate concentrations were similar in the SR and F+P and levels were greater than formula alone.

Lastly, we were particularly interested in the differences in butyrate concentrations between SR and F piglets and the effect of prebiotic supplementation. F+P levels did increase and approach levels seen in SR in both days, but none of the treatments were different from the others. However, even small increases could have some affect on gene expression as butyrate is believed to be important in the regulation of the intestine (Kien et al, 2008). As a whole, prebiotics had an effect on the colon length and SCFA content in the colon at day 14. In the case of %DM and SCFA concentrations, prebiotic supplementation resulted shifted the 14-day-old F+P animals closer to that of SR than formula alone. Increased levels of butyrate in ileum and ascending colon of F+P
piglets, suggest the potential for interaction with histones in the colon, which would alter gene expression (Bartova et al, 2008).

Turning to gene expression profiles, we focused on expression of genes in the Wnt and BMP signaling pathways as they represent two major pathways implicated in the development of the fetal intestine are potentially influenced by epigenetic changes the (Scoville et al, 2008). The genes selected encode proteins that act as receptors, receptor antagonists and transcription factors and can either stimulate or inhibit cellular proliferation and differentiation (Scoville et al, 2008).

Greater abundance of gremlin mRNA in the ileum than the colon could indicate that there is more of a need to repress BMP in the ileum than the colon. sFRP-4 and DKK1, which were greater in the colon, both repress the Wnt pathway. Osteopontin, which is involved in programmed cell death and apoptosis and frizzled 6 complicate things as OPN was found in high levels in the ileum and frizzled 6, part of the Wnt pathway, in the colon. Taken together these gene expression levels could indicate that at these time points in intestinal development, the ileum repressed much of the activity of the BMP pathway in order to grow, however apoptosis is still needed for a healthy intestine and so some activity was still present (Scoville et al, 2008). The colon, which lacks the villi of the small intestine (Leser and Molbak, 2009), may have had less need of the proliferation seen in the ileum at this point in development as it does not undergo the same increase in surface area. Perhaps because of this, the Wnt pathway is more closely controlled, but not completely repressed as some proliferation is still necessary for normal growth.
From day 7 to day 14, gremlin, β-catenin, HNF-4α, and TNF-α expression increased in both the ileum and the colon. Only DKK1 and frizzled 6 showed decreased expression during this same time period, and in both cases the decreases were restricted to the colon. HNF-4α controls gene expression of genes that regulate the development of the intestines, among other organs. HNF-4α is known to interact with β-catenin, which up-regulates the Wnt pathway and increases cellular proliferation. Gremlin is part of the BMP pathway, but serves as a repressor. Therefore, gremlin, β-catenin, and HNF-4α are all involved with promoting the activity of the Wnt pathway, which increases cell proliferation (Scoville et al, 2008). Taken together, these genes indicate greater proliferation occurring in the colon and especially in the ileum at day 14 than day 7.

The decrease of DKK1 in the colon at day 14 would serve to increase Wnt activity as well as DKK1 is a negative regulator of Wnt. However, frizzled 6, which would up regulate the Wnt pathway, was also down regulated at day 14 in the colon. This could be due to using whole tissue samples which do not account for gene location.

A response to diet was found in several genes examined. The mRNA expression of gremlin and frizzled 6 were different in SR than F or F+P. Whereas, for HNF-4α, sFRP-and DKK1 expression, F+P differ from both F and SR. Lastly, TNF-α and osteopontin expression differed between SR and F+P, with neither different from F. Overall, gene expression patterns in the F+P piglets was never more similar to that of SR expression than that of F. Gene expression in the F+P group either more closely resembled F than SR or F+P was different from both. Thus, we can conclude that adding prebiotics did not change the expression of the Wnt/BMP pathway of F piglets towards
that of SR, but, for some genes, the addition of prebiotics led to more differences between SR expression than F.

Taken together, the results of this study demonstrate that gene expression varied between regions of the intestine and time points, the latter potentially due to developmental changes. Further, early nutrition impacted gene expression as well. The F+P fed piglets had longer colons, which may allow for more fermentation of prebiotics to occur, but this did not cause gene expression to shift towards that of SR piglets despite the increased amounts of SCFA. The effect of supplemental 4 g/L scFOS and PDX did not cause F+P piglets to more closely resemble SR than F as we had hypothesized. Therefore, the addition of prebiotics to piglet formula did not induce changes in gene expression of the Wnt/BMP pathways that more closely resembled SR expression than formula alone. There were often large amounts of variation that occurred between the animals. Part of this could be due to the use of whole tissue when extracting mRNA. Many of these genes exist in a gradient that runs along the crypt-villi axis and using whole tissue, which includes a combination of cell types, could reduce our ability to detect treatment differences. The next study seeks to address this by collecting exfoliated cells along the crypt-to-villus axis.
### Tables

**Table 3.1. Real Time qPCR Primers**

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Accession Number</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>WNT/BMP Pathway:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-catenin</td>
<td>CAAGGCTACCGTTGGATTTT</td>
<td>CTCTCGCAAAGGTCATGATT</td>
<td>EU431333.1</td>
</tr>
<tr>
<td>DKK1</td>
<td>TCTGTCCTGTCCCGATCATAGA</td>
<td>GGACCAGAAATGTCTTGCAACA</td>
<td>DQ517932.1</td>
</tr>
<tr>
<td>Frizzled6</td>
<td>CAGGGCACTGGCAGTCACT</td>
<td>ACATGAATCCACTTCCCCAGTAACA</td>
<td>XM_001928261.1</td>
</tr>
<tr>
<td>Gremlin</td>
<td>CGAGGAGGGCTGCAACAG</td>
<td>AGTTCGACTGGCCGTAGCA</td>
<td>XM_001925261.1</td>
</tr>
<tr>
<td>HNF-4α</td>
<td>GAGTACGCGCTGCTCAAAGC</td>
<td>GCCCCGGGTGGCTCAAC</td>
<td>DQ450900.1</td>
</tr>
<tr>
<td>sFRP-3</td>
<td>ATGACCAAGATGCCCAACCA</td>
<td>ACTGCTCAGTGGCGAGAT</td>
<td>XM_001926299.1</td>
</tr>
<tr>
<td>sFRP-4</td>
<td>GCAAGAGGAGTGGCTGAACGA</td>
<td>GGATTGGTGTAAGAAGACTTAAGA</td>
<td>NM_001128465.1</td>
</tr>
<tr>
<td><strong>Regulators of Intestinal Development:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Osteopontin exon 6</td>
<td>ACTTCTGATCCTCGTTCTTATG</td>
<td>CCATTATCTATGCAAAGGAAATCTACGA</td>
<td>AB530168.1</td>
</tr>
<tr>
<td>TGF β1</td>
<td>CCTGCAAGACCATCGACATG</td>
<td>GCCGAAGCTTGGACAGAAT</td>
<td>NM_214015.1</td>
</tr>
<tr>
<td>TNF α</td>
<td>AACCCTCAGATAAGGCCCGTG</td>
<td>ACCACCAGCTGTTGGCTTT</td>
<td>EU682384.1</td>
</tr>
<tr>
<td><strong>Villus-Crypt Epithelial Cell Markers:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>TCTTTACCTTTGGCGGGTTACA</td>
<td>TGTCCGTTCGCTACCA</td>
<td>AY145131.1</td>
</tr>
<tr>
<td>CDK5</td>
<td>GTCTGCTGCTGCTGCTGTAC</td>
<td>CCACCGAGGAGGGCTTGAG</td>
<td>NM_00110816.2</td>
</tr>
<tr>
<td>v-myb</td>
<td>CGAGGCCGGCATGAG</td>
<td>GGACTTGCGGACTTTCTTGATG</td>
<td>XM_00192436.1</td>
</tr>
<tr>
<td><strong>Loading Controls:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td>CACGGCAATCTCCTGCTGGA</td>
<td>AGCACCGTTGGCGGTAGAG</td>
<td>DQ845171.1</td>
</tr>
<tr>
<td>Tubulin</td>
<td>TCCATGCCCTCCTCTCTTGAC</td>
<td>CAGTAATGGGTGGCCCAATAAGG</td>
<td>XM_001925044.1</td>
</tr>
</tbody>
</table>
Table 3.2. Dry matter (DM), pH and SCFA concentrations (µmol/g) of ileum contents of 7-and 14-day-old piglets fed sow milk (SR), formula (F) or formula+prebiotics (F+P)\(^1,2\).

<table>
<thead>
<tr>
<th></th>
<th>DM (%)</th>
<th>pH</th>
<th>Acetate</th>
<th>Propionate</th>
<th>Butyrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>18.1 ± 3.13(^b)</td>
<td>7.6 ± 0.27(^c)</td>
<td>5.9 ± 3.03(^a)</td>
<td>0.47 ± 0.38(^a)</td>
<td>0.14 ± 0.13(^a)</td>
</tr>
<tr>
<td>F+P</td>
<td>16.3 ± 4.45(^b)</td>
<td>7.3 ± 0.24(^c)</td>
<td>12.6 ± 11.3(^a)</td>
<td>0.82 ± 0.94(^a)</td>
<td>0.25 ± 0.34(^a)</td>
</tr>
<tr>
<td>SR</td>
<td>11.0 ± 2.91(^a)</td>
<td>7.6 ± 0.38(^c)</td>
<td>7.8 ± 4.25(^a)</td>
<td>0.60 ± 0.25(^a)</td>
<td>0.23 ± 0.27(^a)</td>
</tr>
<tr>
<td>Day 14</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>28.3 ± 4.7(^c)</td>
<td>6.6 ± 0.33(^b)</td>
<td>19.1 ± 9.6(^a,b)</td>
<td>2.7 ± 3.3(^b)</td>
<td>1.6 ± 1.4(^b)</td>
</tr>
<tr>
<td>F+P</td>
<td>24.8 ± 7.3(^c)</td>
<td>6.1 ± 0.16(^a)</td>
<td>36.9 ± 14.6(^c)</td>
<td>4.3 ± 2.3(^b)</td>
<td>2.3 ± 1.0(^b)</td>
</tr>
<tr>
<td>SR</td>
<td>22.7 ± 3.6(^c)</td>
<td>6.8 ± 0.31(^b)</td>
<td>24.0 ± 7.2(^c)</td>
<td>6.9 ± 2.7(^c)</td>
<td>3.9 ± 1.70(^c)</td>
</tr>
</tbody>
</table>

\(^1\)Values are presented as means ± SEM

\(^2\)Within day, means in a column without a common letter differ, p < 0.05.
Table 3.3. *Dry matter (DM), pH and SCFA concentrations (µmol/g) of ascending colon contents of 7-and 14-day-old piglets fed sow milk (SR), formula (F) or formula+prebiotics (F+P)¹,².*

<table>
<thead>
<tr>
<th></th>
<th>DM (%)</th>
<th>pH</th>
<th>Acetate</th>
<th>Propionate</th>
<th>Butyrate</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Day 7</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>28.3 ± 4.7&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>6.6 ± 0.33&lt;sup&gt;b&lt;/sup&gt;</td>
<td>19.1 ± 9.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.7 ± 3.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.6 ± 1.4&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>F+P</td>
<td>24.8 ± 7.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.1 ± 0.16&lt;sup&gt;a&lt;/sup&gt;</td>
<td>36.9 ± 14.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.3 ± 2.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.3 ± 1.0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>SR</td>
<td>22.7 ± 3.6&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>6.8 ± 0.31&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>24.0 ± 7.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.9 ± 2.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.9 ± 1.7&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Day 14</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>30.0 ± 2.6&lt;sup&gt;c&lt;/sup&gt;</td>
<td>6.20 ± 0.19&lt;sup&gt;b&lt;/sup&gt;</td>
<td>20.8 ± 11.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.1 ± 2.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.5 ± 1.0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>F+P</td>
<td>25.5 ± 3.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.95 ± 0.23&lt;sup&gt;a&lt;/sup&gt;</td>
<td>32.6 ± 14.9&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>7.0 ± 3.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.4 ± 1.1&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>SR</td>
<td>21.5 ± 3.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.93 ± 0.33&lt;sup&gt;c&lt;/sup&gt;</td>
<td>24.1 ± 9.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.9 ± 3.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.3 ± 1.6&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

¹Values are presented as means ± SEM
²Means in a column without a common letter differ, p < 0.05.
Table 3.4. Gene expression in ileum of 7 and 14-day-old piglets fed sow milk (SR), formula-fed (F) or formula+prebiotics (F+P)\textsuperscript{1,2}.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Age (d)</th>
<th>Treatment Group</th>
<th>Overall P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>F</td>
<td>F+P</td>
</tr>
<tr>
<td>Gremlin</td>
<td>7</td>
<td>3.77 ± 3.58</td>
<td>0.53 ± 0.33</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>14.1 ± 5.87</td>
<td>22.8 ± 8.47</td>
</tr>
<tr>
<td>β-catenin</td>
<td>7</td>
<td>0.10 ± 0.11</td>
<td>0.03 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>3.72 ± 1.30</td>
<td>5.33 ± 2.57</td>
</tr>
<tr>
<td>sFRP-3</td>
<td>7</td>
<td>1.20 ± 1.50</td>
<td>0.17 ± 0.10</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>2.25 ± 1.49</td>
<td>1.96 ± 0.17</td>
</tr>
<tr>
<td>sFRP-4</td>
<td>7</td>
<td>5.44 ± 9.71</td>
<td>0.87 ± 0.52</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>2.13 ± 0.85</td>
<td>1.72 ± 0.54</td>
</tr>
<tr>
<td>frizzled6</td>
<td>7</td>
<td>2.72 ± 4.09</td>
<td>1.41 ± 1.31</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>1.23 ± 0.50</td>
<td>0.98 ± 0.43</td>
</tr>
<tr>
<td>DKK1</td>
<td>7</td>
<td>20.5 ± 37.77</td>
<td>2.90 ± 2.21</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>3.33 ± 1.67</td>
<td>3.02 ± 1.49</td>
</tr>
<tr>
<td>Osteopontin</td>
<td>7</td>
<td>5.96 ± 9.11</td>
<td>0.79 ± 0.61</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>7.68 ± 4.27</td>
<td>4.07 ± 0.46</td>
</tr>
<tr>
<td>HNF-4α</td>
<td>7</td>
<td>0.46 ± 0.20</td>
<td>0.84 ± 0.38</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>3.05 ± 1.67</td>
<td>6.15 ± 3.03</td>
</tr>
<tr>
<td>TNF-α</td>
<td>7</td>
<td>1.94 ± 1.92</td>
<td>1.09 ± 0.55</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>12.41 ± 6.54</td>
<td>11.83 ± 10.24</td>
</tr>
</tbody>
</table>

\textsuperscript{1}Mean ± SEM; \textsuperscript{2}Data are expressed as normalized target relative to β-actin expression
Table 3.5. Gene expression in colon of 7- and 14-day-old piglets fed sow milk (SR), formula-fed (F) or formula+prebiotics (F+P).\(^1\)\(^2\).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Age (d)</th>
<th>Treatment Group</th>
<th>Overall P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>F</td>
<td>F+P</td>
</tr>
<tr>
<td>Gremlin</td>
<td>7</td>
<td>0.13 ± 0.06</td>
<td>0.10 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>3.90 ± 2.71</td>
<td>3.41 ± 0.74</td>
</tr>
<tr>
<td>β-catenin</td>
<td>7</td>
<td>0.09 ± 0.03</td>
<td>0.11 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>2.41 ± 1.45</td>
<td>3.11 ± 0.98</td>
</tr>
<tr>
<td>sFRP-3</td>
<td>7</td>
<td>0.09 ± 0.02</td>
<td>0.08 ±0.02</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>2.62 ± 1.13</td>
<td>6.41 ± 6.94</td>
</tr>
<tr>
<td>sFRP-4</td>
<td>7</td>
<td>37.4 ± 14.33</td>
<td>24.9±22.36</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>8.06 ± 3.80</td>
<td>41.88±71.8</td>
</tr>
<tr>
<td>frizzled6</td>
<td>7</td>
<td>39.9 ± 18.45</td>
<td>25.1 ± 10.66</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>2.62 ± 1.01</td>
<td>5.25 ± 4.82</td>
</tr>
<tr>
<td>DKK1</td>
<td>7</td>
<td>87.4 ± 60.92</td>
<td>31.9 ± 19.37</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>0.99 ± 0.89</td>
<td>1.44 ± 1.17</td>
</tr>
<tr>
<td>Osteopontin</td>
<td>7</td>
<td>0.21 ± 0.08</td>
<td>0.25 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>0.92 ± 1.08</td>
<td>0.74 ± 0.32</td>
</tr>
<tr>
<td>HNF-4α</td>
<td>7</td>
<td>0.09 ± 0.04</td>
<td>0.12 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>3.66 ± 1.76</td>
<td>6.19 ± 2.34</td>
</tr>
<tr>
<td>TNF-α</td>
<td>7</td>
<td>1.11 ± 0.28</td>
<td>0.91 ± 0.13</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>5.36 ± 5.69</td>
<td>10.04±8.06</td>
</tr>
</tbody>
</table>

\(^1\) Mean ± SEM; \(^2\) Data are expressed as normalized target relative to β-actin expression
Figures

Figure 3.1. *Body weight (kg) of piglets through day 14 of age.* Piglets were fed either sow milk (SR), formula (F) or formula+prebiotics (F+P). Means without a common letter differ, $P \leq 0.05$. F and SR piglets were significantly heavier than F+P on d11-14. Data are expressed as means ± SD.
Figure 3.2. Small intestinal weight (Panel A) and length (Panel B) normalized per kg body weight of 7- and 14-day-old piglets fed sow milk (SR), formula (F) or formula+prebiotics (F+P). No significant effect of diet was observed within day. Data are expressed as means ± SD.
Figure 3.3. Analysis of absolute colon length (Panel A) and colon length per kg body weight (Panel B) of 7-day-old piglets fed sow milk (SR), formula (F) or formula+prebiotics (F+P). Means without a common letter differ, P ≤ 0.05. Data are expressed as means ± SD.
Figure 3.4. Analysis of absolute colon length (Panel A) and colon length per kg body weight (Panel B) of 14-day-old piglets fed sow milk (SR), formula (F) or formula+prebiotics (F+P). Means without a common letter differ, $P \leq 0.05$. Data are expressed as means ± SD.
**Figure 3.5.** Analysis of gremlin mRNA expression in ileum (Panel A) and ascending colon (Panel B) of 7- and 14-day-old piglets fed sow milk (SR), formula (F) or formula+prebiotics (F+P). Gremlin expression was normalized using β-actin. Means without a common letter differ, P ≤ 0.05. Data are expressed as means ± SD.
Figure 3.6. Analysis of β-catenin mRNA expression in ileum (Panel A) and ascending colon (Panel B) of 7- and 14-day-old piglets fed sow milk (SR), formula (F) or formula+prebiotics (F+P). β-catenin expression was normalized using β-actin. Means without a common letter differ, P ≤ 0.05. Data are expressed as means ± SD.
Figure 3.7. Analysis of sFRP-3 mRNA expression in ileum (Panel A) and ascending colon (Panel B) of 7- and 14-day-old piglets fed sow milk (SR), formula (F) or formula+prebiotics (F+P). sFRP-3 expression was normalized using β-actin. Means without a common letter differ, P ≤ 0.05. Data are expressed as means ± SD.
Figure 3.8. Analysis of sFRP-4 mRNA expression in ileum (Panel A) and ascending colon (Panel B) of 7- and 14-day-old piglets fed sow milk (SR), formula (F) or formula+prebiotics (F+P). sFRP-4 expression was normalized using β-actin. No differences were found between treatment groups or age, P ≤ 0.05. Data are expressed as means ± SD.
Figure 3.9. Analysis of frizzled 6 mRNA expression in ileum (Panel A) and ascending colon (Panel B) of 7- and 14-day-old piglets fed sow milk (SR), formula (F) or formula+prebiotics (F+P). Frizzled 6 expression was normalized using β-actin. Means without a common letter differ, P ≤ 0.05. Data are expressed as means ± SD.
Analysis of DKK1 mRNA expression in ileum (Panel A) and ascending colon (Panel B) of 7- and 14-day-old piglets fed sow milk (SR), formula (F) or formula+prebiotics (F+P). DKK1 expression was normalized using β-actin. Means without a common letter differ, P ≤ 0.05. Data are expressed as means ± SD.
Figure 3.11. Analysis of osteopontin mRNA expression in ileum (Panel A) and ascending colon (Panel B) of 7- and 14-day-old piglets fed sow milk (SR), formula (F) or formula+prebiotics (F+P). Osteopontin expression was normalized using β-actin. Means without a common letter differ, P ≤ 0.05. Data are expressed as means ± SD.
Figure 3.12. Analysis of HNF-4α mRNA expression in ileum (Panel A) and ascending colon (Panel B) of 7- and 14-day-old piglets fed sow milk (SR), formula (F) or formula+prebiotics (F+P). HNF-4α expression was normalized using β-actin. Means without a common letter differ, P ≤ 0.05. Data are expressed as means ± SD.
Figure 3.13. Analysis of TNF-α mRNA expression in ileum (Panel A) and ascending colon (Panel B) of 7- and 14-day-old piglets fed sow milk (SR), formula (F) or formula+prebiotics (F+P). TNF-α expression was normalized using β-actin. Means without a common letter differ, P ≤ 0.05. Data are expressed as means ± SD.
Chapter 4

Comparison of Wnt and BMP pathways between Neonatal Piglets Fed Sow Milk or Infant Formula

Introduction

The major pathways that have been implemented in the development of the fetal intestine include the Wnt and bone morphogenic protein (BMP) pathways, which regulate intestinal stem cells (ISC) to control the growth, development and maintenance of the intestine. The Wnt pathway plays an important role in the regulation of intestinal ISC which are located at the crypts of the intestine and are responsible for creating all types of mature intestinal cells. The Wnt pathway is most active in the crypts, where proliferation and differentiation takes place (Scoville et al, 2008). The BMP pathway is also involved in the maintenance and control of the intestine and opposes the Wnt pathway to stop proliferation from occurring. It is involved in controlled cell death and its activity increases up the villi as the activity of the Wnt pathway decreases (Scoville et al, 2008).

As the Wnt/BMP pathway is known to exist in a gradient along the crypt-villus axis, with Wnt expression dominating in the crypt region and BMP expression dominating in the villi, it was possible that pooling whole tissue in Chapter 3 reduced our ability to detect treatment effects that would be concentrated in either region. A method was therefore developed to remove intestinal epithelial cells along the crypt-villus axis. Isotonic solution was added to segments and incubated at increasing time points to
remove epithelial cells along the crypt-villus axis in the hopes of removing variation from within our groups to better understand the effect of prebiotics in future studies.

**Materials and Methods**

*Animal Study*

All animal protocols and procedures were approved by the University of Illinois Institutional Animal Care and Use Committee. Pregnant sows from the University of Illinois research farm were allowed to farrow naturally. Piglets remained with the sows for two days after birth in order to receive colostrum. On the second day of life, piglets were transferred from the farm to an on-campus animal facility where they were randomized into sow reared (SR) and formula (F) groups. SR piglets were placed with a sow who was not necessarily their own. Formula was a medicated pig milk replacer (Advance Liqui-Wean, MSC Specialty Nutrition, Dundee, IL). F piglets were given 360ml/kg/day and were fed every hour using an electronic pump. Diets were prepared as 20% solids, following the formula specification sheet. Formula intake and piglet body weight were assessed daily.

*Sample Collection*

Piglets were euthanized on day 23 of study (day 25 of life) as described in Chapter 3.
Isolation of Intestinal Epithelial Cells Along the Crypt-Villus Axis.

The isolation protocol was modified from Fan and colleagues (2001). Briefly, the following changes were made: intestine was cut open instead of filled with fluid, as this made changing the incubation fluid more convenient; PMSF was excluded as proteins were not being analyzed in our study; and the temperature of the exfoliation solution was decreased from 37 °C to 4 °C in order to maintain RNA integrity.

Ileum (~25 cm) was collected and rinsed with ice cold PBS. The ileum was cut open and divided into several segments and placed into a 50 ml conical tube containing an ice cold buffer of 5mM dextrose (Fisher Scientific, Pittsburgh, Pa), 1.5mM EDTA (Sigma Aldrich, St. Louis, Mo), 1mM EGTA (Sigma Aldrich, St. Louis, Mo), and 0.5mM DTT (Fisher Scientific, Pittsburgh, Pa). The buffer was oxygenated and kept on ice. The tube was agitated on ice in duplicate five-minute, ten-minute, fifteen-minute and twenty-minute incubations (Table 4.1). Epithelial cells from each of the two time points were pooled so that four time segments remained. Before the last incubation, the ileal segment was scraped with a glass slide to help disassociate remaining tissue. All incubations were centrifuged and the aqueous layer was decanted. Immediately after collection, cells were washed with cold PBS, centrifuged at 500 x g for 5 minutes at 4º C, decanted, and preserved with 5 ml TRIzol Reagent (Invitrogen, Carlsbad, CA) per time point. Cells were flash frozen in liquid nitrogen before being stored at -80 C. Samples were then used to measure mRNA levels using quantitative real-time PCR. After each time point, a section of ileum was removed from the tube and preserved in 10% formalin for histomorphology.
Isolation of mRNA and Quantitative Real-Time PCR

Messenger RNA was isolated and RT-qPCR performed as described in Chapter 3.

Histomorphology

Ileal sections fixed in 10% formalin were transferred to 70% EtOH and were embedded in paraffin, sliced with a microtome, and stained with hematoxylin and eosin by the Clinical Laboratory at the University of Illinois School of Veterinary Medicine. Images were captured using a NanoZoomer Digital Pathology microscope (Olympus, Center Valley, PA) to determine order and amount of tissue collected in each pooled sample.

Statistical Analyses

Statistics were run as described in Chapter 3.
Results

Body Weight

Piglets grew differently depending on which diet they were given (Figure 4.1). SR piglets were larger by day 5 of the study (day 7 of life) and remained larger throughout the study. The difference in the size of the animals was likely due to there being too few piglets on the sow to account for her milk production. This would allow the SR piglets to receive more milk than the F piglets that were given formula according to their body weights.

Intestinal Weight and Length

The length of the small intestine per body weight differed between F and SR at day 25 of life (Figure 4.2), with F animals having longer small intestines per kg body weight. When segment weight of the small intestine was compared, F animals had more intestinal contents per body weight in duodenum and jejunum. However, in the ileum, SR had greater contents per kg body weight than F.

Isolation of Intestinal Epithelial Cells Along the Crypt-Villus Axis

Sections of the ileum remaining after each incubation are shown in Figure 4.3. From the stained and cut slides, it was determined that the incubations removed the cells from the villi as well as other cell types, including lamina propria and goblet cells. Many crypts remained (Panel 4) despite the removal of other tissues. In Panels 1, 2 and 3
mostly epithelial cells were removed. In panel 4, other non-epithelial tissue was removed as well.

**Gene Expression**

The expression of all genes in the ileum of 25-day-old piglets in the three treatment groups is summarized in Table 4.2. Only Frizzled 6, osteopontin and sFRP-3 did not reach statistical significances.

**Gremlin:** Expression of gremlin in the ileum of 25-day-old pigs is summarized in Figure 4.4. Gremlin expression differed by collection method, with the expression of whole tissue being greater than pooled epithelial cells (p≤0.05). Expression differed by diet in the whole tissue samples with expression higher in SR than F, but did not differ in the pooled epithelial cells. Expression did not differ at any point within the collections.

**β-catenin:** Expression of β-catenin in the ileum of 25-day-old pigs is summarized in Figure 4.5. β-catenin expression differed by collection method, with the expression of whole tissue being less than pooled epithelial cells (p≤0.05). Expression differed by diet in the pooled epithelial samples with expression lower in SR than F, but did not differ in the whole tissue. Expression within the epithelial collections differed, with expression greater in F throughout collection time 3. F and SR were different at both collection time 3 and 4.
sFRP-3: Expression sFRP-3 in the ileum of 25-day-old pigs is summarized in Figure 4.6a. SFRP-3 expression did not differ by collection method, diet or within washes.

sFRP-4: Expression of sFRP-4 in the ileum of 25-day-old pigs is summarized in Figure 4.6b. sFRP-3 expression differed by collection method, with the expression of whole tissue being greater than pooled epithelial cells (p≤0.05). Expression did not differ by diet or through collection points.

Frizzled 6: Expression of frizzled 6 in the ileum of 25-day-old piglets is summarized in Figure 4.7. Frizzled 6 expression did not differ by collection method or by diet. Expression within the epithelial collections differed, with expression between time point 1 and 3 dropping significantly in both diets. At time point 3, SR expression was greater than F. At time point 4, F expression rose as SR dropped.

DKK1: Expression of DKK1 in the ileum of 25-day-old pigs is summarized in Figure 4.8. DKK1 expression differed by collection method, with the expression of SR whole tissue being greater than SR pooled epithelial cells (p≤0.05). There was no difference between collection methods of F. Expression differed by diet in the whole tissue samples with expression greater in SR than F, but did not differ in the pooled epithelial cells. Expression within the epithelial collections did not differ.

Osteopontin: Expression of osteopontin in the ileum of 25-day-old pigs is summarized in Figure 4.9. Osteopontin expression did not differ by collection method, by diet, or within the epithelial collections.
HNF-4α: Expression of HNF-4α in the ileum of 25-day-old pigs is summarized in Figure 4.10. HNF-4α expression differed by collection method, with the expression of whole tissue being less than that of pooled epithelial cells (p≤0.05). Expression did not differ by diet. Expression within the epithelial collections did differ, with expression increasing significantly through collection time 3. At time point 4, F expression remained steady while SR expression fell. The diets differed only at collection point 4.

TNF-α: Expression of TNF-α in the ileum of 25-day-old pigs is summarized in Figure 4.11. TNFα expression differed by collection method, with the expression of F whole tissue being greater than F pooled epithelial cells (p≤0.05). There was no difference of collection method for SR. Expression differed by diet, with whole tissue F being greater than SR. Within the epithelial cell collections, TNF-α expression in both treatment groups dropped between collection time 1 and 2, rose at collection time 3 and dropped again at time 4. The two treatment groups differed at collection time 2, with F expression being greater than SR.

TGF-β: Expression of TGF-β in the ileum of 25-day-old pigs is summarized in Figure 4.12. TGF-β expression differed by collection method, with the expression of F whole tissue being greater than F pooled epithelial cells (p≤0.05). There was no difference of collection method for SR. Expression differed by diet, with whole tissue F being greater than SR. Within the epithelial cell collections, expression of TGF-β dropped through the collection points, with time point 4 being different than 1 for both diet groups. F and SR were different from each other only at collection point 1.
Crypt markers: CKD5 and v-myb were chosen as crypt markers (Figure 4.13). Whole tissue expression was not determined. Within the epithelial cell collections, CDK5 expression varied little through the time points and only differed between the treatment groups after the first incubation time. V-myb mRNA expression increased through the incubation times and differed significantly between the treatment groups at collection point 3.
Discussion

The goal of Chapter 4 was to determine if removing cells along the villus-to-crypt axis would allow for more sensitive determination of whether early nutrition impacts expression of genes in the Wnt/BMP pathways. For this purpose, a published protocol for exfoliating epithelial cells (Fan et al, 2001) was modified in several ways. The most significant change was to reduce the incubation temperature from 37 °C to 4 °C.

This change was necessary in order to maintain high quality mRNA. However, examination of the morphology of the ileum after being subjected to the epithelial cell collections, made it evident that the incubation times did not remove tissue entirely in the desired manner. Epithelial cells from the villi were removed, but so too were other parts of the intestine, including from the lamina propria and goblet cells. While the epithelial cell collections allowed for collection of cells located along the villi, most crypts were still intact after the final incubation. Thus, much of the stem cell material was not removed from the tissue, therefore, genes expressed highly in the crypt were not well represented in the exfoliated cells.

No differences were seen within or between whole tissue and collected epithelial cells for osteopontin and sFRP-3. This could indicate that these two genes are expressed more at the villi of the intestine than at the crypt, which would account for our ability to collect them more evenly. Osteopontin, which is involved in controlled cell death, and sFRP-3, which represses the Wnt pathway were expected to be expressed at higher levels in villus than crypt cells, as the top of the villi is primarily where apoptosis and the repression of Wnt are most needed. No differences were seen within the collections,
however, that could be indicative of how cells pulled off and our failure to reach the crypt more than the actual lack of decreasing mRNA levels towards the crypt.

Several genes were expressed differently when comparing whole tissue vs. a pool of exfoliated cells. Most genes had lower expression in the pooled epithelial cells than in whole tissue. Only HNF-4α and β-catenin showed higher expression in the pooled epithelial cells than in the whole tissue. Neither of these would be expected to be confined to the crypt (Scoville et al, 2008), and so perhaps it is not surprising that expression was higher without crypt collection. DKK1 and sFRP-4 are both Wnt inhibitors and were found in higher amounts in whole tissue. DKK1 is mostly located in the crypts and so it is not unexpected that expression would be higher in whole tissue where crypts were included in mRNA isolation. sFRP-4 would be expected to be located in the villi, which was successfully collected. This could indicate a bias that was introduced by the exclusion of crypts while including surrounding non-enterocyte tissue. This expression could also be a result of degraded mRNA, particularly of housekeeping gene tubulin. Lower levels of tublin would cause a higher normalized target of the gene of interest. TNF-α and TGF-β expression was also greater in whole tissue. These genes are not localized at one point in intestine and are expressed in non-enterocyte cells as well (Zabielski et al, 2008), which could account for greater whole tissue expression. Overall these results seem to indicate that the cell collection method did not allow for complete epithelial cell isolation.

In whole tissue samples, differences between SR and F diet treatments were present. TNF-α, which is involved in inflammatory processes, had greater expression of F than SR, indicating that these animals were at a higher inflammatory state. The SR
animals were somewhat protected from this inflammation and expressed lower levels of TNF-α. TGF-β, which is involved in proliferation and cellular differentiation, was also increased in F. This indicates, along with increased expression of gremlin, a BMP repressor, and lower DKK1, a Wnt repressor, that F animals were undergoing greater rates of proliferation than SR animals at day 25 of age.

Within the pooled epithelial cell collections, only β-catenin (Figure 4.5) showed a difference between diet treatments F and SR. Although expression was higher than the whole tissue results, the trend was the same with F animals having greater amounts of ileal β-catenin than SR. This is consistent with whole tissue findings and suggests that F animals have greater proliferation than SR at day 25 of life. As β-catenin was the only gene that showed any difference in the pooled epithelial cell collection, we must consider that this may be due to the failure to collect many of the cells where differences would be seen, particularly the crypts.

Differences between the collection points of the epithelial cell collections were seen in the expression of β-catenin, HNF-4α, frizzled 6, TNF-α and TGF-β. β-catenin and HNF-4α (which interacts with β-catenin) were both found to increase through collection time 3. Incubation 4 was the most variable, which is not surprising as no crypts and large amounts of non-enterocyte tissue were collected. β-catenin showed higher expression of F than SR, which follows with trend seen in whole tissue collection. F and SR were different at collection times 3 and 4. HNF-4α had similar expression of F and SR which is consistent with the whole tissue findings. The only difference between the diet groups was at incubation 4.
Frizzled 6, which we would expect to find in the crypt, demonstrated decreased expression from collection times 1 to 3. This is not in line with what we would have expected. F and SR showed similar expression through the collection times, only differing at time point 3. This is consistent with what we saw in whole tissue. The decrease through the crypt-villus axis was unexpected, but could be due to cells pulling off at differing rates.

TNF-α and TGF-β expression was fairly consistent through the collection times, with the exception of TGF-β expression in cells from F piglets, which showed decreased expression between incubations 1 and 2. Expression was consistent with whole tissue results, which showed F piglets as having a higher expression of TGF-β than SR. TNF-α showed diet effects, which was consistent with whole tissue results where F was greater than SR. These genes were not expected to be localized at one place through the villi, and we did not see a consistent trend in expression in cells from incubations 1-3.

The crypt markers, CDK5 and v-myb, were measured in order to assess whether expression of genes that are known to exist in the crypt would express properly in our method. The expression was expected to increase through the incubations as the crypt was approached. While this did seem to be the case to some extent in v-myb, CKD5 expression was mostly flat. This only serves to confirm what was determined from the stained cells, that the incubations failed to fully recover all the crypts.

From the whole tissue data, we found that F piglets had greater rates of proliferation and inflammation than their SR counterparts. This is possibly due to their delayed growth, however, it could be in response diet as well. Future work is needed to
conclude which factors contributed to the increased activity of the Wnt pathway. From this study, we conclude that the epithelial cell collection method used was not completely successful. While this approach did remove cells in the correct order from villus-to-crypt for the first three incubations, the last incubation, which involved scraping the tissue, seemed to pull off much unwanted tissue while leaving the crypts intact. We did not, therefore, see lower variation in our samples which we had hoped to see along with an enrichment of the genes. New isolation methods to extract cells along the crypt-villus axis should be considered, including the use of a laser capture microscope.
### Table 4.1. Description of how pools of exfoliated cells were created

<table>
<thead>
<tr>
<th>Pool Number</th>
<th>Incubation Time (min)</th>
<th>Total Time Exposed to Exfoliation Solution (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2 incubations @ 5 min each</td>
<td>0 - 10</td>
</tr>
<tr>
<td>2</td>
<td>2 incubations @ 10 min each</td>
<td>11 - 30</td>
</tr>
<tr>
<td>3</td>
<td>2 incubations @ 15 min each</td>
<td>31 - 60</td>
</tr>
<tr>
<td>4</td>
<td>2 incubations @ 20 min each</td>
<td>61 - 100</td>
</tr>
</tbody>
</table>
Table 4.2. Gene expression in ileum of 25-day-old piglets fed sow milk (SR) or formula-fed (F)\textsuperscript{1,2}.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Collection</th>
<th>Method</th>
<th>Treatment Group</th>
<th>Overall P-value</th>
<th>Trmt*Collection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gremlin</td>
<td>F</td>
<td>SR</td>
<td>Model</td>
<td>Trmt</td>
<td>Collection</td>
</tr>
<tr>
<td></td>
<td>whole tissue</td>
<td>13.6 ±13.0</td>
<td>1.95 ± 1.05</td>
<td>&lt;0.0001</td>
<td>0.0004</td>
</tr>
<tr>
<td></td>
<td>pooled cells</td>
<td>0.46 ± 0.38</td>
<td>0.10 ± 0.08</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-catenin</td>
<td>whole tissue</td>
<td>0.94 ± 0.70</td>
<td>0.21 ± 0.12</td>
<td>0.0001</td>
<td>0.009</td>
</tr>
<tr>
<td></td>
<td>pooled cells</td>
<td>2.28 ± 1.00</td>
<td>1.41 ± 0.76</td>
<td></td>
<td></td>
</tr>
<tr>
<td>sFRP-3</td>
<td>whole tissue</td>
<td>1.29 ± 1.29</td>
<td>0.46 ± 0.26</td>
<td>0.09</td>
<td></td>
</tr>
<tr>
<td></td>
<td>pooled cells</td>
<td>0.53 ± 0.37</td>
<td>0.52 ± 0.56</td>
<td></td>
<td></td>
</tr>
<tr>
<td>sFRP-4</td>
<td>whole tissue</td>
<td>1.26 ± 0.98</td>
<td>1.25 ± 1.31</td>
<td>0.0003</td>
<td>0.81</td>
</tr>
<tr>
<td></td>
<td>pooled cells</td>
<td>0.25 ± 0.28</td>
<td>0.14 ± 0.34</td>
<td></td>
<td></td>
</tr>
<tr>
<td>frizzled6</td>
<td>whole tissue</td>
<td>1.64 ± 1.23</td>
<td>0.82 ± 0.66</td>
<td>0.39</td>
<td></td>
</tr>
<tr>
<td></td>
<td>pooled cells</td>
<td>1.02 ± 0.69</td>
<td>1.12 ± 0.73</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DKK1</td>
<td>whole tissue</td>
<td>0.83 ± 0.57</td>
<td>2.42 ± 2.55</td>
<td>0.001</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>pooled cells</td>
<td>0.29 ± 0.28</td>
<td>0.44 ± 0.53</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Osteopontin</td>
<td>whole tissue</td>
<td>0.24 ± 0.20</td>
<td>0.72 ± 0.54</td>
<td>0.16</td>
<td></td>
</tr>
<tr>
<td></td>
<td>pooled cells</td>
<td>0.17 ± 0.29</td>
<td>0.52 ± 0.68</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HNF-4α</td>
<td>whole tissue</td>
<td>1.08 ± 0.88</td>
<td>0.14 ± 0.09</td>
<td>0.0042</td>
<td>0.047</td>
</tr>
<tr>
<td></td>
<td>pooled cells</td>
<td>2.33 ± 1.02</td>
<td>1.65 ± 1.27</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>whole tissue</td>
<td>8.96 ± 8.12</td>
<td>2.50 ± 0.80</td>
<td>&lt;0.0001</td>
<td>0.002</td>
</tr>
<tr>
<td></td>
<td>pooled cells</td>
<td>0.91 ± 0.36</td>
<td>0.60 ± 0.33</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TGF-β</td>
<td>whole tissue</td>
<td>5.49 ± 4.25</td>
<td>2.11 ± 0.82</td>
<td>0.0004</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>pooled cells</td>
<td>2.35 ± 0.94</td>
<td>1.76 ± 0.67</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{1}Mean ± SEM;

\textsuperscript{2}Data are expressed as normalized target relative to tubulin expression.
Figures

**Figure 4.1.** Analysis of body weight of piglets through 23 days. Piglets were fed either sow milk (SR), or formula (F). Means without a common letter differ, P ≤ 0.05. Differences between F and SR animals begins at day 5 and continues through day 23. Data are expressed as means ± SD.
Figure 4.2. Analysis of intestinal length per kg body weight (Panel A) and intestinal weight per kg body weight (Panel B) of 25-day-old piglets fed sow milk (SR) or formula (F). Means without a common letter differ, P ≤ 0.05. Data are expressed as means ± SD.
Figure 4.3. Images of stained ileum from after each of the four intestinal epithelial incubations was completed. Tissue was fixed in ethanol and stained with H&E stain. Tissue sections were taken after 10 (Panel 1), 30 (Panel 2), 60 (Panel 3) and 100 (Panel 4) minutes of exposure to the dissociation solution.
Figure 4.4. Analysis of gremlin mRNA expression in ileum of 25-day-old piglets fed sow milk (SR) or formula (F). Gremlin expression was normalized using tubulin. Whole ileal tissue vs. the pool of exfoliated epithelial cells collected along the crypt-villus axis (collection times 1-4) are Means without a common letter differ, P ≤ 0.05. Data are expressed as means ± SD. Incubation time points were not different and so data is not shown.
The β-catenin mRNA expression in ileum of 25-day-old piglets fed sow milk (SR) or formula (F) was analyzed. Panel A compares expression in whole ileal tissue vs. the pool of exfoliated epithelial cells (collection times 1-4). In panel A, means without a common letter differ (P ≤ 0.05). Panel B shows β-catenin expression in exfoliated epithelial cells across the villus-to-crypt axis. Collection times represent: 1 (0-10 min); 2 (11-30 min); 3 (31-60 min); and 4 (61-100 min). Within dietary treatment group, means without a common letter differ between collection times (P ≤ 0.05). Asterisks denote differences between treatments at that collection time point (P ≤ 0.05). Data are expressed as mean ± SD.
Figure 4.6. Analysis of sFRP-3 (Panel A) and sFRP-4 (Panel B) mRNA expression in ileum of 25-day-old piglets fed sow milk (SR) or formula (F). sFRP-3 and sFRP-4 expression was normalized using tubulin. Whole ileal tissue versus the pool of exfoliated epithelial cells collected along the crypt-villus axis (collection times 1-4) are compared. Means without a common letter differ, P ≤ 0.05. Data are expressed as means ± SD. Incubation time points were not different and so data is not shown.
Figure 4.7. Analysis of frizzled 6 mRNA expression in ileum of 25-day-old piglets fed sow milk (SR) or formula (F). Frizzled 6 expression was normalized using tubulin. Panel A compares expression in whole ileal tissue vs. the pool of exfoliated epithelial cells (collection times 1-4). In panel A, means without a common letter differ (P ≤ 0.05). Frizzled 6 expression in exfoliated epithelial cells across the villus-to-crypt axis are shown in Panel B. Collection times represent: 1 (0-10 min); 2 (11-30 min); 3 (31-60 min); and 4 (61-100 min). Within dietary treatment group, means without a common letter differ between collection times (P ≤ 0.05). Asterisks indicate differences between treatments at that collection time point (P ≤ 0.05). Data are expressed as mean ± SD.
Figure 4.8. Analysis of DKK1 mRNA expression in ileum of 25-day-old formula-fed (F) or sow-reared (SR) piglets. DKK1 expression was normalized using tubulin. Whole ileal tissue versus the pool of exfoliated epithelial cells collected along the crypt-villus axis (collection times 1-4) are compared. Means without a common letter differ, P ≤ 0.05. Data are expressed as means ± SD. Incubation time points were not different and so data is not shown.

Figure 4.9. Analysis of osteopontin mRNA expression in ileum of 25-day-old piglets fed sow milk (SR) or formula (F). Osteopontin expression was normalized using tubulin. Whole ileal tissue vs. the pool of exfoliated epithelial cells collected along the crypt-villus axis (collection times 1-4) are compared. No significant differences between dietary treatments or whole vs. exfoliated cells were observed (p > 0.05). Data are expressed as means ± SD. Incubation time points were not different and so data is not shown.
Figure 4.10. Analysis of HNF-4α mRNA expression in ileum of 25-day-old piglets fed sow milk (SR) or formula (F). HNF-4α expression was normalized using tubulin. Panel A compares expression in whole ileal tissue vs. the pool of exfoliated epithelial cells (collection times 1-4). In panel A, means without a common letter differ (P ≤ 0.05). HNF-4α expression in exfoliated epithelial cells across the villus-to-crypt axis are shown in Panel B. Collection times represent: 1 (0-10 min); 2 (11-30 min); 3 (31-60 min); and 4 (61-100 min). Within dietary treatment group, means without a common letter differ between collection times (P ≤ 0.05). Asterisks indicate differences between treatments at that collection time point (P ≤ 0.05). Data are expressed as mean ± SD.
Figure 4.11. Analysis of TNF-α mRNA expression in ileum of 25-day-old piglets fed sow milk (SR) or formula (F). TNF-α expression was normalized using tubulin. Panel A compares expression in whole ileal tissue vs. the pool of exfoliated epithelial cells (collection times 1-4). In panel A, means without a common letter differ (P ≤ 0.05). TNF-α expression in exfoliated epithelial cells across the villus-to-crypt axis are shown in Panel B. Collection times represent: 1 (0-10 min); 2 (11-30 min); 3 (31-60 min); and 4 (61-100 min). Within dietary treatment group, means without a common letter differ between collection times (P ≤ 0.05). Asterisks indicate differences between treatments at that collection time point (P ≤ 0.05). Data are expressed as mean ± SD.
Figure 4.12. Analysis of TGF-β mRNA expression in ileum of 25-day-old 25-day-old piglets fed sow milk (SR) or formula (F). TGF-β expression was normalized using tubulin. Panel A compares expression in whole ileal tissue vs. the pool of exfoliated epithelial cells (collection times 1-4). In panel A, means without a common letter differ (P ≤ 0.05). TGF-β expression in exfoliated epithelial cells across the villus-to-crypt axis are shown in Panel B. Collection times represent: 1 (0-10 min); 2 (11-30 min); 3 (31-60 min); and 4 (61-100 min). Within dietary treatment group, means without a common letter differ between collection times (P ≤ 0.05). Asterisks indicate differences between treatments at that collection time point (P ≤ 0.05). Data are expressed as mean ± SD.
Figure 4.13. Analysis of mRNA expression of crypt markers in ileum of 25-day-old piglets fed sow milk (SR) or formula (F). V-myb expression in exfoliated epithelial cells across the villus-to-crypt axis are shown in Panel A. CDK5 expression is shown in Panel B. Collection times represent: 1 (0-10 min); 2 (11-30 min); 3 (31-60 min); and 4 (61-100 min). CDK5 and v-myb expression was normalized using tubulin. Within dietary treatment group, means without a common letter differ between collection times (P ≤ 0.05). Asterisks indicate differences between treatments at that collection time point (P ≤ 0.05). Data are expressed as mean ± SD.
CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

This study focused on the effect of prebiotics on the expression of the Wnt/BMP pathway of the neonatal intestine. Our objective was to identify differences in gene expression of piglets fed formula containing 4g/L prebiotics when compared to a control formula and sow reared piglets. It is important to explore the effects of prebiotics on the developing intestine as the implications can be far reaching, particularly when epigenetic changes are involved. Overall, our results showed that prebiotics did not change the expression of genes in the Wnt/BMP pathway to more closely parallel the expression of SR piglets. However, the addition of prebiotics did change expression in the gut as F+P expression did not follow that of F piglets. F+P expression was different from either of the controls. Future work should include later time points to see if these differences occur upon and after weaning. Future work should also include histomorphology in order to characterize the small intestine crypt and villi numbers and size and colon crypt depth and number. A larger sample size would help to determine if the F+P group was smaller because of diet or because of other factors. F+P did cause an increase in colon size, which could allow for increased fermentation. pH decreased and SCFA production increased in F+P animals, however butyrate production did not increase to SR levels in day 14 ileum and day 7 AC. In day 14 AC, F+P production of butyrate fell between F and SR animals. Increased amounts of butyrate may have had some influence on gene regulation, however this did not cause F+P to more closely resemble SR animals.
PDX is not as common an additive to food items as GOS and FOS and may not be as effective a prebiotic. For these reasons, future studies should either use a FOS and GOS combination or a mix of all three so that the full benefit of the prebiotics can be achieved and monitored. More work is needed to determine if an optional ratio of prebiotics that most closely reflect sow-reared piglets can be developed.

In this study, we concentrated on analyzing mRNA. Many proteins undergo post translational modifications that render them inactive or quickly mark them for degradation. For this reason, measuring protein levels present as well as mRNA could add information and allow for a better understanding of the underlying processes occurring in the gut in the presence and absence of prebiotics. Western blots and ELISAs (Enzyme-linked immunosorbent assay) could be done to add this information. If consistent differences are found between groups with both protein and mRNA measurements, designing a chromatin immunoprecipitation (ChIP) could help answer if an underlying epigenetic process is responsible for these differences.

Including the assessment of nutrient absorption, particularly that of nitrogen, and transit time would help us to better understand underlying reasons behind growth of piglets and would allow us to assess the impact of prebiotics on nutrient bioavailability. This information is critical to assessing the safety and potential inadequacies of prebiotics as an additive.

The second part of our study involved incubations of the ileum in isotonic solution to remove epithelial cells along the crypt-villus axis. This was done as an attempt to remove variation between samples within a group as many of our genes of
interest occur along a gradient. While we were not successful in removing the crypts, the first three incubations show some progress. For this reason, it may be worth continuing to optimize the method. Another strategy would be to employ the use a laser capture microscope (LCM) to separate parts of the villi and the crypts manually. This process is more time consuming, expensive and will only allow for RNA/DNA isolation as PCR is required to amplify the small amount of sample to a usable amount, however it would provide a means of successful isolation of the crypts and villi.

The incubations could prove a successful way of collecting protein along the crypt-villus axis as higher incubation temperatures could be used to remove epithelial cells without the worry of RNA degradation and would potentially allow for full crypt removal. Proteases, such as phenylmethylsulfonyl fluoride (PMSF), could be added to help maintain protein integrity.

Future research should also include Caco-2BBE cells which are human derived and provide a model for the absorptive enterocyte. Exposing these cells to butyrate at differing concentrations and then isolating mRNA and to quantify expression of Wnt/BMP genes could give an insight into what amounts of butyrate, which is produced by bacteria, would be appropriate for a human intestine. Such research would also help to determine whether prebiotics or isolated butyrate is a better option to add to infant formulas to help modulate intestinal development.
REFERENCES


Availables of calcium, iron, and zinc from dairy infant formulas is affected by soluble dietary fibers and modified starch fractions. *Nutrition, 19*(7-8):641-5.


Author’s Biography

Allyson Bares was born in Logan, Utah where she lived throughout her childhood. She completed her undergraduate degree in biology with a minor in Chemistry from the University of Utah in Salt Lake City. While there, she was placed in an ecology nutrition lab through a scholarship promoting women in science. After graduating, Allyson spent a year working in a pediatric nutrition lab studying epigenetics and early nutrition. It was because of these research experiences that she decided to pursue an advanced degree in the field of nutrition. Allyson plans on continuing to conduct nutritional research with her degree.