## ABSTRACT

HERFEL, TINA MARIE. Supplementing Prebiotics and Probiotics in Early Animal and Infant Nutrition. (Under the direction of Dr. Jack Odle)

Novel prebiotics and probiotics were evaluated for use in infant and weaning pig nutrition. Risk of childhood diseases increases with imbalanced intestinal microflora during early development due to environmental factors. Prebiotics and probiotics may restore intestinal microbial balance via SCFA production, competitively excluding pathogenic bacteria and tolergenic priming of the intestinal immune system. Piglets are an appropriate neonatal model for prebiotic and probiotic supplementation in infant formula due to their anatomical and functional similarities with human infants.

Stabilized rice bran (SRB) is classified as a "functional food" because of its prebiotic characteristics. Two hundred pigs were weaned at ~21 days of age, blocked by weight, and allotted to diets containing 0 or 10% SRB and (-) or (+) Antibiotic (ANT) according to a 2 x 2 factorial design. Antibiotic supplementation improved average daily gain by 6.3% from day 14 to 28 ($P < 0.05$), but other production parameters were affected by ANT. SRB increased feed efficiency beyond that pigs fed growth promoting antibiotics. Cumulatively, pigs fed the ANT-free + 10% SRB diet improved gain:feed by an average of 14% compared to all other treatments ($P < 0.05$). The improvement in production was accompanied by a trend for increased colonic bifidobacteria ($P = 0.098$), but the fermentative activity of the bacteria was not detectably different when SRB was fed. Trends for increased bifidobacteria and improved feed efficiency indicate SRB has prebiotic properties. SRB shows promise as

a replacement of corn in weaning diets, specifically diets that lack antibiotic growth promoters.

Oligosaccharides, the third largest component in human milk, are virtually absent from cow's milk and most infant formula. Prebiotic carbohydrates like polydextrose (PDX) have been proposed as surrogates for human milk oligosaccharides. To substantiate the safety and efficacy of the PDX, one-day old pigs were fed a cow's milk-based formula supplemented with PDX (1.7, 4.3, 8.5 or 17 g/L) for 18 days (n = 13/dose) and compared to control unsupplemented formula and reference groups (day 0 pigs, and sow-reared pigs). Growth rate, formula intake, stool consistency, behavior score, blood chemistry and hematology, relative organ weights (% of body weight), tissue morphology (i.e. liver, kidney and pancreas) and pancreas biochemistry did not differ among formula-fed pigs ($P > 0.1$). Ileal Lactobacillus CFU, but not Bifidobacteria, increased linearly with increasing PDX ($P = 0.02$). Lactic acid increased linearly by five-fold with increasing PDX ($P = 0.001$). Accordingly, digesta pH decreased linearly ($P < 0.05$) as PDX increased. Polydextrose mimicked other prebiotic carbohydrates and did not produce any adverse affects when evaluated in a neonatal pig model.

Studies were conducted to determine the impact of two novel probiotic strains, B. breve AH1205 (BB) and B. longum AH1206 (BL) at two doses, on the health, growth and development of neonatal pigs as a surrogate for human infants. One day old pigs were fed a milk-based formula containing BB or BL at both a low (109 CFU/day) or high (1011 CFU/day) dose for 18 days (n=10/treatment). Additional groups included 20 newborn pigs sampled at baseline (NB) and 20 sow-reared (SR), reference pigs. Bacterial translocation was

not affected by probiotic treatment ($P > 0.1$). Feeding high levels of BB had a minor but significant impact on feed intake, increasing 5% over controls ($P < 0.05$). Strain BL had no measurable effect on fecal and cecal total bifidobacteria populations; whereas, B. longum increased in response to increasing BL supplementation. Dietary supplementation with novel probiotic strains BB and BL is safe for human infants based on a lack of animal growth, development and immune-related effects on an appropriate surrogate animal model, neonatal pigs.

# Supplementing Prebiotics and Probiotics in Early Animal and Infant Nutrition

by
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## BIOGRAPHY

Tina Marie Herfel is originally from Mount Horeb, WI. She received a Bachelor of Natural Sciences degree in Poultry Science from the University of Wisconsin-Madison in 2001. Tina received a Master's of Science degree in Animal and Poultry Science at Virginia Polytechnic and State University under the direction of Dr. Curtis Novak in 2005. In the fall of 2005, Tina began working towards a Doctor of Philosophy degree in Animal and Poultry Science with a minor in Biotechnology at North Carolina State University under the direction of Dr. Jack Odle. Tina's research focus was on supplementing novel prebiotics and probiotics in young animal and infant nutrition. For the academic year of 2007-2008, Tina was a NCSU provost's fellow. As an NCSU graduate student, Tina participated in the Graduate Student Research Symposium, was a member of the Animal Science Graduate Student Association and was a university graduate student library representative. During the summer of 2010, Tina was awarded a National Science Foundation fellowship to conduct research in Beijing, China through the East Asian Pacific Summer Research Institute under the direction of Dr. Yuming Guo at the College of Animal Science and Technology at China Agricultural University. Tina is currently a nutritionist in the Teklad Diets division of Harlan Laboratories.

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# CHAPTER 1: INTRODUCTION TO THE CONCEPT OF PREBIOTICS AND PROBIOTICS IN EARLY NUTRITION

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The research presented in this dissertation focuses on evaluating novel prebiotics and probiotics for use in infant and weaning pig nutrition. Prior to evaluating the efficacy and safety of pre- and probiotics, a review of these feed additives is necessary. To investigate potential pre- and probiotics in infant formula, the human surrogate model the piglet has been utilized. Therefore, information regarding both human infant and piglet development are discussed.

**Defining prebiotics and probiotics**

A prebiotic was recently redefined as, "a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microbiota that confers benefits upon host well-being and health" (1). There are generally three criteria for prebiotic classification: 1) resistance to mammalian digestion; 2) fermentation by intestinal microflora; 3) selective stimulation of the growth and/or activity of intestinal bacteria associated with health and wellbeing (2). Currently, only four prebiotics have been identified that fully meet the definition of probiotic, but several candidate prebiotics exist (Table 1).

Probiotics have been defined as "living microorganisms that, on ingestion in sufficient numbers, exert health benefits beyond basic nutrition" (3). Probiotic microorganisms delivered via food or dairy products are mainly members of *Lactobacillus* and *Bifidobacteria* genus (Table 2). Prebiotic and probiotics are similar in that they exert their effects via changes in the intestinal microbiota. Prebiotics change the microbiota via the introduction of a substrate, while probiotics change the microbiota by sheer numbers.

The three major activities prebiotic and probiotics are thought to participate in are 1) changing the microbiota; 2) fermentation producing organic acids; 3) producing a health benefit to their host.

**Table 1. Reported prebiotics and candidate prebiotics.**

Compiled from reference (2).

| | *In vitro* | | | *In vivo* | | |
|---|---|---|---|---|---|---|
| | *Criteria 1* | *Criteria 2* | | *Criteria 2 & 3* | | |
| | Indigestible | Pure Culture | Fecal Inoculation | Animals | Humans | Classification |
| Inulin and oligofructose | Good | Good | Good | Good | Good | Prebiotic |
| Transgalacto-oligosaccharides | Good | Good | Good | Good | Good | Prebiotic |
| Lactulose | Structurally indigestible; limited data | Good | Good | Good | Good | Prebiotic |
| Galacto-oligosaccharides | Good | Good | Good | Good | Good | Prebiotic |
| Isomalto-oigosaccharides | Inconclusive | Good | No data | Good | Good | Candidate |
| Lactosucrose | No data | Inconsistent | Inconsistent | Inconsistent | Good | Needs more testing |
| Xylo-oligosaccharides | No data | Inconsistent | Inconsistent | Good | Good | Needs more testing |
| Soybean oligosaccharides | Good | Good | Not selective | No data | Good | Needs more testing |

This review is structured to investigate the definition of pre- and probiotics in the context of both host and bacteria. Although a feedstuff may not adhere strictly to the definition of a prebiotic or probiotic, it may still exert some of the beneficial effects demonstrated by defined pre-and probiotics.

**Table 2. Examples of human probiotic species with research documentation.**

Compiled from reference (4).

| | |
|---|---|
| *Bifidobacteria animali* | *Lactobacillus gasseri* |
| *Bifidobacteria bifidum* | *Lactobacillus plantarum* |
| *Bifidobacteria breve* | *Lactobacillus reutri* |
| *Bifidobacteria clausii* | *Lactobacillus rhamnosus* |
| *Bifidobacteria lactis* | *Lactobacillus sporgenes* |
| *Bifidobacteria longum* | *P. freudenreichii spp. Shermanii JS* |
| *Enterococcus* SF68 | *Streptococcus boulardi* |
| *Lactobacillus acidophilus* | *Streptococcus mitis* |
| *Lactobacillus bulgaricus* | *Streptococcus oralis* |
| *Lactobacillus casei* | *Streptococcus sanguis* |
| *Lactobacillus fermentum* | *Streptococcus thermophilus lactic* |

## Intestinal Microflora

*Classifying the intestinal microbes*

To be defined as a prebiotic or probiotic, a feedstuff must increase the number of beneficial intestinal bacteria. Within the intestine four distinct microbial populations exist: 1) autochthonous microbiota, populates that are present at high levels and permanently colonize the host; 2) normal microbiota, microbes that are frequently present but can vary in number; 3) true pathogens, microbes that are periodically acquired but can persist, causing infection and disease at a high enough concentration; 4) allochthonous microbiota, microbes of another origin that are present temporarily (most probiotics fall into this category) (5). From these four categories, bacteria can further be categorized into their effect on the host into three categories: beneficial bacteria, harmful bacteria and bacteria exhibiting intermediate properties (**Figure 1)** (6). Because lactobacillus and bifidobacteria are

recognized as organisms that are beneficial for health, they are often supplemented as probiotics. Prebiotics are fed to be selectively fermented by these probiotic bacteria. The populations of beneficial bacteria in the intestine limit the growth of harmful bacteria, thus limiting the pathogenicity and toxin production and maintaining healthy gastrointestinal mucosal surfaces (7). Bifidobacteria and lactobacillus are able to thrive in the large intestine by utilizing oligosaccharides for energy via fermentation that otherwise go undigested by mammalian enzymes (8). The adult microflora population is relatively stable. Adut pathogenic bacteria levels are maintained at low levels decreasing their deleterious effects by beneficial and intermediate bacterial groups (9). Unlike the adult microflora, neonatal intestinal populations are dynamic.

*The intestinal microbiota affects health in the neonate*

Risk of childhood diseases increases with imbalanced intestinal microflora during early development due to environmental factors (**Figure 2**) (10). Breastfeeding and vaginal birthing are two mechanisms by which an infant becomes inoculated with beneficial bacterial populations. At birth the fetus is sterile. Several environmental cues affect the newly established bacterial populations. Delivery mode (vaginal versus cesarean) affects bacterial colonization of the infant. Vaginal birth introduces the infant to bacteria from the mother's vagina, skin and feces allowing the infant to begin developing the gut microbiota minutes after birth (11). Infants delivered via cesarean section encounter bacteria first from the delivery staff and birthing equipment delaying the development of microbiota (11). After birthing, feeding strategy greatly affects microbial populations. Mother's milk offers another inoculation opportunity for the infant since it contains microbes, while infant formula is

considered sterile. *B. breve* and *B. longum* are part of the commensal bacterial community of breast-fed infants and inoculation can occur directly through breast milk (12).

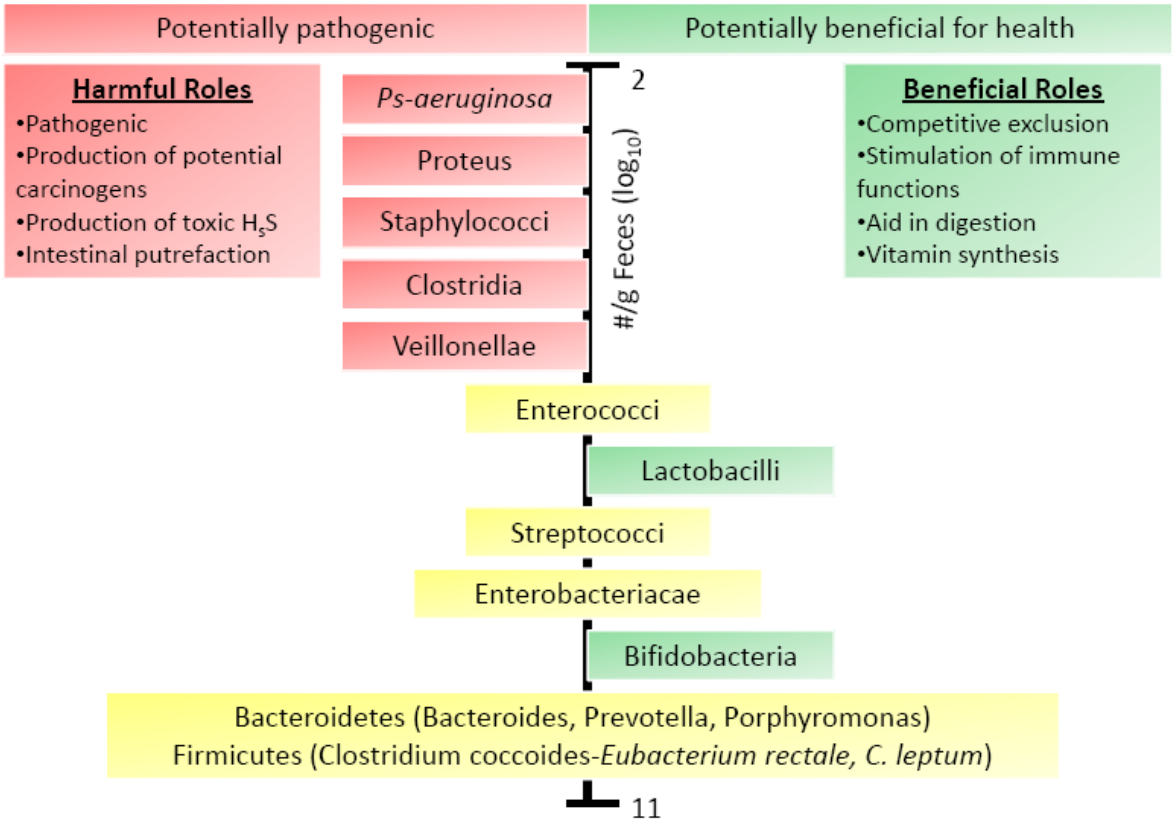


**Figure 1. Average distribution of the components of human fecal microflora between potentially pathogenic and beneficial effects.**

Adapted from reference (9) and (5).

Also, bacteria found on the skin of the mother also inoculate the infant during breast feeding. Breast-feeding during the first 13 weeks of life can decrease gastrointestinal (GI), respiratory and ear infections for the first year of life as compared to formula-feeding (13). After the first week of breast-feeding, bifidobacteria become the dominant GI bacteria, while formula-

fed infants develop a more diverse flora, with *Bacteroides sp.* equaling the number of bifidobacteria (14). Breast feeding results in a bifidobacterial dominated intestinal community with mostly lactic acid bacteria, bifidobacteria, *Bacteroides* and streptococci while formula-feeding leads to a more adult-like diverse bacterial population with a flora with more staphylococci and clostridia (14).

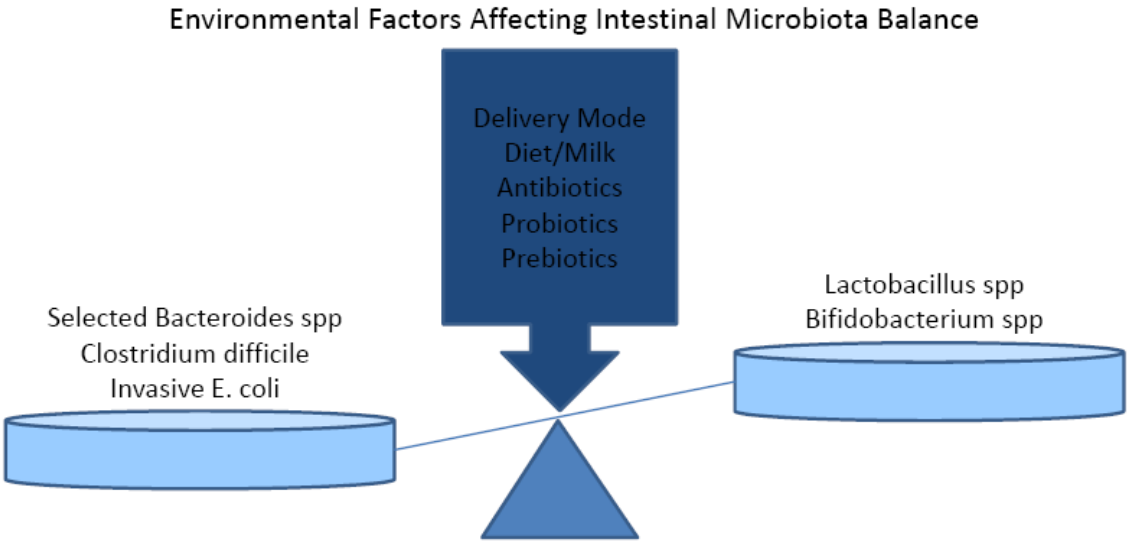


**Figure 2. Environmental factors affect the intestinal microbiota balance of the neonate.**

**Breast milk is a substrate and source of bifidobacteria**

*Bifidobacteria and lactobacillus are able to utilize breast milk carbohydrates*

Bifidobacteria and lactobacilli are considered the most important bacteria for benefiting health (15). Lactobacilli are gram-positive, both homo-and heterfermenter and catalase-negative rods that are part of the *Firmicutes* phylum (16). Bifidobacteria are of the

*Actinobacteria* phylum and are heterofermenters. In the beginning of the 21st century, the genomes of several probiotic bacteria species have been determined enabling researchers to identify genetic mechanisms that might enable these groups to dominate the intestinal ecological niche (Table 3).

**Table 3. Some probiotic bacteria species with a published genome sequence.**

| Bacteria | Year sequence reported | Reference |
|---|---|---|
| *Bifidobacterium longum* (Nestle) | 2002 | (8) |
| *Lactobacillus plantarum* (WCFS) | 2003 | (17) |
| *Lactobacillus johnsonii* (Nestle) | 2004 | (18) |
| *Lactobacillus acidopholis* (NCSU) | 2005 | (19) |
| *Lactobacillus salivarius* (UCC) | 2006 | (20) |
| *Lactobacillus casei* (LABGC) | 2006 | (20) |
| *Lactobacillus gasseri* (LABGC) | 2006 | (20) |
| *Bifidobacteria breve* (UCC) | 2011 | (21) |

*Bifidobacteria are an abundant bacteria group in breast-fed infants*

Bifidobacteria has been recognized as an abundant bacteria group in breast-fed infants for over 100 years (22). *Bifidobacterium longum's* genome represents its metabolic adaption to the large intestine which lacks mono- and di-saccharides. *B. longum* is a strict fermentative anaerobe that has homologs of all enzymes needed for the "fructose-6-phosphate shunt" (8). Fructose, galactose, NAc-glucosamine, NAc-galactosamine, arabinose, xylose, ribose, sucrose, lactose, cellobiose, melibiose, gentobiose, maltose, isomaltose, raffinose and mannose, but not fucose, are able to be fed into the "fructose-6-phosphate shunt" in *B. longum* (8). Bifidobacteria contain proteins specialized for the catabolism of different oligosaccharides found in breast milk (8).

*Breast-milk contains oligosaccharides*

Human breast milk contains 7-12g/L of oligosaccharides, making this the third largest fraction of solid milk (23). Over 100 different oligosaccharides have been isolated from human milk (23). Human milk oligosaccharides (HMO) contain a core molecule with repetitive galactose and *N*-acetylglucosamine in β-glycosidic linkage to lactose (24). The variety of HMO is due to α-glycosidic linkages of fucose or fucose and/or sialic acid, which is based on the Lewis blood group status of the mother (24). Compared to human milk, bovine milk oligosaccharide composition is much simpler with only 10 isolated oligosaccharides (25). The ability of bifidobacteria to metabolize glucose, galactose, N-acetylglucosamine, and sialic acid are indicative of an adaptation to utilizing a milk substrate, as these are monomers that compose the oligosaccharide component of breast milk (26). Although bacteria and indigestible oligosaccharides do not contribute direct nutrition to the infant, adding back non-nutrient components of human breast milk has been a strategy for improving the functionality of milk substitutes.

**Intestinal interactions of prebiotics and probiotics**

Researchers have demonstrated that probiotics and prebiotics represent potential nutritional interventions involved in modulation of gastrointestinal inflammation and are possible agents of protection against pathogenic microbes (27). The gastrointestinal tract is a barrier between the luminal contents and the systemic system **(Figure 3)**. Mucus covered epithelia cells cover the tract in a single layer with blood and lymphatic systems found below this layer in the lamina propria. Due to the proximity of the sterile systemic system and the

non-sterile luminal contents, the immune response of the gastrointestinal tract (GIT) has evolved to not only respond through normal inflammation pathways, but also through tolerance pathways while simultaneously allowing nutrients to enter the body. Dendritic cells (DC) are located throughout the GIT and participate in the sampling and processing of bacteria (which mainly occurs in the M cell layer of Peyer's patches and lymphoid follicles) (28). Mesenteric lymph nodes (MLN) act as a block to the systemic system. DC containing live commensal bacteria migrate to the MLN and remain locally within the gut.

*Organic acids are by-products of fermentation that modulate the GIT*

Within the intestinal lumen, prebiotics and probiotics function to change the environment through SCFA production and competitive exclusion. The homofermentative and heterofermtative pathways utilized by lactic acid bacteria produce organic acids as a by-product (**Figure 3**). Organic acids (butyrate, propionate, acetate, lactic acid, etc.) are fermentative products of lactic acid bacteria (LAB) that can change based on substrate available to the bacteria. Organic acids decrease the pH of the lumen, which can limit less acid tolerant bacteria (29). Organic acids are also able to modulate the intestinal immune system for example by limiting mucosal damage caused by ischemia injury (30). Stereotypical patterns of intestinal organic acids are beginning to emerge with certain diseases, such as allergies (31). Manipulation of substrate and microbiota can change organic acid patterns and therefore illicit various host reactions.

Organic acids can elicit different cytokine profiles. In murine mesenteric lymph nodes, butyrate differentially regulated $T_H2$ cytokines by decreasing $T_H1$ recruiting cytokines IL-2 and IFN-γ and also decreased the anti-inflammatory cytokine IL-10, while acetate and

propionate regulate $T_H1$ cytokines by increasing IFN-γ and IL-10 (32). Butyrate also showed $T_H2$ regulation in porcine PBMC by increasing the expression of suppressor of cytokine signaling (SOCS3), a protein that is several-fold higher in $T_H2$ cells compared to $T_H1$ cells, but butyrate also increased the $T_H1$ cytokines IFN-γ and IL-10 (33). Butyrate has been shown to stimulate MUC-2 production through prostaglandin *in vitro* (34). Further work needs to be conducted to determine the effects of VFA on porcine PBMCs in comparison with other species. Other fermentative products, such as lactic acid, have yet to be studied extensively on their regulation of T cell response *in vitro.*

Lactic acid is an organic acid not commonly reported in studies of prebiotics and probiotics because it must be analyzed separately from VFA, while a panel of over 7 VFA can be run at one time due to differneces in sample preparation. Lactic acid is the major end product of fermentation pathways. Lactic acid serves as an electron sink that oxidizes and recycles reduced coenzymes and ferredoxins that are formed in the initial stages of fermentation allowing fermentation pathways to continue. In the adult intestine, lactic acid levels are low due to the consumption of lactic acid by other colonic bacteria (35). Lactic acid may play a more important role in the infant intestine due to the decreased diversity of microbes (14). Breast fed infants have decreased stool pH due to increased lactic acid, not SCFA (36). In infants, while the lumen is still maturing, increases in SCFA have been implicated in the pathology of necrotizing enterocolitis (NEC) (37, 38). In a study examining the effect of high levels of organic acids on intestinal injuries in a rat model, lactic acid (at 150 and 300 mM) did not cause gross or microscopic colonic lesions, while even the low level of butyrate (150 mM) and acetic acid (150 mM) did, even when pH was held constant

for the treatments (39). In a piglet NEC model, piglets fed lactose had fewer incidences of NEC and had significantly higher colonic levels of lactic acid, but lower levels of butyric acid (37). In infants with NEC, SCFA cause mucosal injury and in the case of butyrate, decrease the recovery of injury due to blocking trefoil factor (TF), a factor important to the maintenance and repair of the intestinal mucosal barrier function (40). Establishing a lactic acid bacterial population early in life may promote maintenance and repair of the mucosal barrier function. At a time when barrier function is maturing, lactic acid may protect the infant from pathogens by decreasing pH while not interfering with barrier function.

As with other SCFA, lactic acid may direct immune responses. Ileal lactic acid is positively correlated to the expression of the $T_H1$ recruiting cytokine IL-12p40 and TNF-α in weaning pigs (41). The expression of IL-6, a cytokine responsible for inhibiting TNF secretion and driving naive T cells to $T_H2$ cell, was positively correlated with lactic acid in the ileum, conflicting with the increased expression of TNF-α (41)**.** Lactic acid response in this experiment could be confounded by VFA in the intestine, so although Pie and colleagues demonstrated a correlation, direct effects of lactic acid on immune function remain unknown (41). Further work is needed to understand the direct effect of lactic acid on the adaptive immune system.

**Competitive exclusion of pathogenic bacteria**

Probiotics and prebiotics work to competitively exclude potentially pathogenic bacteria in the intestine (**Figure 3)**. This concept is highlighted in the formula fed infant. After the first week of breast feeding, bifidobacteria become the dominant GI bacteria, while

formula fed infants develop a more diverse flora, with *Bacteroides sp.* equaling the number of bifidobacteria (14). The differences in microbiota are implicated in the increased risk of disease in formula fed infants compared to breast fed infants (10). Bifidobacteria, dominant organisms among the microbiota of breastfed infants, are less prevalent in that of formula fed infants where they compete with higher levels of other bacterial groups (e.g.*Bacteriodes sp.*) (14). Bifidobacteria have the capacity to inhibit pathogenic bacteria. In a study of over 1,000 infants, fecal counts of *Clostridium difficile* were significantly higher for formula fed infants. *Clostridium difficile* colonization has been associated with diseases such as necrotizing enterocolitis, due to immaturity of the intestine (31, 42). Interestingly, *C. difficile* also was higher in preterm infants than term infants (43). Establishing lactic acid bacteria (LAB) and bifidobacteria commensals early in the life of formula-fed infants may competitively exclude potentially pathogenic bacteria, like *C. difficile.*

**Dendritic cells are primed to be tolergenic via epithelial transported components of commensal bacteria**

Epithelial cells may participate in producing immunological tolerance via transport of bacteria proteins to intestinal dendritic cells (DC; **Figure 3**). The gastrointestinal immune system must interact with many antigens frequently and provide both protection from infection and tolerance. Commensal bacteria like bifidobacteria and lactobacillus are still antigens, but tolerance to these bacteria is beneficial to both the bacteria and the host. Inflammation will cause an increase in host gut permeability and use energy resources, while

the inflammatory process activates the adaptive immune response to affectively kill

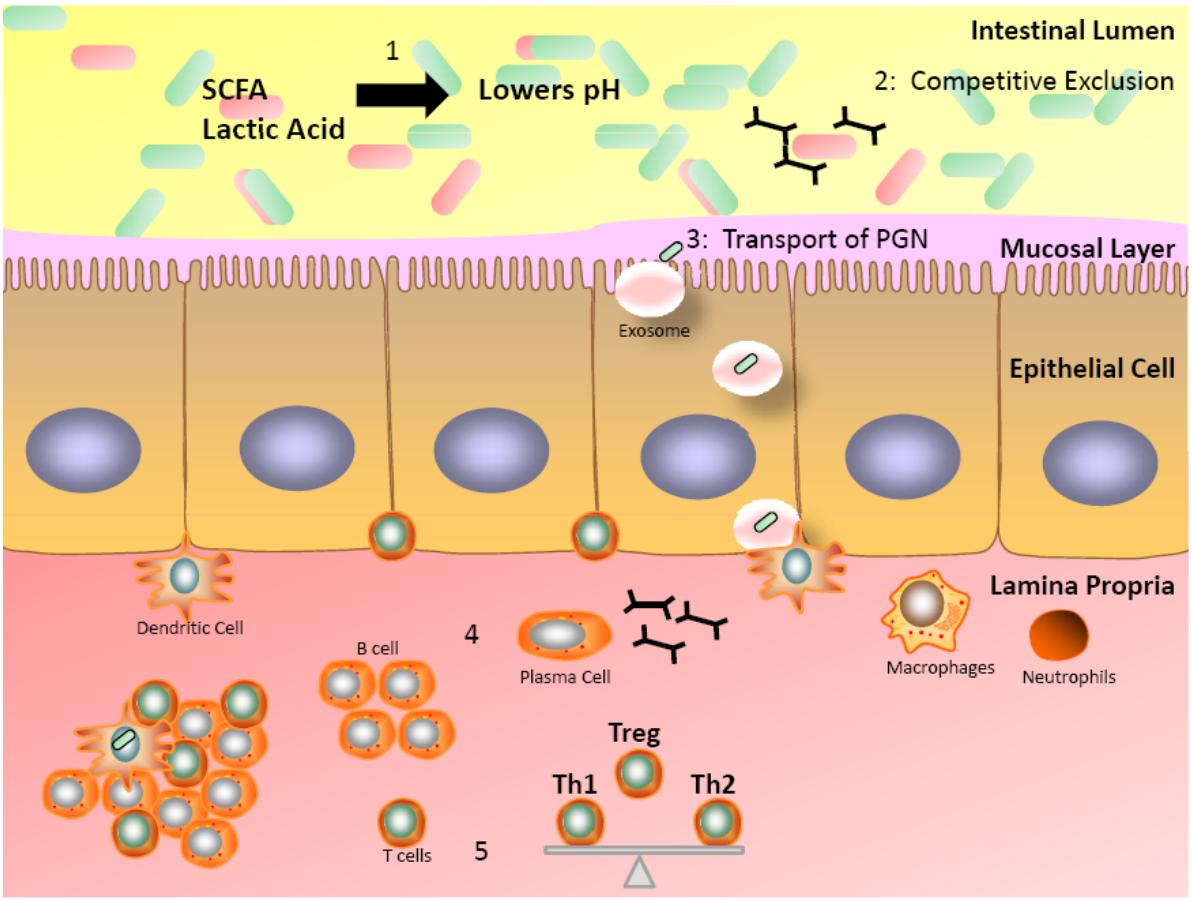


**Figure 3. Interaction of prebiotics and probiotics in the intestine.**

Several mechanisms are utilized by preboitics and probiotics modulate the GIT: 1) formation of organic acids to modulate the GIT immune system and lower pH; 2) competitive exclusion of pathogenic bacteria; 3) transport of peptidoglycan of commensal bacteria to dendritic cells for tolergenic priming; 4) priming of B cells for IgA production; 5) inducing a balanced T cell population.

commensal bacteria and may allow the growth of pathogenic bacteria. Being gram positive bacteria, lactobacillus and bifidobacteria hav e a peptidoglycan (PGN) layer covering their cell membranes. One possible mechanism to induce tolerance to commensal bacteria is through interactions of intestinal epithelia cells and dendritic cells (DC). Macrophages rapidly kill commensal bacteria at the epithelial layer (28). Derivatives of commensal bacteria, such as PGN, have been shown to cross the intestinal barrier via epithelial exosomes under normal physiological conditions (44).

**Commensal bacteria influence tolerance of the adaptive immune system**

DCs are also responsible for sampling the luminal contents for bacteria and are able to determine not only quantity, but also quality of the bacteria. After phagositosis of commensals by DC, DC migrate to the MLN where they are maintained and not allowed to enter the systemic system but still direct the adaptive immune reaction (28). Dendritic cells primed by beneficial bacteria direct the adaptive immune system to an anti-inflammatory state (**Figure 3**). Interleukin-10 is an anti-inflammatory cytokine that directs a T regulatory immune response that is produced due to stimulation via beneficial intestinal bacteria. The microbial molecule polysaccharide A (PSA) from *Bacteriodes fragilis* has been shown to be a stimulator of interleukin-10 production in an inflammation model (47). Treatment of *B. fragilis* in a *Helicobacter hepaticus* induced colitis model reduced symptoms of colitis in mice by increasing the expression of the anti-inflammatory cytokine IL-10 (47). Commensal bacterial molecules are able to directly modulate the host immune system. Wang and

colleagues (2008) demonstrated the ability of *Enterococcus faecalis* to down-regulate IL-8 secretion via its carbohydrate fraction, an additional example of commensal bacterial down-regulating the inflammatory immune respsone (48). Probiotic bacteria cell wall components are able to modify the immune response *in vivo* and *in vitro.*

DC loaded with live bacteria induce Immunoglobulin (Ig) A secretion (**Figure 3**). DC are able to retain live commensal bacteria to activate the adaptive immune system. DC loaded with commensal bacteria do not travel systemically, due to entrapment within the MLN (28). The activated DC induce IgA production by B cells, which after maturation because plasma IgA secreting cells (28). IgA is a secretory Ig that is transported to the intestinal lumen where it binds bacteria, limiting their movement through the intestinal mucosa and interaction with the epithelial layer. Within the lumen, IgA is the first line of defense interacting with bacteria and bacterial products.

**Evidence for the addition of prebiotics and probiotics to infant nutrition**

Human milk is widely considered the optimum food for meeting the nutritional needs of infants but by two months of age the majority of infants in North America have received some quantity of infant formula (49). Though iron-fortified infant formulas are the most appropriate nutritional substitutes, their composition does not fully duplicate that of human milk, particularly with regards to non-nutritive components such as human milk oligosaccharides (HMO) and bifidobacteria that perform functional roles beyond basic nutrition. Oligosaccharides constitute the third largest component in human milk after lactose and lipids, ranging in concentration from 5-10 g/L in mature milk. Oligosaccharides

are virtually absent from cow milk (< 0.08 g/L). Approximately 200 molecular species of oligosaccharides have been identified in human milk which are synthesized from D-glucose, D-galactose, D-N-acetylglucosamine, L-fucose and D-N-acetylneuraminic acid (sialic acid) monomers. In contrast, bovine milk oligosaccharide composition is much simpler as the 10 molecular species that have been identified, consist largely of sialic acid linked with lactose (23, 25, 26, 50). Over 50% of porcine milk oligosaccharides are sialylated and contain 29 distinct oligosaccharides (51). Prebiotic supplementation of pediatric nutritional products is associated with increased levels of lactic acid bacteria and bifidobacteria, decreased diarrhea, improved allergy symptoms and decreased rates of infection in infants and children (52-56). Due to the complexity of HMO and the lack of commercial sources, they cannot be used to supplement infant formulas. Instead, supplementation with prebiotic oligosaccharides is expected to provide some of the functional properties of HMO, such as supporting intestinal commensal bacteria, softening stools, and effecting desirable intestinal immunomodulation.

Bifidobacteria has been recognized as an abundant bacteria group in breast-fed infants for over 100 years (22). Breast milk contains the bifidobacterium species *B. adolescentis*, *B. bifidum* and *B. breve* (**Figure 4**) (12). These three species are also found in the feces of breast fed infants and the abundance of bifidobacteria is greater in breast fed infants compared to their formula fed cohorts (12,14). A strategy to improve the non-nutritive bacteria component of infant formula is the additional of probiotics. In pediatric populations, the administration of specific probiotic bacteria have been shown to reduce the incidence and duration of diarrhea (57), shorten the duration of rotavirus diarrhea (58), reduce the incidence of atopic eczema (in children at high risk) (59) and reduce the risk of

necrotizing enterocolitis and overall mortality (in preterm infants with very low birth weight) (60). In order to more closely approximate both nutritional and functional properties of human milk, efforts are under way to identify novel prebiotics and probiotics with similar functionality of the non-nutritive components in breast milk.

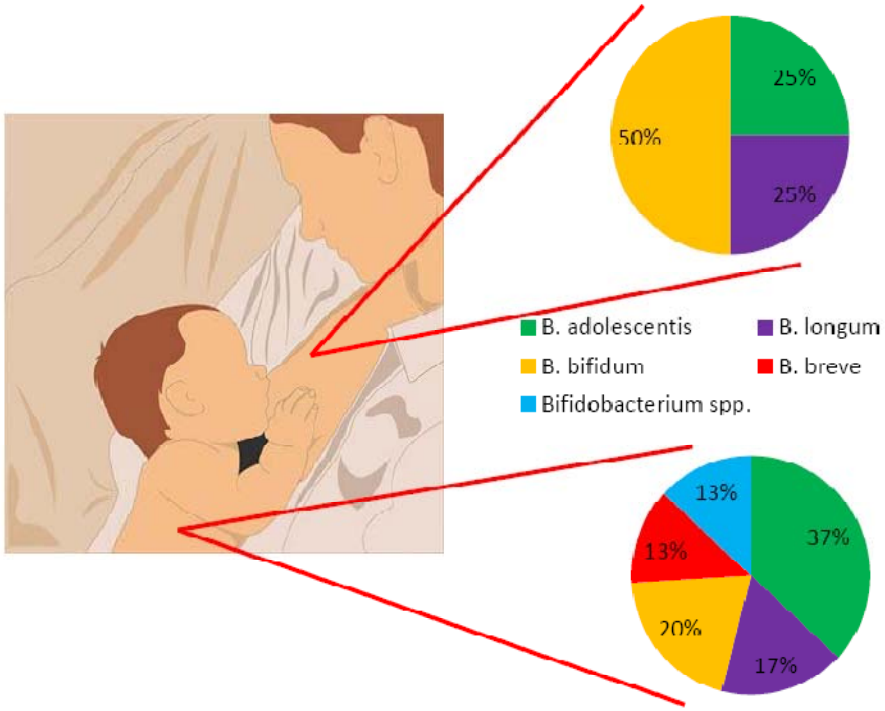


**Figure 4. Populations of bifidobacteria found in breast milk are also found in the feces of breast fed infants.**

The relative abundance of bifidobacteria found in breast milk are found as major populations of bifidobacteria in infant feces. Adapted from reference (12).

**The piglet as a human infant surrogate model for prebiotic and probiotic studies**

Given the importance of gut and immune development of healthy infants and the scarcity of validated, noninvasive markers, piglets are an appropriate neonatal model due to their anatomical and functional similarities with human infants. The neonatal pig is a well established model for infant formula nutrient interactions (61).

Examples of the neonatal pig model establishing the safety of novel ingredients for infant formula include determining the safety of single cell sources of long-chain polyunsaturated fatty acids (62), and more recently the effects of conjugated linoleic acids on lipid accretion and adipose tissue metabolism (63). The pig digestive tract is anatomically and functionally similar to that of the human and is more physiologically relevant for prebiotic studies than a rodent model. The major difference between the neonatal pig and human infant intestinal anatomy is the former's coiled descending colon (although the large intestine is similar in length), while the rodent's large intestine is marked by an enlarged cecum compared to that of the human (64). The accelerated growth rate (~10X) of neonatal pigs relative to human infants enhances the sensitivity of the former to the effects of toxicological agents. In a practical sense, information learned using the piglet as a human surrogate model can benefit commercial production because the microbiota has been shown to affect production. Swine production practices allow for consumption of sow's milk during early life, but differences in husbandry conditions can modify the development of the microbial populations with less intensive, outdoor production having a positive effect of the intestinal microbe by supporting a less diverse and increasing lactobacillus and bifidobacteria populations (65). The similarity of developmental program and anatomy on the large

intestine, in combination with an accelerated growth rate, render the neonatal pig an appropriate surrogate for human infants in nutrition studies focused on the GI impact of prebiotics and probiotics.

In preparation for birth, the porcine intestine increases its ability to absorb macromolecules and intestinal enzyme activity increases (66). This increased absorptive capability and enzyme activity allow for the transfer of large macromolecules (such as immunoglobulins, anti-microbial peptides, lactoferrin, etc.) contained in milk to be transferred to the systemic system of the suckling neonate. This ability to absorb intact macromolecules across the epithelium into the blood ceases after the first 2 to 3 days of life in an event called "intestinal closure" (66).

Newborn swine are beneficial immunological model because they are born immunologically immature because fetal exposure to immunoglobulins (Ig) is limited due to the porcine placental allowing for similar immunological activity of piglets. Immunity is passed to the neonate via milk Ig, first via colostrum with the major Ig being the monomeric IgG form and than with the dimer and pentadimer IgA and IgM isotypes predominating (67). Transfer of Ig via milk occurs via Fc receptors (FcRn) located in the mammary gland capturing sow serum Ig to transfer to the milk and then captured in the piglet intestinal epithelial to transfer from the lumen to the bloodstream. Binding of IgG by the piglet FcRn is dependent on the Fc Rn binding domain of the IgG and is done in a pH-dependent manner, with binding being more affective at acidic pHs (68). Passive uptake of IgG has also been shown *in vitro* (68). Inflammation affects uptake of Ig (69). Pre-term pigs showing signs of

necrotizing enterocolitis (NEC) had decreased serum levels of IgG and IgA compared to pigs not showing NEC symptoms that were the same age and fed the same diet (69).

Serum IgG, IgA and IgM peak 12 hours after birth in conventionally raised piglets (67). This peak is followed by a decrease in levels that peaks at 2 (IgM), 3 (IgA) and 5 (IgG) weeks of age (67). Although artificial raising of pigs with either bovine or swine colostrums changes the profile of serum immunoglobulins by decreasing the early levels of overall serum Ig, but levels increase above that of conventionally raised pigs 1 (IgM), 2 (IgA) and 4 (IgG) weeks of life. As the GI tract is the target site of prebiotic and probiotic activity, and the process of GI maturation in neonatal piglets is closer than other common laboratory animals to that of human infants, neonatal piglets are a favored model for prebiotic and probiotic supplementation of infant formula.

**Conclusion**

Prebiotics are defined as "selectively fermented ingredients that allows specific changes, both in the composition and/or activity in the gastrointestinal microbiota that confer benefits upon host well-being and health" (1). Probiotics have been defined as "living microorganisms that, on ingestion in sufficient numbers, exert health benefits beyond basic nutrition" (3). Risk of childhood diseases increases with imbalanced intestinal microflora during early development due to environmental factors (10). Prebiotics and probiotics may restore intestinal microbial balance via SCFA production, competitively excluding pathogenic bacteria and tolergenic priming the intestinal immune system. Piglets are an appropriate neonatal model for prebiotic and probiotic supplementation in infant formula due

to their anatomical and functional similarities with human infants. The following chapters evaluate novel prebiotics and probiotics in weaning swine and the human surrogate model, the neonatal pig.

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# CHAPTER 2: DIETARY SUPPLEMENTATION OF STABILIZED RICE BRAN IMPROVES PERFORMANCE VIA A PREBIOTIC MECHANISM IN WEANING PIGS

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Abbreviations used: ADFI, average daily feed intake; ADG, average daily gain; ANT, antibiotics; G:F, gain:feed; IEL, intraepithelial lymphocytes; SCFA, short chain fatty acid; SRB, stabilized rice bran; VFA, volatile fatty acid

**Abstract**

Stabilized rice bran (SRB) is classified as a "functional food" because of its prebiotic characteristics. With increasing prices and the possible removal of antibiotics from swine diets due to concern over antibiotic resistance, SRB was investigated as a replacement for corn with and without the addition of antibiotics (ANT). Two hundred pigs were weaned at ~21 days of age, blocked by weight, and allotted to diets containing 0 or 10% SRB and (-) or (+) ANT according to a 2 x 2 factorial design. Five animals were housed per pen throughout a 28 day growth period. At the end of the trial, one pig from each pen was euthanized for measurement of intestinal parameters. Antibiotic supplementation improved average daily gain by 6.3% from day 14 to 28 ($P < 0.05$), but other production parameters were affected by ANT. Cumulatively, pigs fed the ANT-free + 10% SRB diet improved gain:feed by an average of 14% compared to all other treatments ($P < 0.05$). Ileal histology revealed a 28% decrease in crypt depth for pigs fed the ANT-free + 10% SRB and ANT + 0% SRB diets compared to the ANT + 10% SRB diet ($P < 0.05$). Colonic bifidobacteria tended to increase with SRB supplementation. Differences in ileal and cecal digesta short chain fatty acid concentrations were not detected. In conclusion, SRB appears to improve the efficiency of nutrient utilization and tends to increase intestinal bifidobacterial levels.

**Key words:** stabilized rice bran, antibiotics, gastrointestinal, swine, microbiota

## Introduction

In 2010, approximately 243.1 million cwt of rice was produced in the United States (1). A waste product of the rice milling process is rice bran. Rice bran is produced when the outer brown layer of rice is removed from the rice kernel to yield white rice (2). Under normal milling processes, the oil and lipases within the bran come in contact causing degradation of the oil to glycerol and free fatty acids (2). Stabilization of the rice bran involves inactivating the lipase activity and new brands of stabilized rice bran (SRB) have increased shelf life of rice bran to 1 year. With the advent of the stabilization process for rice bran, commercial application in the animal feed industry needs to be investigated.

SRB contains several functional food components. The proximate composition of SRB is 16-22% fat (2). Palmitic, oleic and linoleic acid make up 90% of the fatty acid profile of SRB and it also contains 150 ppm of Vitamin E (2). Additional tocotrienols from the known α-, β-, γ- and δ-tocotrienols have been identified in rice bran and these novel vitamin E isolates have been shown to lower serum cholesterol activity in chickens (3). Additionally, SRB has been shown to improve stress response in rats compared to a standard natural based diet (4).

Although many components of SRB can relay "functional food" properties, our study focused on investigating the ability of the indigestible carbohydrate portion of SRB to interact with the swine intestinal microbiota to determine if SRB has prebiotic properties. Prebiotic, was recently redefined as, "a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microbiota that

confers benefits upon host well-being and health" (5). SRB contains an indigestible carbohydrate component. SRB is 20-25% total dietary fiber and ~2% soluble fiber. This fiber component is able to be utilized by intestinal bacteria and may promote the colonization of a healthy intestinal microbiota. Previously, fecal output has increased with feeding SRB in both humans and dogs, an indication of its fiber component changing fecal parameters (6). The nutrient composition of SRB indicates its potential use as a prebiotic-type dietary ingredient.

Weaning is a period marked with depressed growth in piglets. Weaning stressors include changes in diet from a fat and protein rich liquid diet to a solid carbohydrate rich diet and socialization changes with the introduction of novel pathogens during litter mixing. These stressors can lead to decreased feed intake and scours. Replacing corn with a similar nutrient dense feedstuff that has a higher indigestible carbohydrate fraction may mitigate some of the negative impacts of weaning via changes in the intestinal microbiotia. In this study we compared weaning pigs fed SRB to pigs fed corn diets with and without an antibiotic to determine if feeding SRB was able to change production outcomes and intestinal bacteria in the newly weaned piglet.

**Materials and Methods**

All animal procedures were approved by the Institutional Animal Care and Use Committee of North Carolina State University.

*Animals, experimental design, and diet.*

Two hundred pigs were weaned at approximately 21 days of age and allotted to one of four dietary treatments based on body weight in a randomized complete block design (10 blocks per treatment) for 28 days. Treatments were as follows: 1) control diet (CON), 2) control diet + antibiotics ($A^+/S^-$), 3) control diet + 10% SRB ($A^-/S^+$), 4) control diet + antibiotics + 10% SRB ($A^+/S^+$). Stabilized rice bran replaced corn in SRB diets. The antibiotic used was Aeuromix, a combination of chloretetracycline, sulfamethazine and penicillin that is affective against both gram positive and gram negative bacteria. Diets were fed in two phases (phase 1 from 0 to 14 days post-weaning and phase 2 from 14 to 28 days post-weaning). The formulas for the diets are listed in **Table 1.** Feed was fed as a pellets during phase 1 and as mash in phase 2. Pigs were housed 5 pigs per pen (1.73 m x 0.83 m), using 40 pens in two separate environmentally control nursery rooms (20 pens per room) with raised slate floors. The nursery rooms purposefully had not been cleaned after a previous experiment. Pigs were exposed to artificial light from 6 am to 8 pm. Pens were equipped with two-hole self-feeders and a nipple waterer allowing *ad libitum* access to feed and water. Pig body weight and feed intake were measured weekly.

*Sample collection.*

On day 28 and 29, one pig from each pen was randomly chosen for sampling. Sampling occurred in blocks and 5 blocks were sampled per day. A blood sample was collected via the jugular vein. The animal was then killed by electrocution and exsanguination. The large and small intestines were then collected. Twelve inches proximal to the ileocecal junction, mucosal samples were collected. The section was rinsed with cold PBS and mucosa was

collected using a glass microscope slide. The sample was immediately flash frozen in liquid nitrogen and later stored at -80$^{o}$C. The next 6 inches were sampled for histology. At the center section of the distal sample, approximately two cm of tissue were excised and placed in neutral buffered formalin for fixing. Digesta were collected from the remaining portion of the ileum, the entire colon and duodenum. Ileal digesta were collected, mixed and sub-sampled for short chain fatty acids and digestibility. Colon digesta were collected, mixed and sub-sampled for short chain fatty acids and quantification of bacteria.

*Apparent ileal digestibility.*

During the second phase of feeding, chromic oxide was added to the diet as an indicator of digestibility. After initial collection, digesta and feed samples were frozen until further analysis. Samples were thawed and weighed out into whirlpak bags. Samples of digesta and feed were freeze dried for one week using the Heto PowerDry LL3000. Chromium content was determined via atomic absorption by Analytical Services Lab at North Carolina State University. Digestibility was calculated as a percentage based on modified calculation determining the percentage of Cr in the feed compared to digesta (7).

*Ileal histology*

Ileal samples were fixed in neutral buffered formalin. After 24 hours, the samples were transferred into a 70% ethanol solution and subsequently embedded in wax, sectioned and stained with hematoxylin and eosin. Two slices of the ileum were placed on each slide for analysis. To measure the intestinal morphology, three well defined villi were identified. The length and width of the villi were recorded along with the length of the crypt underneath the

villi. To enumerate the intraepithelial lymphocytes (IEL), five well defined villi were identified. The IELs and epithelia cells were counted on each of these villi. Intestinal slides were read using an Olympus Vanox-S Microscope (Olympus Corporation, Lake Success, NY) and analyzed using SPOT Basic Imaging software (Diagnostic Instruments, Sterling Heights, MI).

*Ileal and colonic short chain fatty acid concentration.*

Short chain fatty acid (SCFA) concentrations in ileal and colonic digesta were determined using a modified gas chromatography method (8). Diethylacetic acid served as the internal standard. Short chain fatty acids were extracted from the digesta overnight in a 1:1 mixture with 0.5N HCl. After extraction, samples were clarified by centrifugation, and the supernatant was injected (1μL) into a Varian CP 3380/3800 with a NUKOL Fused Silica Capillary Column (30 m x 0.25 mm x 0.25 μm) via a Hamilton syringe 10μL (model 701). A flame ionization detector was used to determine SCFA elution.

*Cecal bacterial concentration.*

Ileal and colonic digesta was serially diluted with cold PBS. Serial dilutions were plated for lactobacilli on Difco Rogosa Agar and bifidobacteria on Difco Rogosa Agar and Difico Differential Clostridium Agar. Plates were placed in Bio-Bags (Fisher Scientific) to generate an anaerobic environment and then incubated for 48 hours at 37$^{o}$C. After 48 hours, colonies were counted from 2 plates for each bacteria per animal.

*Digestive enzyme analysis.*

Crude homogenates of ileal mucosal tissue were prepared by homogenizing tissue in sterile phosphate buffered solution. Maltase activity was analyzed based on modifications for a 96 well plate assay (9). The standard control was D-Glucose. One unit of enzyme specific activity was determined as the liberation of one μmol of glucose per min per mg of protein. Leucine aminopeptidase activity was determined through a colormetric assay (10). Total protein was determined (11) using a commercially available bicinchoninic acid kit (Pierce #23225).

*Statistical analysis.*

Data were analyzed according to a randomized complete block design using the general linear models procedure of SAS (SAS, Cary, NC). The experiment was analyzed as a 2 x 2 factorial arrangement. Differences were deemed significant when $P < 0.05$.

**Results**

*Growth performance and apparent ileal digestibility.*

Differences in performance were not observed during the first phase (d 0-14; Table 2). Antibiotic supplementation improved average daily gain during phase 2 (d 12-28) by 6% ($P = 0.024$). Cumulatively (d 0-28), an interaction between ANT and SRB was observed for G:F ($P = 0.017$). G:F increased by 14% in pigs fed $A^-/S^+$ compared to all other treatments and overall SRB improved cumulative G:F by 9% ($P = 0.01$). There was an interaction between ANT and SRB for apparent ileal digestibility ($P = 0.024$). The addition of SRB to

ANT-free diets did not improve digestibility, but SRB supplementation in diets with ANT improved digestibility by 16% ($P < 0.05$). The improvement in digestibility lead to a cumulative improvement in feed efficiency with SRB supplementation compared to ANT (P = 0.01).

*Intestinal microbial concentrations and metabolic products.*

No significant differences or interactions were observed for culturable ileal bifidobacteria (Table 3; $P = 0.69$) or lactobacilli ($P = 0.49$). There was a trend for increased colonic bifidobacteria with SRB supplementation (Table 3; $P = 0.09$). SCFA are fermentative bacterial end products. Although there were trends for increased bifidobacteria, both ileal and colonic SCFA were not affected by dietary treatment (Table 4). Propionic, isobutyric and valeric acid were not detectable in the ileal digesta.

*Ileal morphology and enzyme activity.*

There was an interactive effect of ANT and SRB on ileal crypt depth and IEL (Table 5; $P = 0.038$; $P = 0.003$). Pigs fed $A^+/ S^+$ had increased crypt depth by about 25% compared to all other treatments. IEL were increased in pigs fed $A^-/S^+$ diets by 15% ($P < 0.05$), but were not different in pigs fed $A^+/S^-$ diets. SRB decreased villi:crypt from 3.22 to 2.61 ± 0.210 ($P = 0.044$). Ileal enzyme activity was not affected by dietary treatment.

**Discussion**

Various nutritional approaches have been investigated to minimize the depression in growth during piglet weaning. Non-digestible carbohydrates have been shown to improve

the stability of intestinal microbes and health status of newly weaned piglets (12). Our study did not look at specific health status, but instead focused on growth response as an indication of health. During the first phase of feeding (d 0-14 post-weaning), growth response was not different among treatments. During the second phase (d 14-28 post-weaning), dietary antibiotics improved gain. Previous reports have shown no improvement in gain with the addition of antibiotics in research settings (13, 14). It is believed that nonresponsive growth due to antibiotic feeding in research settings is due to a difference in animal husbandry between research farms and industry settings. For this reason, the building was purposefully not cleaned between weaning groups and therefore there was an observable improvement in gain due to antibiotic feeding.

Feed efficiency was improved in piglets fed $A^-/S^+$ compared to all other treatments. Replacing up to a 60% of a corn-soybean meal diet with SRB also significantly improved feed efficiency in broilers by about 20%, although the improvement in feed efficiency was due to decreased feed intake and body weight gains (Sayre et al., 1987). Replacing 10% of corn with SRB did not decrease feed intake or body weight gains, but improved feed efficiency. Feeding the ANT mixture (Aeuromix) reduces both gram positive and gram negative intestinal bacteria. The trial was designed to focus specifically on the activity of SRB in the intestine and therefore a SRB negative control with ANT supplementation ($A^-/S^+$) was included in the study. We hypothesize the improvement in feed efficiency in $A^-/S^+$ is due to the prebiotic properties of SRB changing the intestinal environment due to the observation that feeding $A^+/S^+$ did not improve feed efficiency compared to $A^-/S^+$ fed pigs.

Intestinal architecture is thought to affect production outcomes. We did not observe differences in villi length, but there was a diet interaction for crypt depth with piglets fed $A^+/S^+$ having the deepest crypt depths. The increased crypt depth lead to a 30% decrease in villi:crypt for $A^+/S^+$ fed piglets compared to all other treatments. The IEL infiltration did not follow a similar pattern as villus height, with CON piglets having a lower number of IEL compared to $A^-/S^+$ fed pigs. Decreased villi:crypt depth have been reported during weaning due to villus atrophy and crypt hyperplasia but in our study decreased villi:crypt was due to increased crypt depth but the villi were not significantly affected (15). Villus atrophy with increased crypt depth is associated with decreased intestinal enzyme activity (15). Villus atrophy and decreased disaccharidase activity are associated with decreased nutrient utilization (16). Although the villi:crypt depth was decreased with feeding $A^+/S^+$, changes in intestinal enzyme activity were not observed and growth was not affected. The pigs fed only SRB improved feed efficiency while not changing intestinal morphology.

Compared to the adult pig, the neonatal and weaning piglet is more susceptible to enteric infections. The adult microbial community is a stable population, but the young pig intestinal microbial population is dynamic within increased risk for enteric infections (12, 17). Piglets weaned in an industrial setting experience several changes in their intestinal microbial environment due to diet, environment and host. Maintaining a balanced microbial community with a large presence of the beneficial bacteria such as lactobacilli and bifidobacteria in early development is hypothesized to improve intestinal health. In this study, feeding SRB tended to increase bifidobacteria, but lactobacilli levels were unaffected. The bacterial metabolites, SCFA, were not different among treatments. Feeding SRB tended

to increase colonic bifidobacteria levels, but changes in the fermentation activity of the bacteria were not detectable.

SRB increased feed efficiency beyond that of conventional weaning diets with growth promoting antibiotic supplementation. The improvement in production was accompanied by a trend for increased colonic bifidobacteria, but the fermentative activity of the bacteria was not detectably different when SRB was fed. Trends for increased bifidobacteria and improved feed efficiency indicate SRB has prebiotic properties. SRB shows promise as a replacement of corn in weaning diets, specifically diets that lack antibiotic growth promoters.

**Table 1. Diet composition during a two phase feeding regime.**

| | Phase One | | | | Phase Two | | | |
|---|---|---|---|---|---|---|---|---|
| | Antibiotics | | | | Antibiotics | | | |
| | - | | + | | - | | + | |
| SRB | - | + | - | + | - | + | - | + |
| Ingredients, % | | | | | | | | |
| Corn Meal | 45.50 | 36.80 | 45.40 | 36.70 | 56.70 | 48.00 | 56.70 | 47.90 |
| Lard | 2.00 | 2.00 | 2.00 | 2.00 | 3.73 | 3.73 | 3.73 | 3.73 |
| Fish Meal | 4.20 | 4.20 | 4.20 | 4.20 | 3.00 | 3.00 | 3.00 | 3.00 |
| Soybean Meal | 25.40 | 24.10 | 25.40 | 24.10 | 27.00 | 25.70 | 27.10 | 25.70 |
| Dried Whey | 15.00 | 15.00 | 15.00 | 15.00 | 4.20 | 4.20 | 4.20 | 4.20 |
| Vitamin Premix | 6.50 | 6.50 | 6.50 | 6.50 | 3.00 | 3.00 | 3.00 | 3.00 |
| Ground limestone | 0.06 | 0.12 | 0.06 | 0.12 | ----- | ----- | ----- | ----- |
| Phosphate Defluoride | 1.05 | 1.00 | 1.06 | 1.00 | ----- | ----- | ----- | ----- |
| Salt | 0.25 | 0.25 | 0.25 | 0.25 | 0.35 | 0.35 | 0.35 | 0.35 |
| Mono dicalcium Phosphate | ----- | ----- | ----- | ----- | 1.00 | 0.96 | 1.00 | 0.96 |
| Calcium carbonate | ----- | ----- | ----- | ----- | 0.86 | 0.89 | 0.86 | 0.89 |
| Sodium selenite | ----- | ----- | ----- | ----- | 0.05 | 0.05 | 0.05 | 0.05 |
| Aureomix[1] | 0.00 | 0.00 | 0.13 | 0.13 | 0.00 | 0.00 | 0.13 | 0.13 |
| SRB | 0.00 | 10.00 | 0.00 | 10.00 | 0.00 | 10.00 | 0.00 | 10.00 |
| Chromium oxide | ----- | ----- | ----- | ----- | 0.10 | 0.10 | 0.10 | 0.10 |
| Calculated Diet Analysis | | | | | | | | |
| CP, % | 23.20 | 23.20 | 23.20 | 23.20 | 20.40 | 20.50 | 20.40 | 20.50 |
| Crude Fat, % | 5.10 | 6.37 | 5.10 | 6.36 | 6.21 | 7.95 | 6.21 | 7.94 |
| Crude Fiber, % | 1.99 | 2.57 | 1.98 | 2.57 | 1.92 | 2.44 | 1.92 | 2.44 |
| Calcium, % | 0.80 | 0.85 | 0.80 | 0.85 | 0.79 | 0.79 | 0.79 | 0.79 |
| Avail. P, % | 0.54 | 0.56 | 0.54 | 0.56 | 0.44 | 0.44 | 0.44 | 0.44 |
| ME, kcal/lb | 1570.00 | 1558.00 | 1568.00 | 1556.00 | 1486.00 | 1471.00 | 1484.00 | 1469.00 |
| Lysine, % | 1.50 | 1.50 | 1.50 | 1.50 | 1.40 | 1.40 | 1.40 | 1.40 |

[1]Chloretetracycline, sulfamethazine and penicillin

**Table 2. Effect of dietary supplementation of stabilized rice bran (SRB) and antibiotics (ANT) on pig growth performance and apparent ileal dry matter digestibility.**

| Antibiotic: | - | | + | | | P-value | | |
|---|---|---|---|---|---|---|---|---|
| SRB: | - | + | - | + | SEM | ANT | SRB | ANT*SRB |
| ADG, g | | | | | | | | |
| d 0 to 7 | 108 | 123 | 131 | 110 | 15 | 0.765 | 0.840 | 0.251 |
| d 7 to 14 | 237 | 204 | 219 | 237 | 14 | 0.622 | 0.615 | 0.087 |
| d 14 to 21 | 370 | 397 | 430 | 392 | 18 | 0.129 | 0.739 | 0.077 |
| d 21 to 28 | 568 | 596 | 608 | 624 | 19 | 0.084 | 0.257 | 0.755 |
| d 0 to 14 | 173 | 164 | 175 | 173 | 12 | 0.640 | 0.678 | 0.751 |
| d 14 to 28 | 469 | 496 | 519 | 508 | 13 | 0.024 | 0.542 | 0.146 |
| d 0 to 28 | 321 | 330 | 347 | 341 | 11 | 0.105 | 0.904 | 0.493 |
| ADFI, g | | | | | | | | |
| d 0 to 7 | 129 | 122 | 127 | 135 | 9 | 0.574 | 0.964 | 0.431 |
| d 7 to 14 | 276 | 230 | 257 | 249 | 16 | 1.000 | 0.106 | 0.246 |
| d 14 to 21 | 571 | 523 | 589 | 560 | 24 | 0.252 | 0.117 | 0.690 |
| d 21 to 28 | 620 | 539 | 665 | 672 | 41 | 0.039 | 0.373 | 0.291 |
| d 0 to 14 | 203 | 176 | 192 | 192 | 10 | 0.787 | 0.174 | 0.177 |
| d 14 to 28 | 595 | 531 | 627 | 616 | 29 | 0.058 | 0.210 | 0.370 |
| d 0 to 28 | 399 | 353 | 409 | 404 | 18 | 0.103 | 0.166 | 0.274 |
| G:F | | | | | | | | |
| d 0 to 7 | 0.790 | 1.025 | 1.012 | 0.930 | 0.119 | 0.600 | 0.525 | 0.193 |
| d 7 to 14 | 0.856 | 0.893 | 0.848 | 1.035 | 0.078 | 0.399 | 0.163 | 0.345 |
| d 14 to 21 | $0.656^{b}$ | $0.765^{a}$ | $0.727^{ab}$ | $0.698^{ab}$ | 0.025 | 0.925 | 0.130 | 0.012 |
| d 21 to 28 | 0.943 | 1.230 | 0.936 | 0.959 | 0.075 | 0.073 | 0.047 | 0.088 |
| d 0 to 14 | 0.840 | 0.936 | 0.906 | 0.922 | 0.053 | 0.627 | 0.297 | 0.451 |
| d 14 to 28 | $0.803^{b}$ | $0.978^{a}$ | $0.836^{b}$ | $0.837^{b}$ | 0.036 | 0.142 | 0.021 | 0.022 |
| d 0 to 28 | $0.816^{b}$ | $0.961^{a}$ | $0.851^{b}$ | $0.857^{b}$ | 0.027 | 0.228 | 0.010 | 0.017 |
| Apparent Digestibility, % | $90.2^{ab}$ | $80.1^{ab}$ | $79.7^{b}$ | $96.0^{a}$ | 6.2 | 0.628 | 0.574 | 0.024 |

**Table 3. Effect of dietary supplementation of stabilized rice bran (SRB) and antibiotics (ANT) on culturable bifidobacteria and lactobacilli in ileal and colonic digesta.**

| | Antibiotic | | | | SRB | | | |
|---|---|---|---|---|---|---|---|---|
| | - | + | SEM | P-value | - | + | SEM | P-value |
| Ileum | CFU/g digesta | | | | CFU/g digesta | | | |
| Bifidobacteria | 9.9E+05 | 9.7E+04 | 4.6E+05 | 0.180 | 3.0E+05 | 7.9E+05 | 5.6E+05 | 0.464 |
| Lactobacilli | 4.9E+06 | 1.7E+06 | 1.5E+06 | 0.147 | 2.5E+06 | 4.1E+06 | 1.4E+06 | 0.410 |
| Colon | | | | | | | | |
| Bifidobacteria | 4.3E+06 | 3.0E+06 | 1.4E+06 | 0.473 | 2.1E+06 | 5.2E+06 | 1.3E+06 | 0.098 |
| Lactobacilli | 1.3E+07 | 1.5E+07 | 2.6E+06 | 0.629 | 1.6E+07 | 1.2E+07 | 2.8E+06 | 0.342 |

**Table 4. Effect of dietary supplementation of stabilized rice bran (SRB) and antibiotics (ANT) on ileal and colonic digesta volatile fatty acid content**

| | Antibiotic | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | - | | + | | | P-value | | |
| SRB | - | + | - | + | SEM | ANT | SRB | ANT*SRB |
| VFA, mg/g dry digesta | | | | | | | | |
| Ileum | | | | | | | | |
| Acetic Acid | 1.46 | 1.10 | 1.18 | 1.19 | 0.18 | 0.580 | 0.324 | 0.295 |
| Butyric Acid | 0.12 | 0.12 | 0.18 | 0.17 | 0.05 | 0.302 | 0.888 | 0.918 |
| Isovaleric Acid | 0.03 | 0.07 | 0.01 | 0.07 | 0.03 | 0.760 | 0.072 | 0.789 |
| Total VFA | 1.64 | 1.29 | 1.37 | 1.43 | 0.18 | 0.712 | 0.407 | 0.239 |
| Colon | | | | | | | | |
| Acetic Acid | 9.06 | 8.27 | 10.20 | 8.80 | 1.12 | 0.459 | 0.336 | 0.784 |
| Propionic Acid | 5.31 | 5.39 | 6.38 | 5.23 | 0.85 | 0.599 | 0.534 | 0.480 |
| Isobutyric Acid | 0.25 | 0.23 | 0.25 | 0.28 | 0.05 | 0.589 | 0.914 | 0.664 |
| Butyric Acid | 4.24 | 3.93 | 4.56 | 3.42 | 0.60 | 0.873 | 0.237 | 0.492 |
| Isovaleric Acid | 0.47 | 0.42 | 0.48 | 0.50 | 0.10 | 0.669 | 0.895 | 0.721 |
| Valeric Acid | 0.63 | 0.94 | 0.65 | 0.47 | 0.20 | 0.286 | 0.739 | 0.247 |
| Total VFA | 19.95 | 19.17 | 22.51 | 18.70 | 2.54 | 0.684 | 0.375 | 0.555 |

**Table 5. Effect of dietary supplementation of stabilized rice bran (SRB) and antibiotics (ANT) on ileal morphology and enzyme activity**

| | Antibiotic | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | - | | + | | | P-value | | |
| SRB | - | + | - | + | SEM | ANT | SRB | ANT*SRB |
| Villi Length (μm) | 500 | 478 | 458 | 444 | 20 | 0.064 | 0.391 | 0.851 |
| Villi Width (μm) | 154 | 150 | 155 | 149 | 6 | 0.946 | 0.421 | 0.805 |
| Crypt Depth (μm) | $196^{ab}$ | $183^{b}$ | $186^{b}$ | $236^{a}$ | 15 | 0.143 | 0.211 | 0.038 |
| Villi:Crypt | $3.35^{a}$ | $3.06^{a}$ | $3.10^{a}$ | $2.18^{b}$ | 0.30 | 0.058 | 0.044 | 0.294 |
| Villi Area ($mm^2$) | 0.528 | 0.505 | 0.484 | 0.449 | 0.031 | 0.112 | 0.355 | 0.847 |
| IEL (IEL/100 enterocytes) | $28.0^{c}$ | $32.1^{a}$ | $31.6^{ab}$ | $28.2^{bc}$ | 1.2 | 0.892 | 0.797 | 0.003 |
| Maltase (U/g protein) | 28.9 | 26.2 | 25.5 | 27.2 | 3.9 | 0.754 | 0.897 | 0.557 |
| Leucine Amino Peptidase (U/g protein) | 1.71 | 0.59 | 1.21 | 1.29 | 0.44 | 0.795 | 0.256 | 0.149 |

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# CHAPTER 3: SAFETY EVALUATION OF POLYDEXTROSE IN INFANT FORMULA USING A SUCKLING PIGLET MODEL

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Abbreviations: ADFI, average daily feed intake; ADG, average daily gain; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BUN, blood urea nitrogen; G:F, body weight gain to feed intake ratio; GI, gastrointestinal; GOS, galactooligosaccharides; GRAS, generally recognized as safe; HMO, human milk oligosaccharides; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; PDX, polydextrose.

**Abstract**

Oligosaccharides, the third largest component in human milk, are virtually absent from cow's milk and most infant formula. Prebiotic carbohydrates like polydextrose (PDX) have been proposed as surrogates for human milk oligosaccharides. Safety assessments of novel infant formula ingredients include dose-response experiments in appropriate neonatal animal models such as the suckling pig. To further substantiate the safety of the ingredient, one-day old pigs were fed a cow's milk-based formula supplemented with PDX (1.7, 4.3, 8.5 or 17 g/L) for 18 days (n = 13/dose) and compared to appropriate control (unsupplemented formula; n = 13) and reference groups (day 0 pigs, and sow-reared pigs; n = 13). Growth rate, formula intake, stool consistency, behavior score, blood chemistry and hematology, relative organ weights (% of body weight), tissue morphology (i.e. liver, kidney and pancreas) and pancreas biochemistry did not differ among formula-fed pigs ($P > 0.1$). Polydextrose mimicked other prebiotic carbohydrates and did not produce any adverse affects when evaluated in a neonatal pig model.

## Introduction

Mother's milk is considered the "gold standard" for infant nutrition. After the first week of breast-feeding, bifidobacteria reportedly become the dominant gastrointestinal (GI) bacteria, while formula-fed infants develop a more diverse microbiota, with *Bacteroides sp.* equaling the number of bifidobacteria (Harmsen et al., 2000). Commensal bacteria, such as bifidobacteria, help maintain healthy gastrointestinal mucosal surfaces (Ismail and Hooper, 2005). Certain commensal bacteria are able to thrive in the large intestine by fermenting oligosaccharides and other resistant carbohydrates, capturing energy that would otherwise escape the human digestive process (Schell et al., 2002). Oligosaccharides constitute the third largest component in human milk after lactose and lipids, ranging in concentration from 5-10 g/L in mature milk. Oligosaccharides are virtually absent from cow's milk (< 0.08 g/L) and most infant formula, which may account in part for the difference in GI microbiota reported between breast and formula fed infants (Kunz et al., 2000; Newburg and Neubauers, 1995). Approximately 200 molecular species of oligosaccharides have been identified in human milk which are synthesized from D-glucose, D-galactose, D-N-acetylglucosamine, L-fucose and D-N-acetylneuraminic acid (sialic acid) monomers. In contrast, bovine milk oligosaccharide composition is much simpler as the 10 molecular species that have been identified, consist largely of sialic acid linked with lactose (Gopal and Gill, 2000; Kunz et al., 2000; Newburg and Neubauers, 1995; Ninonuevo et al., 2006). Given the complexity of human milk oligosaccharides (HMO) and the lack of commercial sources even for the predominant constituents, prebiotic carbohydrates are increasingly added to bovine-milk-

based infant formulas. Such supplementation is intended to substitute for some of the functional properties of HMO (i.e. partial fermentation by GI microbiota, modification of the composition and/or activity of beneficial bacteria, etc.).

Polydextrose (PDX), a randomly bonded glucose polymer with sorbitol end groups and citric acid attached by mono- and diester bonds, was first developed as a bulking agent for foods. Though the random structure of PDX contains numerous glycosidic bonds, those in the β(1-6) configuration predominate. The degree of polymerization, an indication of molecular size, is known to range from a few glucose units to more than 30, but an average of 12 is typically reported (Mitchell, 1996; Craig, 2001). The PDX structure accordingly limits digestion by mammalian enzymes allowing it to reach the large intestine and stimulate fermentation by the commensal microbiota. The indigestible nature of PDX has lead to its consideration as a prebiotic, which was recently redefined by Roberfroid (2007) as, "a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microbiota that confers benefits upon host well-being and health."

Polydextrose has been approved as a food additive in the US since 1982 (Food and Drug Administration 21 CFR 172.841). While PDX is not considered toxic at high dosages, laxation effects have been observed when intakes are elevated, similarly to other non-digestible carbohydrates (i.e., fiber). Children are considered no more sensitive than adults to PDX when given at the same level on a per body weight basis (Flood et al., 2004). Although PDX is approved for consumption by adults and children, its use in infant formula and baby food has yet to be allowed by the FDA. In a recent clinical study, infants were fed formula

supplemented with one of two prebiotic blends: 1) 4 g/L PDX and galactooligosaccharide (GOS) (50:50 ratio); or 2) 8 g/L PDX, GOS and lactulose (50:33:17 ratio). Over the course of 106 days, infants fed prebiotic-supplemented formula achieved normal growth and produced softer/looser stools, a laxation pattern more characteristic of breast fed infants (Ziegler et al., 2007). As reported in a compendium by Burdoch and Flamm (1999), an extensive array of toxicological studies, conducted in a variety of animal models (i.e. mice, rats, rabbits, dogs) for extensive periods of time (3-24 months), have fully supported the safety of PDX as a food ingredient. It should be noted that specific adaptive responses in GI morphology including increased cecal weight and decreased cecal muscular thickness have been reported in suckling (Harada et al., 1995) and adult (Yoshioka et al., 1994) rat populations. Because GI development in neonatal rats is significantly delayed and programmed in a different manner from that of human infants, neonatal rats do not constitute the best model for evaluating the safety of ingredients intended for addition to infant formula. Neonatal pigs are considered more appropriate for such studies as their GI developmental program is intermediate between those of rats and humans.

The neonatal pig is a well established model for infant formula nutrient interactions (Reeds and Odle, 1996). Examples of the neonatal pig model establishing the safety of novel ingredients for infant formula include determining the safety of single cell sources of long-chain polyunsaturated fatty acids (Matthews et al., 2002; Huang et al., 2002), and more recently the effects of conjugated linoleic acids on lipid accretion and adipose tissue metabolism (Corl et al., 2008). The pig digestive tract is anatomically and functionally similar to that of the human and is more physiologically relevant for prebiotic studies than a

rodent model. The major difference between the neonatal pig and human infant intestinal anatomy is the former's coiled descending colon (although the large intestine is similar in length), while the rodent's large intestine is marked by an enlarged cecum compared to that of the human (Moughan et al., 1992). The accelerated growth rate (~10X) of neonatal pigs relative to human infants enhances the sensitivity of the former to the effects of toxicological agents. The similarity of developmental program and anatomy of the large intestine, in combination with an accelerated growth rate, render the neonatal pig an appropriate surrogate for human infants in nutrition studies focused on the GI impact of fermentable carbohydrates. To further substantiate the safety of PDX in a neonatal pig model, increasing doses up to 8.35 g/kg BW per day were fed from days 2 to 19 of life. Body and organ weights and serum and whole blood chemistries were determined. Histological assessment of the kidney, liver, pancreas, cecum and colon were examined by light microscopy after fixation and hematoxylin and eosin staining. Amylase activity and DNA and protein concentrations of the pancreas were analyzed to determine pancreatic effects of PDX. Collectively, these data will substantiate the safety of PDX for inclusion in infant formula, with no adverse effects detected within the suckling pig model.

## Material and Methods

### *Animals*

Full-term pigs were vaginally delivered at the North Carolina State University Swine Education Unit and allowed colostrum for 24 hours. Pigs were randomly allotted to one of

seven dietary interventions (n = 13 pigs per dietary intervention, 1.71 ± 0.27 kg). Pigs that were allotted to the sow-reared reference group were ear-tagged and remained with their sow for the duration of the experiment. Pigs assigned to the other dietary interventions were transported to the Laboratory of Developmental Nutrition at North Carolina State University. All formula-fed pigs were housed in individual cages in an environmentally controlled room (32°C) programmed to deliver a light/dark cycle of 16 hours/8 hours, respectively. For the first 24 hours, all formula-fed pigs were trained to suckle from bottles using the control diet. Pigs were fed at ~60% *ad libitum* with fresh diet offered three times daily for 18 days in order to achieve growth rates similar to sow-fed pigs (Hess et al., 2008). Each day, formula-fed pigs were weighed, their stool consistency evaluated, and both appearance and behavior (gross motor activity, feeding) observed. Stool consistency was rated on a scale of 1 to 5 with 1 representing well-formed stools and 5 representing watery stools with no form. Sow-reared pigs were weighed on days 0, 6, 12 and 18. Body temperature was recorded on day 18 via the ear (6 replicates) or rectally (7 replicates).

*Dietary Interventions*

A basal diet patterned after term human infant formula was prepared to meet the nutrient requirements (National Research Council, 1998) of neonatal pigs. The ingredients of the dry basal diet, previously reported by Hess et al. (2008), were amended to provide one of four levels of PDX (Litesse® Two, Danisco USA Inc, Terre Haute, IN) (1.7 g/L, 4.3 g/L, 8.5 g/L and 17.0 g/L). Dry ingredients were mixed and reconstituted with water, fat and the dietary intervention level of PDX, homogenized and then refrigerated.

*Sampling*

Blood samples were collected from the jugular vein for whole blood analysis and for serum chemistry (Antech Diagnostics, Farmingdale, NY) on day 0 (reference group) and 18 (all dietary intervention groups and sow-milk reference group). Whole blood hematology included the measurement of hemoglobin, hematocrit, white blood cell count, red blood cell count, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, platelet count, neutrophil count and lymphocyte count. The serum analysis included measurements of glucose, blood urea nitrogen (BUN), creatinine, total protein, albumin, total bilirubin, alkaline phosphatase, alanine aminotransferase (ALT), aspartate aminotransferase (AST), cholesterol, calcium, potassium, sodium, phosphorus, chloride, globulin, albumin:globulin ratio, and BUN:creatinine ratio. After blood collection, piglets were euthanized using an American-Veterinary-Medical-Association-approved electrocution device followed by exsanguination. Urine samples were collected via bladder puncture for urinanalysis which included measurements of appearance, color, osmolarity, specific gravity, bilirubin, blood, glucose, ketones, pH, proteins, urobilinogen, casts, cells, and crystals (Antech Diagnostics, Farmindale, NY). Organs were collected for weight (liver, kidney, pancreas, spleen, gallbladder, heart, brain, left eye, lungs, cecum and colon) and histology (liver, kidney, pancreas, cecum and colon) measurements. Pancreas samples were also collected for biochemical analysis and stored on dry ice during sampling and then transferred to a $-80^{0}$C freezer for storage. Cecum and colon contents (digesta) were collected and placed on ice until pH could be measured (approximately 0-20 minutes).

*Liver, kidney, intestine, pancreas, cecum and colon morphology*

Tissue samples for morphological assessment were collected from similar areas on each organ. Samples were fixed in neutral buffered formalin. After 24 hours, the samples were transferred into a 70% ethanol solution and subsequently embedded in wax, sectioned and stained with hematoxylin and eosin. One section of liver, kidney and pancreas were stained, while two sections of the cecum and colon were stained. The cecum and colon muscularis externa was measured in 18 locations throughout the two sections. Intestinal slides were read using an Olympus Vanox-S Microscope (Olympus Corporation, Lake Success, NY) and analyzed using SPOT Basic Imaging software (Diagnostic Instruments, Sterling Heights, MI). A board certified veterinary pathologist who was unaware of dietary interventions evaluated the liver, kidney and pancreas sections. Tissue examinations were made using an Olympus BH-2 microscope with 2, 4, 10, 20 and 40X objectives (Olympus Corporation, Lake Success, NY). Each slide was examined for histological abnormalities. The main observation of hepatocellular vacuolization was classified using a subjective scale from 0 to 3 with 0 used for no apparent hepatocellular vacuolization, 1 for minimal evidence, 2 for mild and 3 for moderate evidence of vacuolization. The zone of the lobule involved with vacuoles (i.e., periportal, centrilobular or massive) also was indicated. A count of mitotic figures for the liver was established by counting all evident mitotic figures in five high power fields (40X objective) for each liver. For the pancreas, the count of mitotic figures was performed on 10 high power fields for each of the samples. The extent of apoptosis was graded subjectively. Other histological changes were evaluated but not scored as they were not evident. The kidney was scored subjectively based on the presence of

vacuoles within the renal tubular epithelium using a scale from 0 to 3, similar to that used for the pancreas apoptosis.

*Pancreatic biochemistry*

Crude homogenates of pancreatic tissue were prepared by homogenizing tissue in sterile phosphate buffered solution. Amylase activity was analyzed using a commercially available kit (Invitrogen, #E33641), based on the method described by Bernfeld (1951). The standard control was α-amylase from *Bacillus sp.* (Sigma #A-6380). Total protein was determined using a commercially available bicinchoninic acid kit (Pierce #23225), based on the method described by Smith and colleagues (1985). Total DNA was determined from crude homogenates using the Labarca and Paigen (1980) method (Sigma #DNA-QF).

*Statistical Analysis*

Data were analyzed according to a completely randomized block design using the general linear models procedure of SAS (SAS, Cary, NC). Linear and quadratic contrasts were analyzed for formula-fed piglets (Steel and Torrie, 1980). Data from organ morphology scores were analyzed using the Proc Genmod procedure of SAS (SAS, Cary, NC). Differences were deemed significant when $P < 0.05$.

**Results**

*Growth Rate and Formula Consumption*

Average daily body weight gains (ADG), the ratio of body weight gain to dry feed intake (G:F) and energy consumption (kcal/kg bw) did not differ among dietary interventions

(Table 1). The ADG of sow-reared pigs (325 g/d) did not differ from formula-fed pigs. Pigs fed PDX at the lowest (1.7 g/L) and highest level (17 g/L) displayed a significant increase in dry weight average daily feed intakes (ADFI) relative to the control animals though no dose-related trend was evident.

*Behavior, Health and Blood Parameters*

All pigs were apperceptive and feeding throughout the experiment. No differences in behavior were observed between groups of formula-fed pigs. Stool consistency was softer for pigs fed 17 g PDX/L compared to pigs fed 4.3 and 8.5 g PDX/L ($P < 0.05$; Table 1), but was not significantly different from control pigs or those fed PDX at 1.7 g/L. No differences were observed in body temperature among groups of formula-fed pigs ($P = 0.283$). Specific gravity, pH, and other measurements included in the urinalysis panel did not vary among dietary interventions (data not shown). With the exception of blood urea nitrogen (BUN), serum chemistry values did not differ significantly among groups of formula-fed pigs (Table 2). BUN decreased in pigs fed formula supplemented at 8.5 g/L and 17g/L versus control and those supplemented at 4.3 g/L ($P = 0.007$), however the values were not different when normalized to creatinine. Compared to formula-fed pigs, sow-reared pigs experienced decreased BUN and BUN:Creatinine levels and increases in creatinine, total bilirubin, alkaline phosphatase, alanine aminotransferase and cholesterol ($P < 0.05$). Whole blood analysis revealed an increase in red blood cells for pigs receiving 4.3 g/L and 17 g/L PDX compared to 1.7 g/L PDX ($P < 0.05$; Table 3), but neither linear nor quadratic relationships were detected. Mean corpuscular hemoglobin concentration was increased in pigs fed 8.5

g/L compared to pigs fed 0, 4.3 and 17 g/L PDX and a quadratic (P = 0.039), but no linear relationship was observed.

*Body Weight, Organ Weights, and Digesta pH*

As a percentage of total body weight, the kidneys of pigs fed PDX at the highest level (17 g /L) were smaller than those of control pigs (P < 0.05) (Table 4). Pancreas weight was increased in pigs fed formula supplemented with 17 g/L PDX when compared to those consuming 1.7 g/L and 8.5 g/L (P < 0.05) but no differences were noted versus control pigs or those fed an intermediate level of PDX (4.3 g/L) (Table 5). No other organ weights differed among groups of formula-fed pigs. When compared to the weights of liver, kidney and pancreas observed in sow-reared piglets, those in all formula groups were significantly heavier (P < 0.05) (Tables 4 and 5). Ileal digesta pH declined linearly from 6.63 to 6.47 with increasing PDX (Linear P-value = 0.021).

*Organ Morphology*

There was no evidence of inflammation or necrosis in liver, kidney or pancreatic tissue. The degrees of change were modest overall in all organs from the majority of animals, and within normal physiological bounds. Evidence of pancreatic hyperplasia was not observed and measures of zymogen content, mitotic index and apoptosis did not vary significantly among any formula group (Table 6). Evaluation of the liver found no differences in vacuolization (Table 4), zonal vacuolization, mitosis, pigmentation, bile ducts, hepatocyte numbers, portal monocyte and neutrophil numbers among formula-fed pigs (data not shown). Kidney vacuolization did not differ between any dietary interventions (Table 4).

*Pancreas Biochemistry*

Though pancreatic DNA and protein concentrations did not vary in response to PDX, both values were significantly higher in piglets fed sow's milk ($P < 0.05$; Table 5). Similarly, pancreatic amylase activity, whether measured relative to tissue, DNA or protein, did not vary significantly across any formula-fed group. As with the morphology measurements described above, a lack of difference in DNA and protein levels and digestive enzyme activity in the formula-fed groups indicates that PDX consumption was not associated with pancreatic hyperplasia in neonatal pigs. These findings are in contrast to those reported by Harada et al. (1995) where the consumption of a high concentration of PDX by suckling rats resulted in increased levels of pancreatic DNA and protein.

*Gastrointestinal Morphology and Weight*

The morphological response of the GI tract to PDX was evaluated by comparing cecum and colon weights, calculated as a percentage of body weight, and the relative thickness of the muscularis externa (muscle layer) (Table 7). The cecum and colon were significantly smaller in sow-reared pigs compared to all formula-fed pigs, following the trend for reduced organ weights (liver, kidney and pancreas) in the former group ($P < 0.05$). No significant differences in cecum or colon weight were observed between any formula-fed group. Likewise, muscle layer thickness did not vary among the various formula-fed groups indicating that PDX did not influence GI morphology in neonatal pigs. This finding contrasts the results of Yoshioka et al. (1994) who reported that PDX reduced muscle layer thickness in adult rats when fed at rates of 5% and 10% of total diet.

**Discussion**

In the present study, the maximal PDX dose examined was 8.35 g/kg BW per day which was approximately 20 times the lowest dose evaluated in human infants (Ziegler et al., 2007). Average daily gain and energy consumption were not different among formula-fed pigs. Average daily feed intake increased in pigs consuming 1.7 and 17 g PDX/day compared to control pigs. Digesta pH varied inversely with PDX concentration indicating that the prebiotic carbohydrate supported fermentation by the GI microbiota. Stools were softened in a quadratic relationship with PDX inclusion, but did not result in diarrhea. Similarly, infants fed a prebiotic blend of PDX and GOS have been shown to grow normally and produce softer stools more characteristic of those reported for breast fed infants (Ziegler et al., 2007).

Incrementally increasing the PDX supplementation rate from 0 g/L to 17 g/L in formula-fed pigs had no impact on the weight of most organs (i.e. lungs, eye, brain, heart, gallbladder, spleen, liver, and pancreas). At the highest supplementation level (17 g/L), PDX consumption was associated with a slight but significant reduction in kidney weight relative to the controls but this had no effect on the urinalysis values. The cecum, colon, liver, kidney and pancreas weights were increased in all formula-fed pigs compared to the sow-reared reference group. Our lab has observed similar increases in relative liver weight in neonatal pigs fed formula supplemented with various long-chain polyunsaturated fatty acids in comparison to those raised by the sow (Mathews et al., 2002). Such variation is most likely due to differences in meal patterns as sow-reared pigs consume frequent small meals while formula-fed pigs receive three large meals per day. In order to match the growth rate of sow-

reared pigs, artificially-reared pigs are fed at ~60% *ad libitum*, typically at 8-hour intervals, and as a result are able to consume their allotted formula before their next feeding. Previous research in rats comparing one meal feeding to continuous feeding found that body weight was not affected, but organ weights related to metabolism were increased in meal-fed rats (Pocknee and Heaton, 1978). The liver, small intestine, kidneys and empty stomach weights increased in rats fed a single daily meal compared to continuously fed rats (Pocknee and Heaton, 1976). Increasing feedings to every 8 hours results in decreased stomach weight compared to rats fed once per day, but the weight was still elevated compared to continuously fed rats (Pocknee and Heaton, 1976). Similarly, Allee and colleagues (1972) observed increased stomach and small intestine weight, but not total body weights in pigs that were meal-fed once per day compared to pigs allowed continuous access to the same amount of feed all day.

Further investigation into liver, kidney, pancreas and intestine morphology and function did not demonstrate any toxicologically relevant changes associated with PDX supplementation or meal feeding in formula-fed pigs. Few significant differences were noted among the hematology and serum chemistry measurements of formula-fed pigs regardless of PDX concentration. BUN, representing the sole exception, was decreased at the 8.5 g/L and 17 g/L supplementation levels but when normalized to creatinine did not vary from the control group. Meal feeding had a much greater impact than PDX as indicated by reduced levels of alkaline phosphatase in all formula-fed pigs compared to those in the sow-reared reference group. As gross and histological measures of the liver detected no hypotrophy or other toxicological effects, the difference in alkaline phosphatase activity appeared to be

without physiological impact. In addition to alkaline phosphatase, creatinine, bilirubin, ALT, and cholesterol were increased in sow-reared pigs while BUN and BUN:creatinine were reduced. We previously have observed a similar decrease in BUN and increases in creatinine, total bilrubin and cholesterol in sow-reared pigs compared to formula-fed pigs in previous experiments (Hess et al., 2008). Heaton and Loveless (1973) also observed increased levels of serum alkaline phosphatase in rats fed continuously compared to those fed one meal per day. In total, most differences observed in organ morphology and function likely resulted from feeding schedule differences and not PDX supplementation. This is most evident in neonatal pigs fed the highest level of PDX (17 g/L; approximately 30 g/d) where liver and renal function, as reflected in serum and urinalysis profiles, were comparable to those of the control group. A comparable safety profile was demonstrated in adult humans where supplementation with PDX (12 g/d) did not affect biochemical measures of liver and renal function, blood electrolytes, triacylgycerol or cholesterol (Jie et al., 2000).

Even though polydextrose evoked physiological responses typical of prebiotic carbohydrates in the current study (i.e. acidification of GI contents and stool softening), other GI effects previously reported to occur in neonatal and adult rodents were not observed. As an example, there were no significant differences in the empty weights of the cecum and colon of neonatal pigs across the full range of PDX concentrations. In contrast, Harada et al. (1995) found that cecal wet weight increased from 116.5 ± 11.9 mg in control animals to 157.0 ± 6.3 mg ($P < 0.05$) and 458.8 ± 50.2 mg ($P < 0.001$) in neonatal rats fed pectin (0.5 mg/g bw/d) and PDX (8 mg/g bw/d), respectively for 7 days. Recent long-term studies of rats fed other slowly digested carbohydrates (i.e., oligofructose and dextrin) have

documented dose-dependent increases in cecal weight (Boyle et al., 2008; Wils et al., 2008). The cecal enlargement seen in rats fed high concentrations of slowly digested carbohydrates is widely considered an adaptive response of no toxicological relevance (Dybing et al., 2002) and likely derives from efficient hindgut fermentation, a trait that is functional as early as 15 days of age and allows rodents to recover calories from resistant carbohydrates that would otherwise escape digestion (O'Connor and Diamond, 1999). In addition to changes in cecal weight, Harada and colleagues (1995) also reported that when a high concentration of PDX (8 mg/g bw/d) was fed to neonatal rats, digestive enzyme activity (trypsin) and the pancreatic concentrations of both DNA and protein increased significantly compared to controls. These changes, which the authors termed pancreatic hyperplasia, were not evident in those fed a lower concentration of PDX (4 mg/g bw/d). In the current study, no indication of pancreatic hyperplasia was evident under thorough histological examination and the levels of pancreatic DNA, protein, and digestive enzyme (amylase) did not vary between groups of neonatal pigs, further supporting the safety of PDX.

Adaptation of the GI tract in response to high concentrations of slowly digested carbohydrates also has been documented in adult animals. Yoshioka et al., (1994) compared the effects of three slowly digested carbohydrates with very different physical properties (cellulose, galactomannan derivatives and PDX) on large intestine morphology in adult male rats. After 52 days of feeding at concentrations of 5% and 10%, cecal muscular layer thickness was greatest with cellulose, which is insoluble and inert to many bacteria, intermediate with galactomannan which is soluble, readily fermented but viscous, and thinnest with PDX which is soluble and fermentable. Unlike these early finding in adult rats,

in the current study PDX had no effect on GI morphology in neonatal pigs as neither weight nor muscular layer thickness of the cecum or colon varied significantly between formula-fed groups.

In summary, measurements across a range of morphological, histological, biochemical parameters indicate that the supplementation of formula with polydextrose between 1.7 g/L and 17 g/L was without toxicological effect on neonatal pigs, further supporting the safety of this prebiotic carbohydrate for the human neonate. Though PDX displayed properties similar to other prebiotics and fibers, dietary supplementation at 8.35 g/kg BW per day for 19 days was safe in an animal model considered an appropriate surrogate for human infants.

**Conflict of Interest Statement**

D.C. Walker and Z.E. Jouni are employees of Mead Johnson and Company, the sponsor of this research. J. Odle was compensated by Mead Johnson and Company to provided expert testimony on the subject of this research.

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**Table 1. Effects of incremental PDX on sucling pig growth rate and stool consistency.**

| | PDX, g/L | | | | | | | Contrast P-values | |
|---|---|---|---|---|---|---|---|---|---|
| | 0 | 1.7 | 4.3 | 8.5 | 17 | SEM | P-value | Linear | Quadratic |
| ADG, g | 304 | 313 | 309 | 299 | 316 | 12.9 | 0.738 | 0.770 | 0.665 |
| ADFI, g | 259[b] | 294[a] | 276[ab] | 277[ab] | 289[a] | 7.70 | 0.014 | 0.073 | 0.324 |
| G:F | 1.17 | 1.07 | 1.11 | 1.07 | 1.09 | 0.034 | 0.213 | 0.182 | 0.175 |
| Stool consistency[1] | 1.79[ab] | 1.81[ab] | 1.68[b] | 1.61[b] | 2.06[a] | 0.113 | 0.043 | 0.312 | 0.025 |
| Body temperature, $^0$C | 39.4 | 38.8 | 38.2 | 38.7 | 38.7 | 0.38 | 0.284 | 0.2043 | 0.1300 |
| Energy consumption, kcal/kg BW | 369 | 399 | 378 | 371 | 365 | 11.0 | 0.146 | 0.276 | 0.129 |
| Average PDX consumed, g/d | 0.00[e] | 3.71[d] | 8.27[c] | 16.40[b] | 31.95[a] | 0.661 | <0.001 | <0.001 | <0.001 |

[1] Stool consistency was rated on a 1 to 5 scale with 1 representing well-formed stools and 5 representing watery stool with no form.
[a-e] Means within a row lacking a common superscript differ ($P < 0.05$).

**Table 2. serum profiles of pigs fed formula containing incremental PDX for 19 days versus sow-fed and day-0 reference pigs.**

| | | | PDX Level g/L | | | | | | | Formula Contrasts | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | D0 CON | SOW | 0 | 1.7 | 4.3 | 8.5 | 17 | SEM | P-value | Linear | Quadratic |
| Glucose (mg/dL) | 95$^{c}$ | 129$^{b}$ | 163$^{a}$ | 156$^{a}$ | 169$^{a}$ | 155$^{a}$ | 153$^{ab}$ | 13 | <0.001 | 0.306 | 0.491 |
| BUN[1] (mg/dL) | 10.0$^{c}$ | 7.7$^{c}$ | 17.5$^{a}$ | 15.3$^{ab}$ | 16.8$^{a}$ | 14.1$^{b}$ | 14.4$^{b}$ | 1 | <0.001 | 0.007 | 0.734 |
| Creatinine (mg/dL) | 0.454$^{b}$ | 0.970$^{a}$ | 0.560$^{b}$ | 0.579$^{b}$ | 0.585$^{b}$ | 0.541$^{b}$ | 0.554$^{b}$ | 0.063 | <0.001 | 0.581 | 0.493 |
| Total Protein (g/dL) | 4.73 | 4.69 | 4.72 | 4.65 | 4.71 | 4.65 | 4.78 | 0.325 | 1.000 | 0.752 | 0.364 |
| Albumin (g/dL) | 2.72 | 2.92 | 3.16 | 3.06 | 3.18 | 3.03 | 3.17 | 0.603 | 0.995 | 0.952 | 0.589 |
| Total Bilrubin (mg/dL) | 0.300$^{b}$ | 0.552$^{a}$ | 0.166$^{b}$ | 0.209$^{b}$ | 0.208$^{b}$ | 0.250$^{b}$ | 0.192$^{b}$ | 0.067 | <0.001 | 0.293 | 0.092 |
| Alkaline Phosphatase (U/L) | 1148$^{a}$ | 1428$^{a}$ | 492$^{b}$ | 459$^{b}$ | 497$^{b}$ | 507$^{b}$ | 478$^{b}$ | 122 | <0.001 | 0.917 | 0.928 |
| ALT[2] (U/L) | 37.2$^{a}$ | 35.7$^{a}$ | 21.2$^{b}$ | 21.5$^{b}$ | 22.9$^{b}$ | 21.3$^{b}$ | 23.4$^{b}$ | 2.6 | <0.001 | 0.434 | 0.947 |
| AST[3] (U/L) | 40.6 | 40.7 | 30.1 | 36.2 | 44.6 | 36.2 | 36.8 | 9.2 | 0.822 | 0.452 | 0.177 |
| Cholesterol (mg/dL) | 84$^{b}$ | 200$^{a}$ | 100$^{b}$ | 94$^{b}$ | 96$^{b}$ | 94$^{b}$ | 106$^{b}$ | 21 | <0.001 | 0.487 | 0.094 |
| Ca (mg/dL) | 8.0 | 12.7 | 11.0 | 19.8 | 11.3 | 20.2 | 11.2 | 6.3 | 0.519 | 0.964 | 0.388 |
| P (mg/dL) | 6.3$^{c}$ | 8.8$^{b}$ | 11.0$^{a}$ | 11.6$^{a}$ | 11.6$^{a}$ | 11.1$^{a}$ | 11.1$^{a}$ | 0.622 | <0.001 | 0.753 | 0.250 |
| Na (mEq/L) | 140$^{b}$ | 144$^{a}$ | 142$^{ab}$ | 142$^{ab}$ | 141$^{b}$ | 141$^{b}$ | 142$^{b}$ | 1 | 0.010 | 0.141 | 0.162 |
| K (mEq/L) | 4.83$^{b}$ | 5.50$^{b}$ | 8.87$^{a}$ | 8.94$^{a}$ | 8.62$^{a}$ | 8.53$^{a}$ | 8.66$^{a}$ | 0.321 | <0.001 | 0.363 | 0.768 |
| Cl (mEq/L) | 107 | 101 | 99 | 97 | 97 | 105 | 97 | 8 | 0.922 | 0.868 | 0.873 |
| Albumin:Globulin | 0.15$^{c}$ | 1.54$^{b}$ | 2.07$^{a}$ | 2.00$^{a}$ | 2.13$^{a}$ | 2.01$^{a}$ | 2.11$^{a}$ | 0.216 | <0.001 | 0.843 | 0.868 |
| BUN:Creatinine | 24.0$^{b}$ | 8.8$^{c}$ | 32.4$^{a}$ | 27.8$^{ab}$ | 29.2$^{ab}$ | 26.6$^{ab}$ | 26.8$^{ab}$ | 2.9 | <0.001 | 0.076 | 0.434 |
| Globulin (g/dL) | 3.78$^{a}$ | 1.88$^{b}$ | 1.55$^{c}$ | 1.59$^{c}$ | 1.52$^{c}$ | 1.58$^{c}$ | 1.62$^{c}$ | 0.115 | <0.001 | 0.702 | 0.717 |

[a-b] Means within a row lacking a common superscript differ (P < 0.05).
[1]Blood urea nitrogen
[2]Alanine aminotransferase
[3]Aspartate aminotransferase

**Table 3. Whole blood hematology of pigs fed formula containing incremental PDX for 19 days versus sow-fed and day-0 reference pigs.**

| | | | PDX Level g/L | | | | | | | Formula Contrasts | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | D0 CON | SOW | 0 | 1.7 | 4.3 | 8.5 | 17 | SEM | P-value | Linear | Quadratic |
| Hemoglobin (g/dL) | 7.47[b] | 10.6[a] | 11.2[a] | 11.1[a] | 11.8[a] | 11.3[a] | 11.9[a] | 0.575 | 0.001 | 0.269 | 0.873 |
| Hematocrit (%) | 23.5[c] | 31.2[b] | 35.3[ab] | 33.9[ab] | 36.9[a] | 33.8[ab] | 37.5[a] | 2 | <0.001 | 0.339 | 0.444 |
| White Blood Count ($10^3/\mu L$) | 15.8 | 6.2 | 9.8 | 7.7 | 10.2 | 10.3 | 11.9 | 4 | 0.501 | 0.120 | 0.395 |
| Red Blood Count ($10^6/\mu L$) | 3.51[c] | 5.83[a] | 5.35[ab] | 5.05[b] | 5.74[a] | 5.19[ab] | 5.67[a] | 0.271 | <0.001 | 0.256 | 0.723 |
| MCV[1] (fL) | 67.3[a] | 53.6[b] | 66.0[a] | 67.5[a] | 64.5[a] | 65.2[a] | 66.5[a] | 2 | <0.001 | 0.632 | 0.375 |
| MCH[2] (pg) | 21.3[a] | 18.2[b] | 21.0[a] | 22.0[a] | 20.6[a] | 21.7[a] | 21.0[a] | 0.785 | 0.006 | 0.658 | 0.485 |
| MCHC[3] (g/dL) | 31.8[b] | 33.8[a] | 31.9[b] | 32.8[ab] | 32.0[b] | 33.5[a] | 31.8[b] | 0.517 | 0.004 | 0.689 | 0.039 |
| Platelet Count ($10^3/\mu L$) | 364[b] | 584[a] | 508[a] | 518[a] | 579[a] | 537[a] | 530[a] | 58.436 | 0.033 | 0.699 | 0.483 |
| Neutrophils ($10^9/L$) | 4796 | 3538 | 3594 | 2445 | 4382 | 4361 | 5563 | 1259 | 0.395 | 0.085 | 0.515 |
| Lymphocytes ($10^9/L$) | 3024[b] | 2621[b] | 5286[a] | 4703[a] | 5086[a] | 5302[a] | 5374[a] | 724 | 0.004 | 0.597 | 0.578 |

[a-b] Means within a row lacking a common superscript differ ($P < 0.05$).
[1] Mean corpuscular volume
[2] Mean corpuscular hemoglobin
[3] Mean corpuscular hemoglobin concentration

**Table 4. Relative weights[1] and morphologies of organs from pigs fed formula containing incremental PDX for 19 days versus sow-fed and day-0 reference pigs.**

| | | | PDX, g/L | | | | | | | Contrast P-values | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | D0 CON | SOW | 0 | 1.7 | 4.3 | 8.5 | 17 | SEM | P-value | Linear | Quadratic |
| **Liver weight and Morphology** | | | | | | | | | | | |
| Weight | 3.06$^{bc}$ | 2.30$^{c}$ | 3.31$^{a}$ | 3.12$^{ab}$ | 3.18$^{ab}$ | 3.18$^{ab}$ | 3.17$^{ab}$ | 0.100 | <0.001 | 0.282 | 0.703 |
| Vacuolization | 1.63$^{a}$ | 2.33$^{a}$ | 0.91$^{b}$ | 0.77$^{b}$ | 1.00$^{b}$ | 0.82$^{b}$ | 1.00$^{b}$ | ------ | <0.001 | ------- | --------- |
| **Kidney weight and Morphology** | | | | | | | | | | | |
| Weight | 0.389$^{ab}$ | 0.260$^{c}$ | 0.434$^{a}$ | 0.398$^{ab}$ | 0.405$^{ab}$ | 0.405$^{ab}$ | 0.373$^{b}$ | 0.018 | <0.001 | 0.057 | 0.683 |
| Vacuolization | 0.91 | 0.92 | 1.00 | 1.00 | 1.00 | 1.00 | 1.09 | ------ | 0.928 | ------ | ------ |
| **Other Organ Weights** | | | | | | | | | | | |
| Spleen | 0.109$^{b}$ | 0.187$^{a}$ | 0.181$^{a}$ | 0.188$^{a}$ | 0.190$^{a}$ | 0.190$^{a}$ | 0.182$^{a}$ | 0.015 | <0.001 | 0.922 | 0.487 |
| Gallbladder | 0.045 | 0.052 | 0.046 | 0.055 | 0.072 | 0.038 | 0.060 | 0.011 | 0.178 | 0.771 | 0.407 |
| Heart | 0.792$^{b}$ | 0.616$^{a}$ | 0.581$^{a}$ | 0.578$^{a}$ | 0.575$^{a}$ | 0.568$^{a}$ | 0.599$^{a}$ | 0.034 | <0.001 | 0.878 | 0.675 |
| Brain | 1.686$^{a}$ | 0.596$^{b}$ | 0.562$^{b}$ | 0.541$^{b}$ | 0.564$^{b}$ | 0.611$^{b}$ | 0.572$^{b}$ | 0.076 | <0.001 | 0.620 | 0.571 |
| Left Eye | 0.137$^{a}$ | 0.044$^{b}$ | 0.042$^{b}$ | 0.041$^{b}$ | 0.041$^{b}$ | 0.043$^{b}$ | 0.043$^{b}$ | 0.006 | <0.001 | 0.590 | 0.885 |
| Lungs | 1.61$^{a}$ | 1.33$^{b}$ | 1.16$^{b}$ | 1.17$^{b}$ | 1.12$^{b}$ | 1.16$^{b}$ | 1.14$^{b}$ | 0.098 | 0.001 | 0.651 | 0.932 |

[1]Organ weights are percentage of body weights.
[a-b]Means within a row lacking a common superscript differ ($P < 0.05$).

**Table 5. Weight and biochemical measures of pancreatic tissue from pigs fed fromula containing incremental PDX for 19 days versus sow-fed and day-o reference pigs.**

| | | | PDX, g/L | | | | | | | Contrast P-values | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | D0 CON | SOW | 0 | 1.7 | 4.3 | 8.5 | 17 | SEM | P-value | Linear | Quadratic |
| Pancreas (% BW) | $0.118^{c}$ | $0.101^{c}$ | $0.173^{ab}$ | $0.158^{b}$ | $0.174^{ab}$ | $0.163^{ab}$ | $0.194^{a}$ | 0.014 | <0.001 | 0.238 | 0.128 |
| DNA (ng/mg pancreas) | $3.81^{b}$ | $5.18^{a}$ | $3.77^{b}$ | $4.12^{b}$ | $3.78^{b}$ | $3.85^{b}$ | $3.41^{b}$ | 0.358 | 0.036 | 0.324 | 0.265 |
| Protein (mg/g pancreas) | $98.8^{c}$ | $174^{a}$ | $133^{bc}$ | $134^{bc}$ | $146^{ab}$ | $156^{ab}$ | $145^{ab}$ | 14.6 | 0.0169 | 0.624 | 0.844 |
| Amylase Activity | | | | | | | | | | | |
| U/mg Tissue | $0.71^{c}$ | $22.50^{a}$ | $6.76^{bc}$ | $7.79^{bc}$ | $13.67^{ab}$ | $11.83^{b}$ | $6.69^{bc}$ | 4.3 | 0.0078 | 0.857 | 0.456 |
| U/μg DNA | $0.36^{c}$ | $4.26^{a}$ | $1.79^{bc}$ | $1.91^{bc}$ | $3.42^{ab}$ | $3.04^{ab}$ | $1.94^{bc}$ | 0.907 | 0.032 | 0.947 | 0.525 |
| U/mg protein | $10.1^{c}$ | $124.4^{a}$ | $50.0^{bc}$ | $55.9^{bc}$ | $78.5^{ab}$ | $72.4^{abc}$ | $58.4^{bc}$ | 25.1 | 0.044 | 0.922 | 0.857 |
| Whole Tissue Levels | | | | | | | | | | | |
| Pancreas (g) | $2.4^{d}$ | $7.8^{c}$ | $13.2^{ab}$ | $12.6^{b}$ | $13.9^{ab}$ | $12.3^{b}$ | $14.8^{a}$ | 0.766 | <0.001 | 0.297 | 0.270 |
| DNA (mg) | $8.3^{c}$ | $40.4^{b}$ | $49.4^{ab}$ | $51.9^{a}$ | $52.5^{a}$ | $47.2^{ab}$ | $51.1^{ab}$ | 4.75 | <0.001 | 0.935 | 0.806 |
| Protein (g) | $0.27^{d}$ | $1.33^{c}$ | $1.74^{abc}$ | $1.70^{bc}$ | $2.14^{a}$ | $1.90^{ab}$ | $1.89^{ab}$ | 0.196 | <0.001 | 0.318 | 0.357 |
| Amylase (kU) | $2^{b}$ | $170^{a}$ | $90^{ab}$ | $100^{ab}$ | $168^{a}$ | $135^{a}$ | $110^{ab}$ | 49.1 | 0.151 | 0.586 | 0.288 |

[a-d] Means within a row lacking a common superscript differ ($P < 0.05$).

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# CHAPTER 4: POLYDEXTROSE ENRICHMENT OF INFANT FORMULA DEMONSTRATES PREBIOTIC CHARACTERISTICS BY ALTERING INTESTINAL MICROBIOTA AND CYTOKINE EXPRESSION IN SUCKLING PIGLETS

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Abbreviations used: HMO, human milk oligosaccharides; IEL, intraepithelial lymphocytes; LAB, lactic acid bacteria; MPO, myeloperoxidase; NEC, necrotizing enterocolitis; NB, newborn; PDX, polydextrose; SR, sow-reared; TMB, tetramethylbenzidine;

**Abstract**

Oligosaccharides, the 3rd most abundant component in human milk, are virtually absent from infant formulas and from the cow milk on which most are based. In breastfed infants human milk oligosaccharides (HMO) act as both receptor analogs, interfering with pathogen adhesion, and as prebiotics, stimulating the growth of certain commensal bacteria (e.g. bifidobacteria) and supporting the innate immunity. In order to further align the functional properties of infant formula with those of human milk, polydextrose (PDX) is proposed as a substitute for HMO. To determine the prebiotic functionality of PDX, one-day old pigs were fed a cow-milk-based formula supplemented with increasing concentrations of PDX (0, 1.7, 4.3, 8.5 or 17 g/L) for 18 d (*n*=13). Additional reference groups included pigs sampled at d 0 and sow-reared pigs sampled at d 18 (*n*=12). Ileal Lactobacillus CFU, but not Bifidobacteria, increased linearly with increasing PDX ($P = 0.02$). Propionic acid concentration in digesta linearly increased with PDX level ($P = 0.045$) and lactic acid increased linearly by five-fold with increasing PDX ($P = 0.001$). Accordingly, digesta pH decreased linearly ($P < 0.05$) as PDX increased, with a maximal reduction approaching 0.5 pH units in pigs fed 17 g/L. Expression of TNFα, IL-1β, and IL-8 showed a negative quadratic pattern in response to PDX supplementation, declining at intermediate concentrations and rebounding at higher concentrations of PDX. In summary, PDX enrichment of infant formula resulted in a prebiotic effect by increasing ileal lactobacilli, propionic and lactic acid concentrations and decreasing pH with associated alterations in ileal cytokine expression.

## Introduction

Human milk is widely considered the optimum food for meeting the nutritional needs of infants, but by two months of age the majority of infants in North America have received some quantity of infant formula (1). Though iron-fortified infant formulas are the most appropriate nutritional substitutes, their composition does not fully duplicate that of human milk, particularly with regards to bioactive components such as human milk oligosaccharides (HMO) that perform functional roles beyond basic nutrition, such as innate immune support. In order to more closely approximate both nutritional and functional properties of human milk, efforts are under way to identify novel ingredients with similar bioactive properties. To that end, polydextrose (PDX) and is thought to function similarly to HMO and is proposed for addition to a new generation of pediatric nutritional products.

Oligosaccharides constitute the third most abundant component in human milk after lactose and lipids, ranging in concentration from 5-10 g/L in milk (2). Oligosaccharides are virtually absent from cow milk (< 0.08 g/L) and most infant formula, which may account in part for the difference in gastrointestinal (GI) microbiota reported among breastfed and formula-fed infants (2-4). Bifidobacteria, dominant organisms among the microbiota of breastfed infants, are less prevalent in the gut of formula-fed infants where they compete with higher levels of other bacterial groups (e.g.*Bacteroides sp.*) (5). Bifidobacteria are considered beneficial commensal bacteria, as they help maintain healthy mucosal surfaces in the GI tract (6) and have the capacity to inhibit pathogenic bacteria, populations of which also differ among breastfed and formula-fed infants. In a study of over 1,000 infants, fecal

counts of *Clostridium difficile* were significantly higher for formula-fed infants. *C. difficile* colonization has been associated with diseases such as necrotizing enterocolitis (NEC), due to immaturity of the intestine (7, 8). Interestingly, *C. difficile* also was higher in preterm infants than term infants (9). Establishing lactic acid bacteria (LAB) and bifidobacteria commensals early in the life of formula-fed infants may competitively exclude potentially pathogenic bacteria, like *C. difficile.* The presence of fermentable oligosaccharides (e.g. HMO, prebiotics) that are not digested by the host aid in the establishment of commensal bacteria that are able to capture energy from the carbohydrates that would otherwise escape the human digestive process (10).

Approximately 200 molecular species of oligosaccharides have been identified in human milk and are synthesized from D-glucose, D-galactose, D-N-acetylglucosamine, L-fucose and D-N-acetylneuraminic acid (sialic acid) monomers. In contrast, bovine milk oligosaccharide composition is much simpler as the 10 molecular species that have been identified consist largely of sialic acid linked with lactose (2, 4, 11, 12). Similarly, only 29 molecular species have been identified among porcine milk oligosaccharides, of which over 50% are sialylated (13). Due to the complexity of HMO and the lack of commercial sources, they are not presently used to supplement infant formulas. Instead, supplementation with alternative prebiotic oligosaccharides is expected to provide some of the functional properties of HMO, such as supporting intestinal commensal bacteria, softening stools, and effecting desirable intestinal immunomodulation.

Prebiotic supplementation of pediatric nutritional products is associated with increased levels of LAB and bifidobacteria, decreased diarrhea, improved allergy symptoms

and decreased rates of infection in infants and children (14-18). Decreased incidence of disease may be related to changes in immune regulation through cytokine secretion. Although changes to the immune system have been shown, the exact mechanism of immunomodulation remains unknown. Commensal bacteria fed as isolated strains also can have varying effects ranging from anti-inflammatory to pro-inflammatory (19). The mechanisms through which commensal bacteria modulate the host immune system are poorly understood, rendering the prediction of specific immune-related effects challenging, especially for newer prebiotics like PDX.

PDX is an indigestible, selectively fermented carbohydrate that is a candidate prebiotic. First developed as a bulking agent for foods, PDX's structure of randomly bonded glucose polymer with some sorbitol end groups allows for uses that include both fiber and prebiotic applications. Though the random structure of PDX contains numerous glycosidic bonds, those in the β (1-6) configuration predominate, rendering the material resistant to mammalian digestive enzymes and allowing it to reach the large intestine, stimulating fermentation by the commensal microbiota. Both the indigestible nature and selective fermentation of PDX support its utilization as a prebiotic, which was recently redefined as, "a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microbiota that confers benefits upon host well-being and health", but currently evidence is too sparse to define PDX as a prebiotic (20, 21). Our research was designed to investigate the prebiotic nature of PDX by determining changes in intestinal microflora and health status of neonatal pigs supplemented with various levels of PDX.

The supplementation of PDX with up to 17 g/L of PDX has been previously shown to be safe by our laboratory, supporting normal piglet growth and development (22). Research into the efficacy of PDX supplementation is ongoing. The objective of this study was to determine the enteric responses of incremental dietary PDX in infant formula, including effects on the intestinal immune responses using a piglet model.

## Methods

*Pigs and study design.*

Full-term pigs were vaginally delivered at the North Carolina State University Swine Education Unit and allowed colostrum for 24 hours. Reference sow-reared piglets (SR) remained with their respective sow at the Swine Education Unit. After colostrum consumption, treatment pigs were transferred to the Laboratory of Developmental Nutrition at North Carolina State University. A baseline reference group of newborn pigs (NB) were sampled after colostrum consumption. Treatment pigs were housed in individual pens in an environmentally controlled room (32°C) programmed to deliver a light/dark cycle of 16/8 hr, respectively. For the first 24 hr, pigs were trained to suckle from bottles using the control diet. Pigs were fed at ~60% *ad libitum* with fresh diet offered three times daily for 18 d in order to achieve growth rates similar to sow-fed pigs (22). The experiment was run in two replicates. The first replicate included 6 pigs/treatment, and the second included 7, yielding a total of 13 pigs/treatment. All animal procedures were approved by the North Carolina State University Institutional Animal Care and Use Committee.

*Diet.*

Pigs were randomly allotted to one of five dietary treatments or two reference groups. (n = 13 pigs per treatment, initial weight 1.71 ± 0.27 kg). A basal diet patterned after term human infant formula was prepared to meet the nutrient requirements of neonatal pigs (National Research Council, 1998). The ingredients of the dry basal diet amended to provide one of four levels of PDX (1.7, 4.3, 8.5 and 17.0 g/L) (Supplemental Table 1; Litesse® Two, Danisco USA Inc, Terre Haute, IN) (22). The form of PDX had a pH = 3.9 and a reduced citric acid content compared with the original Litesse formulation. The oligosaccharides of porcine milk contain at least 29 distinct molecular species (compared to approximately 200 identified in human milk) of which 50% are sialylated (13). Although the quantitative content of oligosaccharides in sow milk is unknown, piglets consuming sow's milk were considered a positive reference group. Dry ingredients were mixed and reconstituted with water, fat and the dietary intervention level of PDX, and then homogenized and refrigerated. Commercially, PDX and other prebiotics are included directly in both dry and liquid preparations of supplemented infant formulas, so our diets were created to mimic this supplementation pattern at multiple dietary levels. Calculated total dietary fiber for the basal diet was 1.05% and for the four PDX diets were: 1.06% (1.7 g/L PDX), 1.08% (4.3 g/L PDX), 1.10% (8.5 g/L PDX) and 1.15% (17.0 g/L PDX). A dietary fat blend, including docosahexaenoic (DHA) and arachidonic (ARA) acids, was added to the dry basal diet at 26% of the diet. Excluding the SR reference group, the total caloric intake between treatments ranged 365-399 ± 11 kcal/kg of body weight and did not differ significantly ($P$ = 0.146).

*Sampling.*

Piglets were allowed free access to feed on the day of euthanasia. Piglets were euthanized using American-Veterinary-Medical-Association-approved electrocution followed by exsanguination. The intestine was then removed. The first meter proximal to the ileocecal junction was removed. The first proximal 0.5 m were rinsed with cold PBS and mucosa was collected, immediately flash frozen in liquid nitrogen and later stored at -80$^{o}$C. The distal 0.5 m was collected for digesta and histology measurements. At the center section of the distal sample, approximately two cm of tissue were excised and placed in neutral buffered formalin for fixing. The proximal colon and cecum digesta were collected for pH measurement which was performed at the time of sampling. After pH measurement a cecal digesta subsample was frozen on dry ice and an additional subsample was collected and placed on ice for bacterial enumeration. Colon digesta was only collected for pH measurement.

*Ileal histology.*

Ileal histology was performed as previously described (23). To enumerate the intraepithelial lymphocytes (IEL), five well defined villi were identified. The IELs and epithelia cells were counted on each of these villi.

*Ileal mucosal enzyme assays.*

To determine maturation of the ileum, maltase and lactase activities were assessed in the formula-fed and SR piglets. Ileal lactase and maltase activity were analyzed based on modifications for a 96 well plate assay (24). To determine neutrophil infiltration in the

ileum, myloperoxidase (MPO) activity was determined with modifications for a 96 well plate (25). Substrate concentration was calculated based on the Beer's-Lambert equation with the molecular absorption coefficient of tetramethylbenzidine (TMB; Sigma #860336) being 3.9 x $10^4$ L●$mol^{-1}$●$cm^{-1}$. One unit of specific MPO activity was defined as that degrading one mmol of TMB/min. Total protein was determined (26) using a commercially available bicinchoninic acid kit (Pierce #23225).

*Cecal short chain fatty acid concentration.*

Short chain fatty acid (SCFA) concentrations in cecal digesta from the first replicate were determined using a modified gas chromatography method (27) using a Varian CP 3380/3800 with a NUKOL Fused Silica Capillary Column (30 m x 0.25 mm x 0.25 μm).

*Lactic acid concentration.*

Lactic acid concentrations in cecal digesta from the second replicate were determined by gas chromatography (28). Methylated DL-lactate was detected on a Hewlett Packard Series II 5890 Gas Chromatograph with HP 6890 Series Injector with an HP Innowax column (30 m long with 0.32 mm internal diameter).

*Cecal bacterial concentration.*

Serial dilutions of cecal digesta were plated for lactobacilli and bifidobacteria concentrations on Difco Rogosa Agar and Difico Differential Clostridium Agar. Plates were placed in Bio-Bags to generate an anaerobic environment and then incubated for 48 hr at 37$^o$C. After 48 hr, colonies were counted from 2 plates for each bacteria per animal.

*RNA isolation and Real-Time RT-PCR.*

Total RNA was isolated and treated with DNase I (Qiagen, Valencia, CA) from ileal mucosal scrapings using a commercially available kit (Qiagen, Valencia, CA). Purity was assessed by determining the ratio of the absorbance at 260 and 280 and the 18S and 28S ribosomal bands visualized on an agarose gel. Total RNA (1 μg) was reverse transcribed using a commercially available cDNA synthesis kit (iScript Select, BioRad Laboratories, Hercules, CA). Real-time PCR detection of mRNA was conducted utilizing the SYBR Green assay. Primer sequences are listed in Supplemental Table 2. Amplification was carried out in a total volume of 25 μL containing 1X iQ SYBR Green Supermix (BioRad Laboratories, Hercules, CA), forward and reverse primers (400 nmol/L each) and 100 ng of the reverse transcribed cDNA. The PCR program consisted of an initial 5 minute denaturation step at 95$^{o}$C followed by 39 cycles of: 95 $^{o}$C for 30 s, 60 $^{o}$C for 30 s and 72 $^{o}$C for 30 s (BioRad Laboratories, Hercules, CA). At the end of the PCR, melt curve analysis was conducted to validate the specificity of the primers. Both "no-template" and "no-reverse- transcribed-RNA" controls were used with every assay and all determinations were performed in at least duplicates. External cDNA standards were constructed by cloning the corresponding RT-PCR product into a PCR TOPO vector (Invitrogen, Carlsbad, CA) and the resulting plasmids were sequenced at Operon MWG for verification. Serial dilutions of a known amount of plasmid containing the gene of interest were included on each 96-well plate. The abundance of each gene product was calculated by regressing against the plasmid standard curve. The housekeeping gene, GAPDH, was not different among treatments or animals.

*Statistical analysis.*

Data were analyzed according to a randomized complete block design using the general linear models procedure of SAS (SAS, Cary, NC). Comparisons between formula-fed pigs and reference pigs were determined using a one-way ANOVA and least square means. Formula-fed groups of pigs were compared to one another using orthogonal contrasts (29). Differences were deemed significant when $P < 0.05$.

**Results**

*Ileal morphology and enzyme activity.*

Maltase, lactase, villi height and width were not different among treatments and the reference group (Table 1). Crypt depth was affected by dietary treatment, with decreased crypt depth observed for pigs fed 8.5 g/L PDX compared to piglets fed less PDX ($P < 0.01$). The piglets fed the highest level of PDX were not different than the other formula-fed piglets. Although there were significant differences in crypt depth between formula-fed pigs, there was not a linear or quadratic relationship observed for the changes in crypt depth. Myeloperoxidase activity was not different among treatments ($P = 0.657$). IEL were not different among dietary treatments (Table 1).

*Digesta pH changes.*

Both cecal and colon digesta pH decreased linearly with increasing PDX concentration (Table 2). The digesta pH of piglets fed 0 g/L PDX was not significantly different than the NB pigs, but was higher than the SR piglets ($P < 0.001$). Feeding 4.3 g/L PDX or more decreased pH to that of the SR reference group. For colon digesta, piglets fed

8.5 and 17 g/L PDX did not have significantly different pH values compared to SR piglets. Reported in our previous study, ileal digesta pH also declined linearly from 6.63 to 6.47 with increasing PDX (Linear $P = 0.021$) (22). Overall, as PDX increased, the pH of the digesta lowered to that of digesta in SR piglets (Table 2).

*Digesta metabolites.*

The pH of digesta in the cecum and colon decreased with increasing PDX suggesting that fermentation of the material by gut microbes formed organic acids as end-products. To determine if the pH changes of digesta were due to bacteria, lactic acid and SCFA were measured in the cecum digesta. Total SCFA did not change linearly with increasing PDX ($P < 0.05$; Table 3). Both propionic acid and lactic acid increased linearly with increasing PDX ($P < 0.05$ and $P = 0.001$ respectively). Piglets fed 8.5 and 17 g/L PDX had lactic acid concentrations that exceeded 0 g/L PDX fed pigs by more than five-fold and a linear increase of lactic acid with PDX supplementation was observed ($P = 0.02$; *Linear* $P < 0.01$; SEM = 0.81). The reference SR piglets contained 3.17 μmol/g lactic acid, which was similar to that of the piglets fed 8.5 and 17 g/L PDX. The SR pigs had significantly higher levels of lactic acid compared to the 0 g/L PDX fed piglets ($P = 0.036$) and tended to be higher than piglets fed 1.7 and 4.5 g/L PDX ($P < 0.10$).

*Cecal bacterial contents.*

To further confirm the hypothesis that pH changes were due to lactic acid produced by intestinal bacteria, cecal lactobacillus and bifidobacteria were enumerated (Table 4). Cecal lactobacilli concentrations increased linearly with increasing dietary PDX

concentration ($P$ = 0.01). No differences in the number of cecal bifidobacteria were detected ($P$ = 0.93).

*Ileal mRNA expression.*

Message abundance of the IgG receptor (FcRn; NM_214197.2) was compared between the 0 and 17 g/L PDX piglets only. The copy number per 100ng cDNA was 5170 and 6510 (SEM = 540) for piglets fed 0 and 17 g/L PDX, respectively. There was a trend for increased FcRn expression with piglets fed PDX compared to 0 g/l PDX fed pigs ($P$ = 0.10). The inflammatory cytokines, TNFα, IL-8 and IL-1β showed similar trends for expression with the lowest expression observed in piglets fed 4.3 and 8.5 g/L PDX (Figure 1; *Quadratic* $P$ = 0.09; *Quadratic* $P$ = 0.06, $P$ = 0.05, respectively). Feeding 17 g/L PDX cytokine expression levels were similar to that of the 0 g/L PDX piglets for these cytokines. There were no significant quadratic trends for expression of the pro-inflammatory cytokines, but there were for TNFα and IL-8 ($P$ = 0.09, $P$ = 0.06). Message abundance of the anti-inflammatory cytokine IL-10 also tended to be lower in piglets fed 4.3 and 8.5 g/L PDX, but this level was not significantly different than the 0 g/L PDX fed pigs ($P$ > 0.05). Feeding 1.7 and 17 g/L PDX significantly increased IL-10 message compared to control piglets ($P$ = 0.015).

**Discussion**

The purpose of this study was to examine potential prebiotic characteristics of PDX in an infant pig model. The FAO defines a prebiotic "as a non-viable food component that

confers a health benefit on the host associated with modulation of the microbiota" (30). To determine if PDX meets the FAO definition, gut development, the metabolic response of the GI microbiota and the impact of supplementation on select markers of mucosal immunity were evaluated in suckling swine fed PDX. Given the importance of gut and immune development to healthy infants and the scarcity of validated, non-invasive markers, piglets were selected as an appropriate neonatal model due their anatomical and functional similarities with human infants. Previously, our laboratory observed that formula inclusion levels of PDX up to 17 g/L were safe as determined in our pig model (22). In the present study, indices of ileal health (MPO activity and IEL numbers) were not affected by formula feeding (compared to SR animals) or by PDX supplementation, indicating that piglets fed formula had similar ileal health status as the SR reference group. Maturation of the ileum, as indicated by maltase and lactase activity, did not differ among formula-fed and sow-fed groups. Furthermore, villi height and width did not differ. Formula-fed pigs were healthy and maturing properly.

Polydextrose supplementation changed the cecal and colonic environment by increasing lactobacilli, the most likely source of the increased lactic acid and SCFAs and decreased luminal pH that was observed (31). Cecal and colonic pH both decreased linearly with increased PDX ($P < 0.001$). Piglets fed 8.5 and 17 g/L PDX had similar cecal and colonic pH as the SR reference group. The decrease in cecal and colonic pH is consistent with the fermentation pattern of PDX in weaned pigs (32). Decreased luminal pH due to increased organic acid concentration has been frequently reported with feeding of non-digestible carbohydrates (33-35). In this study, the drop in pH accompanied an increase in

total lactobacillus in the cecum and increased lactic acid. *Lactobacillus* strains from porcine mucosa produce high levels of lactic acid relative to other organic acids in the absence of excess fermentable carbohydrates (36). Our findings demonstrate not only increases in lactic acid, but also propionic acid in response to PDX supplementation. Comparable stimulation of lactic acid has not been uniformly observed, particularly in studies involving *in vitro* models (36, 37). Typically in *in vitro* studies a substrate like PDX is introduced and acid production measured during 24 hr, an interval that may not allow the bacterial community sufficient time to fully respond and metabolize the material. Such studies have shown that the fermentation of PDX takes longer than other less complex prebiotics (36, 37). Similar to our results, others have reported decreased luminal pH in response to increased lactic acid in weaning piglets fed a diet supplemented with PDX (32). Our study findings indicate the intestine acclimated to PDX after 18 d feeding with increased lactobacilli and lactic acid and SCFA.

Although SCFA have been reported to increase with prebiotic feeding (33, 34), the neonatal environment may be more conducive to changes in lactic acid, the main product of homofermentation. This is supported by the observation that breast-fed infants have decreased stool pH due to increased lactic acid, not SCFA (38). In infants, while the intestine is still maturing, increases in SCFA have been implicated in the pathology of NEC (39, 40). In a study examining the effect of high levels of organic acids on intestinal injuries in a rat model, lactic acid (at 150 and 300 mmol/L) did not cause gross or microscopic colonic lesions, while even the low level of butyrate (150 mmol/L) and acetic acid (150 mmol/L) did, even when pH was held constant for the treatments (41). In a piglet NEC

model, piglets fed lactose had fewer incidences of NEC and had significantly higher colonic levels of lactic acid, but lower levels of butyric acid (39). In infants with NEC, SCFA cause mucosal injury and in the case of butyrate, decrease the recovery from injury due to blocking trefoil factor, which is important to the maintenance and repair of the intestinal mucosal barrier function (42). A potential benefit of feeding PDX, compared to other prebiotics such as fructooligosaccharide and inulin, is that PDX takes longer to achieve maximum rate of gas production and therefore is more slowly fermented with lactic acid being a primary product (37, 43). Establishing a lactic acid-producing bacterial population early in life may promote maintenance and repair of the mucosal barrier function. As an example, when 300 mmol/L of butyric acid was administered luminally in rats, TF expression decreased after 1 h and remained decreased after 24 h (42). In comparison, administration of lactic acid at the same concentration caused a decrease in TF expression after 1 h, but expression continued to increase at 3, 6 and 24 h until its expression was 90% of that of the control (42). Lactic acid may protect infants from pathogens by decreasing pH without interfering with barrier function while it is undergoing maturation.

In general, ileal cytokine expression tended to lower with increasing dietary PDX, except at the highest supplementation level (17 g/L) where cytokine expression rebounded. This trend occurred for both pro-inflammatory and anti-inflammatory cytokines. The other tested parameters did not follow this pattern and therefore cannot be adequately explained based on the data in hand. One possibility is that at the highest PDX supplementation level the concentration of *Lactobacillus* and/or lactic acid reached a threshold value sufficient to induce increased cytokine expression. Prior to reaching that threshold value, decreased

cytokine expression could result from decreased pathogen load, although this aspect was not specifically evaluated. Lactic acid, the predominant end product of *Lactobacillus* fermentation, acts as an antimicrobial by inhibiting the growth of pH sensitive gram-negative bacteria, which can include pathogenic species such as *E. coli* O157:H7 (44). Isolated *Lactobacillus* strains from porcine ileal mucosa have been shown to inhibit a range of pathogenic bacteria including *Staphylococcus aureus, Bacillus cereus, E. coli* and *Clostridium perfringens* (36). Although not evaluated in this study, we hypothesize that at the lower, physiologic levels of PDX supplementation, increases in *Lactobacillus*-associated lactic acid and other antimicrobials were able to competitively exclude pathogenic bacteria, decreasing the need for inflammatory response-driven cytokine production. In young pigs, a lack of overall intestinal microbial diversity, but increased lactobacilli is associated with decreases in expression of IFN inducible genes and antigen presentation MHC class I genes (including the chemokines CCL2, CCL8 (IL-8), CCL28, CCR1 and CXCR4) (45). Similarly, in the current study, increased lactobacilli and decreased cytokine expression occurred in response to PDX supplementation, while all piglets remained healthy.

In contrast to the cytokine data, expression of the neonatal FcRn was increased in piglets fed 17 g/L PDX as compared to the 0 g/L PDX piglets. To our knowledge, changes in FcRn expression when feeding prebiotics have not been previously reported. Albumin and IgG are the two ligands for FcRn (46, 47). FcRn has several functions, which includes: extension of IgG half life, intestinal delivery of IgG and translocation of IgG and albumin from one cell compartment to another (46, 47). The translocation of FcRn to the intestinal apical membrane and binding of IgG are pH dependent (47, 48). Although not fully confirmed by

this study, it appears that changes in pH may affect expression and not just function of FcRn. Further work is needed to confirm this observation.

PDX supplementation in infant formula mimics some of the functional properties of HMO. The results from the current study demonstrate the ability of PDX-supplemented formula to reduce ileal IL-1β expression ($P = 0.015$), while the cytokines TNFα and IL-8 tended to decrease at low levels but returned to levels similar to that of control pigs with high level supplementation (Quadratic $P = 0.09$; Quadratic $P = 0.06$, respectively). The reduced cytokine expression in pigs fed low levels of PDX was accompanied by increased cecal lactobacilli and lactic acid concentrations. Lactic acid can inhibit pH-sensitive pathogenic bacteria while not compromising the neonatal intestinal barrier function as compared to other organic acids which may be contributing to this finding (41, 42). In conclusion, PDX potentially modulates the intestinal immune system by increasing luminal lactobacilli and lactic acid.

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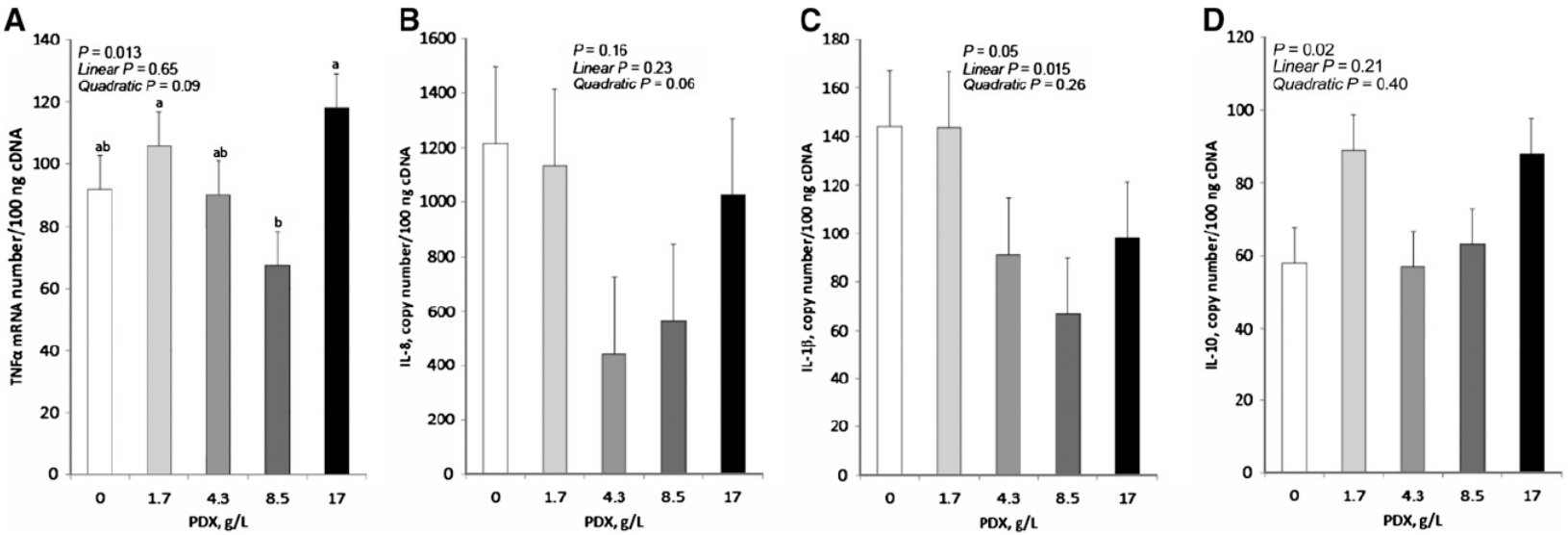


**Figure 1. Effects of formula PDX concentration on TNFα (A), IL-8 (B), IL-1β (C) and IL-10 (D) mRNA expression in piglet ileal mucosa (copy number/100 ng of cDNA) fed PDX for 18 d. Values are mean + SEM, n = 13. [a-c] Means for a variable without a common letter differ, $P < 0.05$.**

**Table 1. Effects of formula PDX concentration on ileal enzyme activity and morphology compared to SR piglets[1].**

| | | PDX g/L | | | | | | | Orthogonal *P* | |
|---|---|---|---|---|---|---|---|---|---|---|
| | SR | 0 | 1.7 | 4.3 | 8.5 | 17 | SEM | *P* | Linear | Quadratic |
| Maltase (U/g protein) | 18 | 21 | 21 | 24 | 22 | 23 | 2.29 | 0.44 | 0.69 | 0.72 |
| Lactase (U/g protein) | 36 | 28 | 14 | 40 | 19 | 12 | 18 | 0.62 | 0.47 | 0.53 |
| Villi Height (µm) | 557 | 445 | 457 | 480 | 447 | 483 | 31 | 0.10 | 0.35 | 0.91 |
| Villi Width (µm) | 155 | 159 | 156 | 149 | 152 | 147 | 6.51 | 0.71 | 0.11 | 0.78 |
| Crypt Depth (µm) | $90^{c}$ | $159^{a}$ | $156^{a}$ | $154^{a}$ | $121^{bc}$ | $145^{ab}$ | 12 | 0.001 | 0.11 | 0.54 |
| IEL (IEL/100 enterocytes) | 13 | 13 | 14 | 12 | 12 | 14 | 0.98 | 0.39 | 0.68 | 0.53 |
| MPO (U/g protein) | 37 | 46 | 33 | 39 | 47 | 46 | 11.3 | 0.66 | 0.73 | 0.50 |

[1] Values represent least-square means, n=12-13.

[a-c] Means in a row without a common letter differ ($P < 0.05$).

**Table 2. Effects of formula PDX concentration on suckling pig digesta pH compared to SR and day-old piglet reference groups[1].**

| | Reference Groups | | PDX Level g/L | | | | | | | Orthogonal *P* | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | SR | NB | 0 | 1.7 | 4.3 | 8.5 | 17 | SEM | *P* | Linear | Quadratic |
| Cecum | 6.04$^{bc}$ | 6.32$^{a}$ | 6.46$^{a}$ | 6.43$^{a}$ | 6.24$^{ab}$ | 6.12$^{b}$ | 5.84$^{c}$ | 0.081 | <0.001 | <0.001 | 0.12 |
| Colon | 5.93$^{de}$ | 6.58$^{a}$ | 6.41$^{ab}$ | 6.47$^{ab}$ | 6.31$^{bc}$ | 6.13$^{cd}$ | 5.85$^{e}$ | 0.077 | <0.001 | <0.001 | 0.01 |

[1]Values represent least-square means, n=12-13.

[a-e]Means in a row without a common letter differ ($P < 0.05$).

**Table 3. Effects of formula PDX concentration on cecal organic acid concentration in suckling pigs[1].**

| | PDX g/L | | | | | | | Orthogonal *P* | |
|---|---|---|---|---|---|---|---|---|---|
| µmol/g of wet digesta | 0 | 1.7 | 4.3 | 8.5 | 17 | SEM | *P* | Linear | Quadratic |
| Acetic | 53.3 | 56.6 | 66.6 | 64.9 | 58.3 | 8.5 | 0.79 | 0.50 | 0.33 |
| Propionic | 11.6 | 11.6 | 16.3 | 15.9 | 16.6 | 2.2 | 0.24 | 0.05 | 0.63 |
| Isobutyric | 1.02 | 1.02 | 3.29 | 0.57 | 1.82 | 1.13 | 0.55 | 0.74 | 0.59 |
| Butyric | 7.38 | 7.72 | 7.38 | 8.63 | 8.29 | 1.36 | 0.93 | 0.47 | 0.95 |
| Isovaleric | 1.17 | 1.57 | 1.86 | 2.15 | 2.35 | 1.08 | 0.94 | 0.39 | 0.90 |
| Valeric | 2.15 | 1.57 | 1.57 | 2.55 | 2.45 | 0.69 | 0.69 | 0.44 | 0.42 |
| SCFA Total | 77.4 | 80.8 | 97.5 | 95.1 | 90.5 | 11.3 | 0.65 | 0.25 | 0.40 |
| Lactic | 0.63[b] | 1.12[b] | 0.60[b] | 3.52[a] | 3.83[a] | 0.81 | 0.02 | <0.01 | 0.26 |

[1]Values represent least-square means, n=6-7.

[a-b]Means in a row without a common letter differ ($P < 0.05$).

**Table 4. Effects of formula PDX concentratio non cecal bacteria concentrations ($log_{10}$ CFU/g wet digesta) compared to SR piglets[1].**

| | | PDX g/L | | | | | | | Orthogonal *P* | |
|---|---|---|---|---|---|---|---|---|---|---|
| | SR | 0 | 1.7 | 4.3 | 8.5 | 17 | SEM | *P* | Linear | Quadratic |
| Lactobacilli | 10 | 4.3 | 16 | 13 | 34 | 50 | 10 | 0.12 | 0.01 | 0.53 |
| Bifidobacteria | 72 | 37 | 48 | 28 | 47 | 33 | 20 | 0.78 | 0.93 | 0.69 |

[1]Values represent least-square means, n=12-13.

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**Supplemental Material**

**Supplemental Table 1. Diet composition**

| Ingredient | g/kg of Dry Basal Diet |
|---|---|
| Non-fat dry milk[1] | 708 |
| Sodium caseinate[2] | 112 |
| Whey protein[3] | 74.3 |
| Lactose[4] | 48.3 |
| L-Arginine HCl[5] | 5.9 |
| L-Histadine[5] | 0.54 |
| Xanthan gum[5] | 13.5 |
| Calcium carbonate[5] | 5.1 |
| Calcium phosphate dibasic[5] | 17.4 |
| Mineral premix[6] | 6.6 |
| Vitamin premix[7] | 1.8 |
| Salt | 6.8 |

[1]Milk Specialties (Dundee, IL)
[2]International Ingredient Corporation (St. Louis, MO)
[3]Hilmar Ingredients (Hilmar, CA)
[4]Akey, Inc. (Lewisburg, OH)
[5]Dyets, Inc. (Bethlehem, PA)
[6]Mineral premix (Milk Specialties) contained: 1.002 g/100 g Ca, 0.549 g/100 g P, 0.284 g/100 g Na, 0.04 g/100 g CI, 2.024 g/100 g K, 0.102 g/100 g Mg, 20,000 mg/g Fe, 200 mg /100 g Co, 1850 mg/g Cu, 400 mg/g I, 5000 mg/g Mn, 60 mg/g Se, 23,500 mg/g Zn.
[7] Vitamin premix (Milk Specialties) contained: 9.9 g/kg retinol, 0.165 g/kg cholecalciferol, 55 g/kg α-tocopherol, 117,000 mg/g ascorbic acid, 29,983 mg/g D-pantothenic acid, 33,069 mg/g niacin, 8378 mg/g riboflavin, 5115 mg/g menadione, 66 mg/g biotin, 44000 mg/g vitamin B-12, 2038 mg/g thiamin, 3996 mg/g vitamin B-6, 2756 mg/g folic acid.

**Supplemental Table 2. List of primer sequences.**

| Gene | Primer* | Oligonucleotide Sequence (5'-3') | Reference |
|---|---|---|---|
| TNFα | S | CCCAAGGACTCAGATCATCG | 1 |
| | AS | ATACCCACTCTGCCATTGGA | |
| IL-8 | S | AGTTTTCCTGCTTTCTGCAGCT | 2 |
| | AS | TGGCATCGAAGTTCTGCACT | |
| IL-1β | S | CCTCCTCCCAGGCCTTCTGT | 1 |
| | AS | GGGCCAGCCAGCACTAGAGA | |
| IL-10 | S | ATGGGCGACTTGTTGCTGAC | 3 |
| | AS | CACAGGGCAGAAATTGATGACA | |
| GAPDH | S | CATCCATGACAACTTCGGCA | 4 |
| | AS | GCATGGACTGTGGTCATGAGTC | |
| FcRn+ | S | CCTCCTGATATACATGGC | 5 |
| | AS | TGAAACAATGAGAACACAAA | |

*Sense and antisense primer sequences are indicated by S and AS, respectively.
+FcRn (FCGRT; NM_214197.2)

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# CHAPTER 5: SAFETY OF *BIFIDOBACTERIUM* STRAINS DEMONSTRATED IN A HUMAN INFANT SURROGATE, THE NEONATAL PIG

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Abbreviations used: BB, *B. breve* AH1205; BL, *B. longum* AH1206; gastrointestinal, GI; IEL, intraepithelial lymphocytes; MPO, myeloperoxidase; NB, newborn; SR, sow-reared; TMB, tetramethylbenzidine;

**Abstract**

The intestinal microbiota of infants reportedly differ in response to gestational age, delivery mode and feeding regimen, and supplementation with probiotic bacteria is one method of promoting healthy populations. Studies were conducted to determine the impact of two novel probiotic strains, *B. breve* AH1205 (BB) and *B. longum* AH1206 (BL) at two doses, on the health, growth and development of neonatal pigs as a surrogate for human infants. One day old pigs were fed a milk-based formula containing BB or BL at both a low ($10^9$ CFU/day) or high ($10^{11}$ CFU/day) dose for 18 days (n=10/treatment). Additional groups included 20 newborn pigs sampled at baseline (NB) and 20 sow-reared (SR), reference pigs. Differences were not detected in piglet organ weights and body temperatures ($P > 0.1$). Bacterial translocation was not affected by probiotic treatment as indicated by total anaerobic and aerobic counts (CFU) in samples of spleen, liver and mesenteric lymph nodes ($P > 0.1$). Feeding high levels of BB had a minor but significant impact on feed intake, increasing 5% over controls ($P < 0.05$). Feeding BB at $10^9$ increased ileal lactase activity and decreased maltase activity, but did not affect ileal myeloperoxidase, intraepithelial lymphocytes or cytokine expression. Strain BL had no measurable effect on fecal and cecal total bifidobacteria populations; whereas, *B. longum* increased in response to increasing BL supplementation. BL tended to decrease ileal maltase activity (*Linear P* = 0.10) while increasing lactase activity (*Linear P* = 0.09). Ileal TNFα and IL-10 expression also tended to increase with increasing BL supplementation (*Linear P* = 0.08; *Linear P* = 0.01). We conclude that dietary supplementation with novel probiotic strains BB and BL is safe for

human infants based on a lack of developmental and immune-related effects on an appropriate surrogate animal model, neonatal pigs

**Introduction**

Although human milk is widely considered the optimum food for meeting the nutritional needs of infants, by two months of age the majority of infants in North America have received some quantity of infant formula (1). Bovine-milk and soy-based infant formulas are the most common substitute for human milk, although their composition does not fully duplicate that of human milk, particularly with regards to non-nutritive bioactive components which perform functional roles beyond basic nutrition, such as innate immune support. Breast-feeding during the first 13 weeks of life can decrease gastrointestinal (GI), respiratory and ear infections for the first year of life as compared to formula-feeding (2). After the first week of breast-feeding, bifidobacteria become the dominant GI bacteria, while formula-fed infants develop a more diverse flora, with *Bacteroides sp.* equaling the number of bifidobacteria (3). Commensal bacteria, such as bifidobacteria, help maintain healthy gastrointestinal mucosal surfaces (4). *B. breve* and *B. longum* are part of the commensal bacterial community of breast-fed infants and inoculation can occur directly through breast milk (5). In order to more closely approximate both nutritional and functional properties of human milk, efforts are under way to identify novel ingredients to more closely align the bacterial community development of formula-fed infants to that of their breast-fed counterparts. To that end, supplementation of probiotic bifidobacteria strains is hypothesized

to modify the bacterial community of formula-fed infants to be similar to that of breast-fed infants.

Probiotics have been defined as "living microorganisms that, on ingestion in sufficient numbers, exert health benefits beyond basic nutrition" (6). In pediatric populations, the administration of specific probiotic bacteria have been shown to reduce the incidence and duration of diarrhea (7), shorten the duration of rotavirus diarrhea (8), reduce the incidence of atopic eczema (in children at high risk) (9) and reduce the risk of necrotizing enterocolitis and overall mortality (in preterm infants with very low birth weight) (10). Probiotics are typically food-grade microorganisms that are isolated from food materials and the digestive tract of humans and other animals. Species and strains derived from two bacterial genera (*Lactobacillus* and *Bifidobacterium*) comprise the largest groups of commercially available probiotic bacteria. *Bifidobacterium* strains are considered particularly important in pediatric applications as members of this genus reportedly dominate the GI microbiota of breastfed infants (11). The introduction of novel ingredients to infant formula, including probiotic *Lactobacillus* and *Bifidobacterium* isolates depends in part on the establishment of a thorough safety profile (12). As the GI tract is the target site of probiotic activity, and the process of GI maturation in neonatal piglets is closer than other animals to that of human infants, neonatal piglets were selected as a favored model in which to evaluate the safety and immunological impact of a new probiotic *Bifidobacterium* strain intended for possible use in infant formula.

Bifidobacteria are the dominant organisms among the microbiota of breastfed infants and are less prevalent in that of formula-fed infants where they compete with higher levels of other

bacterial groups (e.g.*Bacteroides sp.*) (3). Bifidobacteria are considered beneficial commensal bacteria, as they help maintain healthy mucosal surfaces in the GI tract (4) and have the capacity to inhibit pathogenic bacteria, populations which also differ among breastfed and formula-fed infants. In a study of over 1,000 infants, fecal counts of *Clostridium difficile* were significantly higher for formula-fed infants. *Clostridium difficile* colonization has been associated with diseases such as necrotizing enterocolitis, due to immaturity of the intestine (13, 14). Interestingly, *C. difficile* also was higher in preterm infants than term infants (15). Establishing bifidobacteria commensals early in the life of formula-fed infants may competitively exclude potentially pathogenic bacteria, like *C. difficile.* Some bifidobacteria strains are present in human milk and through milk consumption it is thought that infants become inoculated with these strains, while infant formulas lack this bacterial species(5). It is therefore proposed that in supplementing bifidobacteria within infant formula, bifidobacteria strains would establish within the formula-fed infant intestine. We conducted this study to determine the safety of two novel bifidobacteria species and identify changes in immunological messages associated with the probiotics.

## Methods

*Animals and experimental diets.*

All animal procedures were approved by the Institutional Animal Care and Use Committee of North Carolina State University. Colostrum fed piglets were acquired from the

North Carolina State University Swine Education Unit within 12 to 24 hours of birth and housed in an environmentally controlled room at Laboratory of Developmental Nutrition at North Carolina State University. All pigs were injected with iron dextran and penicillin prior to arriving at the Laboratory of Developmental Nutrition. Piglets were randomly assigned to one of three treatments and two reference groups (sow-reared (SR) and newborn, day 0 controls (NB)) based on body weight (n = 10). Dietary treatments included the basal diet and the basal diet with $10^9$ or $10^{11}$ CFU of *B. breve* AH1205 (BB) or *B. longum* AH1206 (BL) added once per day. Dietary treatments (milk formulas) were fed through a gravity flow system for accurate measurement of formula consumption. The basal dry diet was designed as a sow milk replacer diet and is detailed in **Supplementary Table 1.** Once the diet was reconstituted with water it was stored at $4^oC$ for a maximum of 48 hours before use. The lyophilized probiotic was added to the reconstituted diet once daily. The probiotic dose was stored in individual packets for each day and stored at $4^0C$ prior to use. After the probiotic was added to the diet, the diet was offered to the pigs within less than 30 min. For 2 days prior to starting the probiotic treatments, animals were fed the basal diet with antibiotics included (Gentamycin sulfate) and treated with penicillin daily. After treatments were started, antibiotics were removed from the feed. NB piglets in the BB study were sampled prior to the two-d acclimation period, while NB piglets in the *B. longum* study were sampled after the two d of acclimation.

*Animal feeding and management.*

Fresh formula was provided 3 times per day (0800, 1400 and 2100), prepared daily and stored under refrigeration until fed. Diets were reconstituted at 180g dry diet in 1 L of

water. The probiotic was added to the diet during the 1400 feeding period and pigs were allowed access to the diet until the 0800 feeding period. Pig weights and feed intakes were recorded daily. Rectal temperatures and rectal bacteria were determined on days 0, 7, 14 and 18. Stool consistency was evaluated daily and rated on a 1 to 5 scale with 1 representing well-formed stools and 5 representing watery stool with no form. Sow-reared pigs were weighed, rectal temperatures recorded and fecal samples taken on days 0, 7, 14 and 18. Pigs were euthanized on d 0 (10 pigs) and d 20 by AVMA-approved electrocution and exsanguination. Tissue collection occurred immediately following exsanguination.

*Sample collection.*

Prior to euthanasia three blood samples were collected for plasma, serum and whole blood analysis. Samples were kept on ice until all samples had been collected and then were centrifuged for plasma and serum separation. Plasma and serum (sub-samples) were then stored at -20$^{o}$C. Serum and whole blood samples were placed on ice and transported to Antech Diagnostic Laboratories in Cary, NC for clinical blood parameter measurement (Vet Screen and Whole Blood Analysis). After blood sampling, animals were killed by AVMA-approved electrocution. A urine sample was collected via bladder puncture, stored on ice and transported to Antech Diagnostic Laboratories in Cary, NC for urinanalysis and Cr analysis. The large and small intestines were then collected. Proximal to the ileocecal junction, mucosal and histological samples were collected. Digesta was collected from the remaining portion of the ileum, the entire colon and cecum. Ileal, colon and cecal digesta was collected, mixed and sub-sampled for various analyses. Fresh cecal digesta was sampled for bacterial colony forming units (CFU). The pH of digesta was determined on fresh samples. Organ

weights were recorded for the liver, gallbladder, kidney, spleen, pancreas, cecum, colon, thymus, pancreas, lung, heart, brain and eye. Histology samples of these tissues and the mesenteric lymph nodes, jejunum, and ileum were collected. Ileal mucosa was collected and snapped frozen for later analysis of cytokine message and enzyme activity. Microbiology swabs were taken from the liver, spleen and mesenteric lymph nodes.

*Fecal and cecal bacteria colony forming units.*

Immediately after collection, samples of feces and cecal digesta were placed in a sterile 0.1x MRS agar with 0.05% cysteine solution. This diluent was then used to make serial dilutions of the samples for plating on Rogosa, Differential Reinforced Clostridium and Anaerobic CDC Blood Agars. Several dilutions were plated in duplicate. Plates were incubated in anaerobic conditions at $37^0$C for 48 h and then colonies were counted.

*Bacteria translocation.*

The liver, spleen and mesenteric lymph nodes were swabbed with an anaerobic and aerobic transport swab in duplicate and swabs were placed on ice. Swabs were then streaked in duplicate on Nutrient Agar (aerobic bacteria) and Anaerobic CDC Blood Agar (total anaerobic bacteria). Samples plated on Anaerobic CDC Blood Agar were incubated in anaerobic conditions. All plates were incubated at $37^o$C for 48 h. Organs were considered positive if CFU were $\geq$ 30 per plate (16).

*Bacterial determination via quantitative PCR.*

Intestinal bifidobacteria were quantified as previously described by (17). DNA was isolated following the manufacturer's instructions (Qiagen QIAamp DNA Stool Mini Kit

Catalog #51504). Amplification was carried out in a total volume of 25 µL containing 1X iQ SYBR Green Supermix (BioRad Laboratories, Hercules, CA), forward and reverse primers (200 nM each) and 5 µL of the isolated DNA (BioRad Laboratories, Hercules, CA). At the end of the PCR, melt curve analysis was conducted to validate the specificity of the primers. All determinations were performed in at least duplicate. To determine if identified microbes were viable, digesta samples were inoculated in RCM and grown over night. DNA was isolated from the growth cultures and compared to levels in non-incubated digesta. *B. breve* and *B. longum* are not autochollontous to the swine intestine, and therefore identifying these species within the digesta was attributable to feeding.

*Jejunal and ileal histology.*

Ileal and jejunal samples were fixed in neutral buffered formalin. After 24 h, the samples were transferred into a 70% ethanol solution and subsequently embedded in wax, sectioned and stained with hematoxylin and eosin. Two slices of the ileum and jejunum were placed on each slide for analysis. To measure the intestinal morphology, three well defined villi were identified. The length and width of the villi were recorded along with the length of the crypt underneath the villi. To enumerate the intraepithelial lymphocytes (IEL), five well defined ileal villi were identified. The IELs and epithelia cells were counted on each of these villi. Intestinal slides were read using an Olympus Vanox-S Microscope (Olympus Corporation, Lake Success, NY) and analyzed using SPOT Basic Imaging software (Diagnostic Instruments, Sterling Heights, MI).

*Organ histology.*

Morphological tissue samples were collected from similar areas on each organ. Samples were fixed in neutral buffered formalin. After 24 h, the tissues were transferred into a 70% ethanol solution and subsequently embedded in wax, sectioned and stained with hematoxylin and eosin. A board certified veterinary pathologist who was unaware of dietary treatments evaluated the organ sections. Tissue examinations were made using an Olympus BH-2 microscope with 2, 4, 10, 20 and 40X objectives (Olympus Corporation, Lake Success, NY). Each slide was examined for histological abnormalities. The grading scheme used was different for each tissue.

*Ileal mucosal enzyme assays.*

Crude homogenates of ileal mucosal tissue were prepared by homogenizing tissue in sterile phosphate buffered solution. Lactase and maltase activity were analyzed based on modifications for a 96 well plate assay (18). The standard control was D-glucose. One unit of specific enzyme activity was determined as the liberation of one μmol of glucose per min per mg of protein. To determine neutrophil infiltration in the ileum, myloperoxidase (MPO) activity was determined with modifications for a 96 well plate (19). Substrate concentration was calculated based on the Beer's-Lambert equation with the molecular absorption coefficient of tetramethylbenzidine (TMB; Sigma #860336) being $3.9 \times 10^4$ $M^{-1}cm^{-1}$. One unit of specific MPO activity was defined as that degrading one μmol of TMB per min per mg of protein. Total protein was determined (20) using a commercially available bicinchoninic acid kit (Pierce #23225).

*Serum ELISA.*

Serum was stored at $-20^0$ and thawed on ice prior to use. Serum IgG and IgM were determined via the manufacture's protocol (Bethyl Laboratories, #E100-104 and #E100-100).IL-4, IL-10 and IFNγ were determined via ELISA using the manufacture's protocol for the BL experiment (Invitrogen #KSC0041, KSC0102, KSC4021). All cytokine and immunoglobulin levels were normalized to serum protein determined via BCA (Thermo Scientific Pierce BCA Protein Assay Kit).

*RNA isolation and Real-Time RT-PCR.*

RNA isolation and real-time RT-PCR to determine cytokine expression in ileal mucosal was determined as previously described (21).

*Statistical analysis.*

Data were analyzed according to a completely randomized block design using the general linear models procedure of SAS (SAS, Cary, NC). Linear contrasts were analyzed for formula-fed piglets (22). Data from bacterial translocation were analyzed using the Proc Glimmix procedure of SAS (SAS, Cary, NC). Organ histology was analyzed using the Proc Genmod procedure of SAS. Differences were deemed significant when $P < 0.05$.

## Results

### ***B. breve AH1205 experiment***

*Piglet performance and body temperatures.*

Growth was not different among formula-fed pigs (**Table 1**). Overall, feed intake was increased in the low level probiotic group ($10^9$ CFU/d) compared to other formula fed pigs ($P < 0.05$), but ADG and G:F were not different. Fecal consistency did not differ among treatments. Rectal temperatures were not different among formula fed pigs (data not shown).

*Fecal and cecal microbial content.*

Concentrations of culturable bifidobacteria and lactobacilli(CFU) were not altered by BB supplementation (**Table 2**), nor were total anaerobes except for a small reduction in cecal digesta of pigs supplemented with $10^9$ BB for 20 d. Cecal concentrations of total bifidobacteria determined via quantitative PCR were lower than concentrations measured via culture techniques. Total bifidobacteria measured by qPCR increased with increasing BB feeding (*Linear P* = 0.02). Piglets fed $10^{11}$ CFU/d BB had higher levels of total bifidobacteria and BB than that of the other treatment groups (*P* = 0.02;*Linear P*< 0.01). The difference in BB levels between $10^{11}$ and $10^9$ CFU/d piglets was ~1 log, instead the 2 log difference in feeding levels. To determine if the BB observed in the cecum was viable, frozen cecal samples were inoculated in RCM overnight at 37$^o$C, DNA isolated and compared to the non-incubated cecal BB levels. Incubating the cecal digesta increased BB levels, indicating the probiotic was viable in the digesta (*P* = 0.054; $10^9$: 3.10E04 cells/g non-

incubated digesta versus 9.9E05 cells/g incubated digesta ± 10.6E05; $10^{11}$: 1.21E05 cells/g non-incubated digesta versus 3.5E06 cells/g incubated digesta ± 11.3E05).

*Safety evaluation of B. breve.*

There were no differences between formula-fed pigs in relative organ weights (**Supplementary Table 2**). There were no significant lesions identified in any of the pigs associated with their treatment group (**Supplementary Table 3**). A few pigs had background lesions such as focal renal inflammation, but this was uncommon and not associated with any treatment. There was some variation in the number of lymphocytes within the mesenteric lymph nodes, but this was judged to be within the limits of normal variation, as was the varying degree of lymphoid follicle activation and number. Variable numbers of eosinophils were found in the mesenteric lymph nodes, but this was similar in all groups and consistent with typical findings in young pigs. Differences in the histological appearance of the thymus and spleen tissues were also judged to be within the limits of normal variation. Hepatic vacuolization was also within the range of normal variation. Other organs, the heart, the kidneys, the pancreas and the lungs were within normal limits. Overall, differences between treatments in regard to dietary treatment were not observed. Differences between reference groups and the treatments were as expected and follow similar trends observed in past suckling pig trials our laboratory has conducted. Whole blood and urine analysis did not reveal differences between formula-fed piglets (**Supplementary Table 4**).The serum profile did not reveal any significant differences between dietary treatments (**Table 3**). A linear increase was observed in serum creatinine concentration with increasing BB feeding (*Linear P* = 0.03); the concentration increased to levels approaching that of the

SR piglets with increasing BB. Bacterial translocation to the liver, spleen and mesenteric lymph nodes were not different among treatment groups or the sow reared reference controls for both anaerobic and aerobic bacteria (**Table 4**). Collectively, no toxicological differences were observed with the addition of BB up to $10^{11}$ CFU per day.

*Intestinal morphology, disaccharidases and digesta pH.*

No effects of dietary BB were detected on ileal and jejunal morphology (**Table 5**).Maltase activity was decreased in piglets fed $10^{9}$BB CFU/d compared to control pigs, but was not different from piglets fed $10^{11}$. There were no linear trends in cecal and colonic digesta pH, values were higher in piglets fed $10^{9}$ CFU/d compared to piglets fed $10^{11}$.

***Systemic and ileal immunologic measures.*** Serum levels of IgM and IgG were not affected by BB feeding (**Table 6**). No differences or trends were observed in ileal cytokine expression. IEL were lower in the NB and SR pigs compared to formula fed piglets ($P < 0.01$).

***B. longum AH1206 experiment***

*Piglet performance and stool consistency.*

Feed intake decreased with increasing BL supplementation (*Linear P* = 0.003; **Table 1**). The decrease in feed intake was first significantly different during days 8 to 14 (*Linear P*< 0.01; data not shown). Although feed intake was affected, overall daily gain did not differ between treatment groups. BL supplementation did not affect cumulative stool consistency ($P = 0.12$). Body temperature also was not affected by probiotic supplementation (data not shown).

*Fecal and cecal microbial content.*

Concentrations of culturable lactobacilli and total anaerobes(CFU) were not altered by BL supplementation (**Table 7**); however, bifidobacteria CFU decreased linearly in cecal digesta of pigs fed increasing BL for 20 d. Cecal concentrations of total bifidobacteria determined via qPCR were lower than that observed for culture techniques. Total bifidobacteria measured via qPCR did not differ among formula fed piglets ($P = 0.46$). Cecal concentrations of BL measured by qPCR increase progressively with increasing BL feeding ($P< 0.01$), but the increase in cecal BL was <1 log compared with the 2 log difference in feeding level. One control animal had detectable BL in the digesta. The result was confirmed by isolating DNA two separate times. This animal was removed from the data set. Viability of the probiotic in digesta was determined. Viability could not be confirmed because there was no difference in cell numbers between non-incubated and incubated digesta ($P = 0.39$; $10^9$: 1.17E05 cells/g non-incubated digesta versus 2.80E04 cells/g incubated digesta ± 1.25E06; $10^{11}$: 2.44E05 cells/g non-incubated digesta versus 1.51E06 cells/g incubated digesta ± 1.02E06).

*Safety evaluation.*

Organ weights were not affected by supplementation of BL (**Supplementary Table 5**). The liver, kidney and pancreas were smaller in the sow reared reference group compared to the formula feed pigs ($P< 0.01$). In a previous study using a suckling swine model similar results were seen between sow reared pigs and formula fed pig organ weights and was explained as differences in feeding frequency between the reference group and treatment group (23). The liver sections varied in the extent of hepatocellular vacuolization with a

linear trend for increased vacuolization with increasing BL (**Table 8**). Most animals were similar with two animals fed $10^{11}$ CFU/d having periarteriolar lymphoid sheaths in apposition. The vacuoles were typically characterized by a ragged outline suggesting glycogen accumulation. Although the extent of lipid could not be assessed in hematoxylin and eosin-stained sections, there were no animals with prominent round, clear, distinct cytoplasmic vacuoles typical of lipid, suggesting that lipid accumulation was not a prominent feature. Mixtures of lipid and glycogen within hepatocytes may have occurred. Kidneys generally had no significant findings. Several animals had renal cysts, a recognized developmental disorder common in pigs. Occasional animals had epicardial thickening of unknown etiology but the thickening was found within blocks and was distributed evenly throughout treatments (results not shown).Overall, organ histology was normal throughout dietary treatments. Serum profiles revealed a linear decrease in total bilirubin (**Table 9;** *Linear P* = 0.05). Whole blood did not reveal differences between formula-fed piglets (**Table 10**). No differences between formula-fed pigs were observed in urinalysis (data not shown). Bacterial translocation to the liver, spleen and mesenteric lymph nodes were not different among treatment groups or the sow reared reference controls for both anaerobic and aerobic bacteria (**Table 11**).

*Intestinal morphology, disaccharidases and digesta pH.*

No effects of dietary BL were detected on ileal and jejunal morphology (**Table 12**). Maltase activity tended to decrease with increasing BL(*Linear P* = 0.10)while lactase activity tended to increase (*Linear P* = 0.09).There were no differences or linear trends in cecal and colonic digesta pH.

*Systemic and ileal immunologic measures.*

Serum levels of IgM did not differ among formula fed piglets (**Table 13**). There was a linear trend for decreasing serum IgG with increasing BL (*Linear P* = 0.09). Serum IL-4, IL-10 and IFNγ levels were not within detectable range of the assays. Ileal TNFα expression tended to increase with probiotic feeding (*Linear P* = 0.08). Ileal IL-10 expression increased with increasing BL(*Linear P* = 0.01). Intestinal IEL and MPO were not different between formula-fed pigs.

**Discussion**

Probiotics have been defined as "living microorganisms that, on ingestion in sufficient numbers, exert health benefits beyond basic nutrition" (6). Bifidobacteria has been recognized as an abundant bacteria group in breast-fed infants for over 100 years (24). Improved health status of breast-fed versus formula-fed infants is hypothesized to be linked to the difference in intestinal microbial populations. Bifidobacteria are the dominant organisms among the microbiota of breastfed infants and are less prevalent in the more diverse microbial population of formula-fed infants(3). Although these differences have been well documented, the mechanism by which bifidobacteria improves infant health remains unknown. We conducted this study to determine the safety of two novel bifidobacteria species and identify changes in immunological measures associated with the probiotics.

Overall, both probiotics were determined to be safely fed to neonates up to $10^{11}$CFU/day. Bacterial translocation was not affected by feeding additional microbes.

Organ weights were not different among formula fed piglets, but differences between SR and formula fed piglets did exist. Previously, our laboratory has observed similar differences in organ weights that we believe are due to the decreased number of feedings in the formula fed piglets compared to the SR piglets (23, 25). Piglets fed BB had typical organ histology, while piglets fed BL had increased liver vacuolization that is hypothesized to be due to increased glycogen but not fat content in the liver. Whole blood and urine clinical measures were not affected by bifidobacteria feeding. The blood metabolite panel found creatine levels different in piglets fed BB than controls, but that these levels were approaching SR piglets and therefore these differences are not deemed a safety concern. Piglets fed BL had decreasing bilirubin. The level of serum bilirubin for SR was ten times the level of that of NB and formula fed piglets. As with our study, previous work with neonatal rabbits has shown parenteral feeding of Bifidobacteria to lower serum bilirubin levels by stabilizing the intestinal microbiota (26). Neither probiotic demonstrated toxic effects in the neonatal piglet model.

Changes in the intestinal microbiota were observed via PCR, but not via traditional culturing techniques. Previous work has shown discrepancies between culturing and PCR techniques on the level of fecal and digesta Bifidobacteria in young pigs. Using PCR detection, Regmi and colleagues (27) found the level of Bifidobacteria in the ileum and feces to be ~10e5 cells/g of wet weight. Using fresh feces inoculated onto Tryptone Phytone Yeast medium, Zhang and colleagues (28) found the level of Bifidobacteria in weaning pigs to be 10e8 CFU/g of wet feces. In 17 d old piglets, fecal Bifidobacteria counts were 10e5.32/g of wet feces (29). As with these previous studies, our results show larger numbers of

Bifidobacteria via culturing techniques compared to PCR. BB in the cecum differed by 1 log between animals fed $10^9$ and $10^{11}$ CFU/d using PCR. The probiotic was viable within the cecum. Based on the total Bifidobacteria numbers, feeding BB may replace other bifidobacteria in the intestine because BB levels were less than 1 log different from total Bifidobacteria. BL did not have the same robustness in the intestine and a log difference was not able to be seen between the $10^9$ and $10^{11}$ fed animals. Furthermore, viability of the probiotic within the cecum could not be confirmed. Because the sample was stored at -80$^0$C and a fresh sample was not used to determine viability, it is unknown if the probiotic was truly unviable in the cecum. Although the viability of BL is uncertain, differences in cytokine expression, feed intake and intestinal development indicate activity of the probiotic.

Probiotics have been shown to affect growth performance differently. In this study, BB fed at $10^9$ increased feed intake, while *B. longum* decreased intake. Similar to our finding with BL, feeding a probiotic mixture of *Lactobacillus amylovorus* and *Enterococcus faecium*(3E08/day) decreased feed intake in weaning piglets but body weight gains were not different (30). As with the increased intake observed with feeding BB at $10^9$, feeding a probiotic mixture to weaning pigs increased feed intake (31). In weaning pigs, various probiotics and combinations of probiotics have shown no affect on weight gain, feed intake or feed efficiency (32). Creep feeding piglets that remained with their sow did not affect performance when probiotics were added to the feed (33). Although perhaps generally thought as improving production, literature on the effects of probiotics in young pigs is inconsistent and differences between strains and single verses multistrain doses are evident. Probiotics have been shown to influence satiety through changes in neuropeptides, such as

leptin (34). The mechanism by which probiotics regulate neuropeptides is still unknown. Although, SCFA are ligands for G-coupled protein receptors that activate peptide hormones, probiotic administration increased leptin levels over that of lactic acid administration in rodent models (35). In our study, pH of the digesta was not changed, which would indicate that SCFA were also not different between treatments, but intake was affected. The exact mechanism by which probiotics affect satiety still needs to be elucidated, especially because these two probiotics of the same genus elicited different satiety responses. Predictions on performance based on probiotic genus alone is not appropriate given the divergent observations seen in this study on feed intake with probiotics of the same genus.

Both probiotic strains affected intestinal development. Greater affects of BB on intestinal development were seen in piglets fed $10^9$versus $10^{11}$. Jejunal widths tended to decrease with BB($P = 0.06$). The intestinal enzymes, maltase and lactase, were used to determine maturation of the intestine. As the intestine matures, maltase activity increases while lactase decreases. Pigs fed $10^9$ had decreased maltase activity ($P< 0.01$) and tended to have increased lactase activity ($P = 0.06$) indicating a less mature intestinal enzyme profile. BL also tended to decrease maltase (*Linear* $P = 0.10$) and increase lactase (*Linear* $P = 0.09$) ileal activities, indicating the probiotic increased maturation time of the intestine. The genome of BL contains several genes that enable the bacteria to breakdown several complex carbohydrates (36). Carbohydrate enzymes of bifidobacteria are hypothesized to be intracellular (37). Changes in host disaccharidase activity may be related to decreased expression due to exogenous disaccharidase activity attributed to microbes, but unfortunately in this study only activity was determined.

Overall, intestinal health was not different with probiotic supplementation from control animals as indicated by the MPO and IEL levels. Changes in systemic and intestinal health status were not observed with feeding BB, but BL affected intestinal cytokine expression. BL activated expression of two cytokines, IL-10 and TNFα. Neonates are born with an immune response biased towards a Th2 response leading to increased risk of infection (38). Microbes are able to activate the maturation process of the immune system in neonates. The supplementation of *B. longum* in this study activated the expression of the Th1 cytokine TNFα, but increased inflammation associated with disease status was not observed. The increase in the inflammatory regulation cytokine IL-10 may prevent inflammatory reactions in the intestine of neonates while the activation of the Th1 response is occurring.

The novel strains of *Bifidobacterium* have been shown to be safe to feed in the human surrogate model, the neonatal piglet. BB was shown to be viable in the cecum, while the viability of BL could not be concluded. The results from the current study demonstrate the ability of BL to increase the expression of the cytokines TNFα and IL-10. The increased expression of these cytokines indicate an improvement in the maturation of the intestinal immune system to overcome the Th2 biases at birth and decrease the risk to bacterial infection. Feeding BB and BL in infant formula may change the microbiota of formula-fed infants to be more similar to that of breast-fed infants. In conclusion, feeding the novel *Bifidobacterium* strains BB and BL in neonatal diets is safe and causes differential intestinal effects.

**Table 1. Effects of incremental *Bifidobacteria* on suckling pig growth and fecal consistency[1]**

| | CFU/d | | | | | |
|---|---|---|---|---|---|---|
| | 0 | $10^9$ | $10^{11}$ | SEM | *P* | *Linear P* |
| *B. breve*AH1205 | | | | | | |
| Body gain, g/d | 298 | 299 | 298 | 9 | 0.99 | 0.96 |
| Intake, g/d | 240[b] | 254[a] | 239[b] | 2 | <0.01 | 0.66 |
| Gain:Intake | 1.24 | 1.18 | 1.25 | 0.03 | 0.21 | 0.89 |
| Fecal consistency | 2.06 | 2.18 | 1.66 | 0.48 | 0.66 | 0.52 |
| *B. longum*AH1206 | | | | | | |
| Body gain, g/d | 297 | 294 | 297 | 11 | 0.96 | 1.00 |
| Intake, g/d | 281[a] | 281[a] | 275[b] | 1 | <0.01 | <0.01 |
| Gain:Intake | 1.06 | 1.08 | 1.04 | 0.04 | 0.81 | 0.67 |
| Fecal consistency | 1.64 | 1.62 | 1.81 | 0.18 | 0.63 | 0.92 |

[1]Values represent least-square means, n=10.
[a-b]Means in a row without a common letter differ ($P < 0.05$).

**Table 2. Digesta bacteria changes after feeding *B. breve* AH1205 to suckling pigs[1].**

| | *B. breve* AH1205 (CFU/d) | | | | | |
|---|---|---|---|---|---|---|
| | 0 | $10^9$ | $10^{11}$ | SEM | *P* | *Linear P* |
| | Cultured Bacteria | | | | | |
| Day 0, CFU/g Feces | | | | | | |
| Bifidobacteria | 1.40E+09 | 9.30E+08 | 7.20E+08 | 4.80E+08 | 0.59 | 0.32 |
| Lactobacilli | None Identified | | | | | |
| Total Anaerobes | 8.00E+08 | 7.40E+08 | 9.10E+08 | 3.60E+08 | 0.90 | 0.82 |
| Day 7, CFU/g Feces | | | | | | |
| Bifidobacteria | 1.10E+10 | 6.60E+09 | 4.80E+09 | 3.70E+09 | 0.40 | 0.20 |
| Lactobacilli | 9.70E+07 | 1.80E+08 | 1.70E+08 | 4.80E+07 | 0.44 | 0.32 |
| Total Anaerobes | 2.60E+10 | 2.90E+10 | 2.10E+10 | 7.20E+09 | 0.71 | 0.60 |
| Day 14, CFU/g Feces | | | | | | |
| Bifidobacteria | 2.60E+10 | 3.40E+10 | 2.30E+10 | 1.30E+10 | 0.83 | 0.91 |
| Lactobacilli | 4.90E+09 | 3.50E+09 | 1.40E+09 | 1.70E+09 | 0.37 | 0.17 |
| Total Anaerobes | 4.50E+10 | 6.10E+10 | 6.80E+10 | 2.90E+10 | 0.84 | 0.57 |
| Day 20, CFU/g Cecal Digesta | | | | | | |
| Bifidobacteria | 1.00E+11 | 6.20E+10 | 1.60E+11 | 6.60E+10 | 0.52 | 0.54 |
| Lactobacilli | 6.90E+07 | 2.50E+09 | 4.40E+09 | 2.50E+09 | 0.47 | 0.23 |
| Total Anaerobes | 1.04E+12[a] | 2.6E+11[b] | 7.1E+11[a] | 2.00E+11 | 0.03 | 0.23 |
| | Quantitative PCR (cells/g cecal digesta) | | | | | |
| Total Bifidobacteria | 1.68E+05[b] | 1.80E+05[b] | 9.23E+05[a] | 2.25E+05 | 0.02 | 0.02 |
| *B.breve* | nd | 3.10E+04[b] | 1.21E+05[a] | 1.69E+04 | <0.01 | ------ |

[1]Values represent least-square means, n=10

[a-b]Means in a row without a common letter differ ($P < 0.05$).

Table 3. Serum profiles of pigs fed formula containing incremental *B. breve* AH1205 versus SR and NB reference pigs[1].

| | | | *B. breve*AH1205 (CFU/d) | | | | | |
|---|---|---|---|---|---|---|---|---|
| | NB | SR | 0 | $10^9$ | $10^{11}$ | SEM | *P* | *Linear P* |
| Glucose (mg/dL) | $95^{b}$ | $145^{a}$ | $145^{a}$ | $139^{a}$ | $150^{a}$ | 8 | <0.01 | 0.81 |
| BUN[2] (mg/dL) | $10.38^{a}$ | $7.90^{ab}$ | $6.70^{b}$ | $8.42^{ab}$ | $7.00^{ab}$ | 1.31 | 0.29 | 0.83 |
| Creatine (mg/dL) | $0.507^{c}$ | $0.820^{a}$ | $0.707^{b}$ | $0.741^{ab}$ | $0.770^{ab}$ | 0.039 | <0.01 | 0.03 |
| Total Protein (g/dL) | $5.76^{a}$ | $4.74^{b}$ | $4.19^{c}$ | $4.33^{c}$ | $4.18^{c}$ | 0.15 | <0.01 | 0.77 |
| Albumin (g/dL) | $1.17^{c}$ | $3.28^{a}$ | $2.82^{b}$ | $2.79^{b}$ | $2.78^{b}$ | 0.1 | <0.01 | 0.70 |
| Total Bilirubin (mg/dL) | $0.559^{b}$ | $0.950^{a}$ | $0.088^{c}$ | $0.121^{c}$ | $0.100^{c}$ | 0.085 | <0.01 | 0.23 |
| Alkaline Phosphatase (U/L) | $1625^{a}$ | $860^{b}$ | $661^{b}$ | $648^{b}$ | $602^{b}$ | 146 | <0.01 | 0.51 |
| ALT[3] (U/L) | $58.6^{a}$ | $39.1^{b}$ | $18.9^{c}$ | $20.4^{c}$ | $19.5^{c}$ | 4.9 | <0.01 | 0.86 |
| AST[4] (U/L) | $97.5^{a}$ | $42.8^{b}$ | $27.8^{b}$ | $34.7^{b}$ | $29.2^{b}$ | 10.2 | <0.01 | 0.96 |
| Cholesterol (mg/dL) | $121^{b}$ | $264^{a}$ | $107^{b}$ | $114^{b}$ | $105^{b}$ | 12 | <0.01 | 0.71 |
| Ca (mg/dL) | 10.6 | 10.6 | 11 | 10.7 | 10.8 | 0.2 | 0.33 | 0.52 |
| P (mg/dL) | 7.43 | 17.71 | 10.69 | 10.12 | 10.56 | 4.6 | 0.51 | 0.38 |
| Na (mEq/L) | 142 | 140 | 143 | 144 | 142 | 1 | 0.06 | 0.41 |
| K (mEq/L) | 5.74 | 13.19 | 10.22 | 8.56 | 8.46 | 3.2 | 0.50 | 0.53 |
| Cl (mEq/L) | $102^{b}$ | $106^{a}$ | $106^{a}$ | $106^{a}$ | $105^{a}$ | 1 | 0.01 | 0.44 |
| Albumin:Globulin | $0.218^{c}$ | $2.290^{a}$ | $2.090^{ab}$ | $1.853^{b}$ | $2.000^{ab}$ | 0.12 | <0.01 | 0.64 |
| BUN:Creatine | $19.8^{a}$ | $10.2^{b}$ | $9.6^{b}$ | $11.1^{b}$ | $9.4^{b}$ | 1.7 | <0.01 | 0.92 |
| Globulin (g/dL) | $4.59^{a}$ | $1.46^{b}$ | $1.37^{b}$ | $1.54^{b}$ | $1.40^{b}$ | 0.14 | <0.01 | 0.98 |

[1]Values represent least-square means, n=10.

[2]Blood urea nitrogen

[3]Alanine aminotransferase

**Table 4. Bacterial translocation was not affected by supplementing *B. breve* AH1205 to suckling piglets[1]**

| | *B. breve*AH1205 (CFU/d) | | | | | |
|---|---|---|---|---|---|---|
| % Positive Cultures | 0 | $10^9$ | $10^{11}$ | Standard Deviation | *P* | *Linear P* |
| Anaerobic Bacteria | | | | | | |
| Spleen | 0 | 0 | 10 | 32 | 1.00 | 0.97 |
| Liver | 11 | 0 | 0 | 33 | 1.00 | 1.00 |
| Mesenteric lymph nodes | 0 | 0 | 0 | 0 | 1.00 | 1.00 |
| Aerobic Bacteria | | | | | | |
| Spleen | 0 | 0 | 0 | 0 | 1.00 | 0.97 |
| Liver | 0 | 0 | 0 | 0 | 1.00 | 1.00 |
| Mesenteric Lymph nodes | 0 | 0 | 0 | 1 | 0.60 | 0.45 |

[1]Values represent least-square means, n=10.

**Table 5. Effects of supplementing *B. breve* AH1205 on intestinal morphology, disaccharidases and digesta pH of suckling piglets[1].**

| | | | *B. breve* AH1205 (CFU/d) | | | | | |
|---|---|---|---|---|---|---|---|---|
| | NB | SR | 0 | $10^9$ | $10^{11}$ | SEM | *P* | *Linear P* |
| Jejunum | | | | | | | | |
| Villi Height (μm) | 553 | 409 | 557 | 633 | 554 | 57 | 0.08 | 0.72 |
| Villi Width (μm) | $107^{bc}$ | $96^{c}$ | $152^{a}$ | $146^{a}$ | $128^{ab}$ | 9 | <0.01 | 0.06 |
| Crypt Depth (μm) | 143 | 145 | 164 | 174 | 166 | 15 | 0.37 | 0.63 |
| Ileum | | | | | | | | |
| Villi Height (μm) | 528 | 477 | 484 | 472 | 440 | 49 | 0.79 | 0.49 |
| Villi Width (μm) | 105 | 131 | 129 | 126 | 138 | 9 | 0.12 | 0.20 |
| Crypt Depth (μm) | 112 | 111 | 137 | 124 | 118 | 9 | 0.31 | 0.25 |
| Maltase (mU/mg protein) | $0.71^{d}$ | $1.45^{bc}$ | $2.28^{a}$ | $1.30^{cd}$ | $2.13^{ab}$ | 0.261 | <0.01 | 0.72 |
| Lactase (mU/mg protein) | 957 | 76 | 124 | 194 | 113 | 250 | 0.06 | 0.62 |
| Cecum pH | $6.38^{abc}$ | $6.44^{a}$ | $6.21^{bc}$ | $6.38^{ab}$ | $6.15^{c}$ | 0.14 | 0.05 | 0.49 |
| Colon pH | $6.69^{a}$ | $6.38^{b}$ | $6.38^{b}$ | $6.50^{ab}$ | $6.35^{b}$ | 0.08 | 0.03 | 0.69 |

[1]Values represent least-square means, n=10.
[a-d]Means within a row lacking a common superscript differ (P < 0.05).

**Table 6. Systemic and ileal immunologic changes due to supplementing *B. breve* AH1205 to suckling piglets[1].**

| | | | *B. breve* AH1205 (CFU/d) | | | | | |
|---|---|---|---|---|---|---|---|---|
| | NB | SR | 0 | $10^9$ | $10^{11}$ | SEM | *P* | *Linear P* |
| Serum | | | | | | | | |
| IgM (μg/mg serum protein) | 51.4[a] | 10.0[b] | 11.2[b] | 13.0[b] | 10.8[b] | 3.7 | <0.01 | 0.77 |
| IgG (μg/mg serum protein) | 418[a] | 104[b] | 123[b] | 118[b] | 128[b] | 17 | <0.01 | 0.79 |
| Ileal | | | | | | | | |
| IEL (IEL/100 enterocytes) | 5[b] | 5[b] | 13[a] | 9[a] | 13[a] | 2 | <0.01 | 0.92 |
| MPO (mole/g protein) | 8.38 | 5.04 | 11.32 | 8.34 | 8.02 | 1.77 | 0.17 | 0.28 |
| TNFα (copy number/100ng cDNA) | 236 | 487 | 255 | 245 | 246 | 73 | 0.08 | 0.94 |
| IL-8 (copy number/100ng cDNA) | 4651 | 2271 | 3758 | 2884 | 2611 | 687 | 0.09 | 0.31 |
| IL-10 (copy number/100ng cDNA) | 341 | 26021 | 1116 | 38331 | 11464 | 15857 | 0.36 | 0.66 |
| IL-1β (copy number/100ng cDNA) | 515 | 579 | 667 | 332 | 296 | 197 | 0.61 | 0.28 |

[1]Values represent least-square means, n=10.
[a-b]Means within a row lacking a common superscript differ (P < 0.05).

**Table 7. Digesta bacteria changes after feeding *B. longum* AH1206 to suckling piglets[1].**

| | *B. longum*AH1206 (CFU/d) | | | | | |
|---|---|---|---|---|---|---|
| | 0 | $10^9$ | $10^{11}$ | SEM | *P* | *Linear P* |
| | Cultured Bacteria | | | | | |
| Day 0, CFU/g Feces | | | | | | |
| Bifidobacteria | 1.35E+10 | 3.96E+10 | 2.24E+09 | 1.48E+10 | 0.20 | 0.55 |
| Lactobacilli | 9.86E+07 | 144590728 | 1.40E+08 | 8.12E+07 | 0.91 | 0.71 |
| Total Anaerobes | 7.69E+09 | 4.44E+10 | 5.46E+09 | 1.87E+10 | 0.27 | 0.93 |
| Day 7, CFU/g Feces | | | | | | |
| Bifidobacteria | 6.96E+09 | 3.18E+10 | 4.57E+09 | 1.51E+10 | 0.33 | 0.91 |
| Lactobacilli | 1.054E+09 | 2.97E+09 | 2.171E+09 | 775220834 | 0.22 | 0.28 |
| Total Anaerobes | 8.81E+09 | 1.02E+10 | 2.07E+10 | 1.22E+10 | 0.73 | 0.47 |
| Day 14, CFU/g Feces | | | | | | |
| Bifidobacteria | 6.02E+09 | 7.30E+10 | 2.13E+11 | 9.05E+10 | 0.27 | 0.11 |
| Lactobacilli[2] | 2.10E+08 | 3.32E+09 | 1.55E+11 | 7.50E+10 | 0.24 | 0.12 |
| Total Anaerobes | 9.11E+09 | 2.38E+10 | 2.21E+11 | 1.15E+11 | 0.36 | 0.20 |
| Day 20, CFU/g Cecal Digesta | | | | | | |
| Bifidobacteria | 3.03E+11[a] | 1.25E+11[ab] | 4.92E+10[b] | 7.60E+10 | 0.05 | 0.02 |
| Lactobacilli | 1.61E+10 | 1.64E+10 | 1.85E+10 | 4.47E+09 | 0.90 | 0.67 |
| Total Anaerobes | 3.521E+10 | 3.794E+10 | 5.47E+10 | 9.62E+09 | 0.28 | 0.14 |
| | Quantitative PCR (cells/g cecal digesta) | | | | | |
| Total Bifidobacteria | 1.65E+07 | 4.04E+05 | 2.11E+06 | 1.00E+07 | 0.46 | 0.33 |
| *B. longum*[3] | nd | 1.17E+05[b] | 2.44E+05[a] | 1.62E+04 | <0.01 | ------ |

[1]Values represent least-square means, n=10.
[2]$N = 6$.
[3]One control animal had observable *B. longum* in the digesta. The result was confirmed by isolating DNA two separate times. This animal has been removed from the data set.
[a-b]Means in a row without a common letter differ ($P < 0.05$).

**Table 8. Effects of feeding *B. longum* AH1206 on organ histology of suckling piglets[1].**

| | | *B. longum* AH1206 (CFU/d) | | | | |
|---|---|---|---|---|---|---|
| | SR | 0 | $10^9$ | $10^{11}$ | SEM | *P* |
| Liver[2] | 1.79[b] | 2.98[a] | 2.87[a] | 3.18[a] | 1.16 | <0.01 |
| Spleen[3] | 1.00 | 1.00 | 1.00 | 1.00 | 0.34 | 1.00 |
| Thymus eosinophilic infiltrates[4] | 0.051 | 0.017 | 0.028 | 0.025 | 0.671 | 0.45 |
| Thymus[5] | 1.00 | 1.00 | 1.00 | 1.00 | 0.34 | 1.00 |
| Pancreas[5] | 1.00 | 1.00 | 1.00 | 1.00 | 0.34 | 1.00 |
| Kidney[5] | 0.99 | 1.09 | 0.98 | 1.09 | 0.05 | 0.22 |
| Heart[5] | 1.10 | 1.00 | 0.99 | 1.00 | 0.05 | 0.26 |
| Kidney cysts[6] | 1.09 | 1.09 | 1.20 | 0.99 | 0.08 | 0.36 |

[1]Values represent least-square means, n=10.
[2]Scoring included: 0; no vacuolization, 1; minimal irregular clear vacuoles, zonal, 2; mild irregular clear vacuolization, sublobular to diffuse, 3; moderate vacuolization, 4; marked vacuolization, 5; marked vacuolization with cell distention.
[3]Extramedullary hematopoiesis scored as 1 if normal (absent to minimal), scored as a 2 if mild.
[4]Eosinophilic aggregates with the medulla of thymic follicles scoring included: 0; absent, 1; minimal foci of eosinophils, 2; milk foci of eosinophils.
[5]Organs without lesions received a score of 1 while those with lesions were graded as a 2. Scores were averaged.
[6]Renal cysts socred as 1; absent, 2; present.
[a-b]Means within a row lacking a common superscript differ ($P < 0.05$).

**Table 9. Hematologic profiles of pigs fed formula containing incremental *B. longum* AH1206 versus sow-reared (SR) and newborn (NB) references.**

| | | | *B. longum* AH1206 (CFU/d) | | | | | |
|---|---|---|---|---|---|---|---|---|
| | NB | SR | 0 | $10^9$ | $10^{11}$ | SEM | *P* | *Linear P* |
| Glucose (mg/dL) | 108[b] | 142[a] | 140[a] | 139[a] | 141[a] | 7.63 | 0.01 | 0.95 |
| BUN[2] (mg/dL) | 6.31[b] | 9.20[b] | 15.00[a] | 13.97[a] | 14.70[a] | 1.32 | <0.01 | 0.83 |
| Creatine (mg/dL) | 0.57[c] | 0.85[a] | 0.72[b] | 0.71[b] | 0.73[b] | 0.03 | <0.01 | 0.83 |
| Total Protein (g/dL) | 5.12 | 5.22 | 4.96 | 4.81 | 4.68 | 0.2 | 0.21 | 0.09 |
| Albumin (g/dL) | 1.14[c] | 3.64[a] | 3.25[b] | 3.09[b] | 3.10[b] | 0.11 | <0.01 | 0.17 |
| Total Bilirubin (mg/dL) | 0.05[b] | 1.04[a] | 0.16[b] | 0.13[b] | 0.12[b] | 0.25 | 0.01 | 0.05 |
| Alkaline Phosphatase (U/L) | 1425[c] | 926[b] | 630[c] | 800[bc] | 761[bc] | 106.3 | <0.01 | 0.09 |
| ALT[3] (U/L) | 28.3[ab] | 28.6[a] | 26.0[ab] | 20.5[b] | 21.1[b] | 2.95 | 0.10 | 0.07 |
| AST[4] (U/L) | 24.9[b] | 38.6[ab] | 46.7[a] | 30.6[ab] | 45.6[a] | 6.74 | 0.13 | 0.91 |
| Cholesterol (mg/dL) | 98[b] | 300[a] | 127[b] | 110[b] | 109[b] | 17 | <0.01 | 0.09 |
| Ca (mg/dL) | 10.6[b] | 11.3[a] | 11.8[a] | 11.6[a] | 11.8[a] | 0.24 | <0.01 | 0.81 |
| P (mg/dL) | 8.32[c] | 11.11[b] | 12.74[a] | 12.42[a] | 12.68[a] | 0.3 | <0.01 | 0.84 |
| Na (mEq/L) | 143[a] | 140[a] | 135[b] | 139[ab] | 139[ab] | 2 | 0.05 | 0.13 |
| K (mEq/L) | 7.55[c] | 9.85[b] | 12.69[a] | 12.31[a] | 11.90[a] | 0.46 | 0.23 | 0.12 |
| Cl (mEq/L) | 105 | 103 | 103 | 103 | 103 | 0.98 | 0.56 | 0.83 |
| Na:K | 28.7[a] | 14.7[b] | 11.0[b] | 11.5[b] | 11.9[b] | 4.1 | 0.02 | 0.07 |
| Albumin:Globulin | 0.32[c] | 2.34[a] | 1.94[b] | 1.81[b] | 1.97[b] | 0.09 | <0.01 | 0.76 |
| BUN:Creatine | 11.5[b] | 10.5[b] | 22.1[a] | 20.4[a] | 20.2[a] | 2 | <0.01 | 0.48 |
| Globulin (g/dL) | 3.95[a] | 1.59[b] | 1.71[b] | 1.72[b] | 1.58[b] | 0.15 | <0.01 | 0.17 |

[1]Values represent least-square means, n=10.
[2]Blood urea nitrogen
[3]Alanine aminotransferase
[4]Aspartate aminotransferase
[a-c]Means within a row lacking a common superscript differ ($P < 0.05$).

**Table 10. Complete blood count of pigs fed formula containing incremental *B. longum* AH1206 versus sow-reared (SR) and newborn (NB) reference pigs[1].**

| | | | *B. longum*AH1206 (CFU/d) | | | | | |
|---|---|---|---|---|---|---|---|---|
| | NB | SR | 0 | $10^9$ | $10^{11}$ | SEM | *P* | *Linear P* |
| | | | | Complete Blood Count | | | | |
| Hemoglobin (g/dL) | 10.8 | 11.7 | 12 | 11.8 | 11.4 | 0.7 | 0.69 | 0.40 |
| Hematocrit (%) | 34.7 | 36.9 | 39.4 | 39 | 37.1 | 2.3 | 0.52 | 0.29 |
| White Blood Count ($10^3$/μL) | 7.9 | 7.5 | 9.9 | 9.3 | 8.2 | 0.9 | 0.14 | 0.17 |
| Red Blood Count ($10^6$/μL) | 5.6 | 5.4 | 5.7 | 5.7 | 5.5 | 0.4 | 0.97 | 0.67 |
| MCV[2] (fL) | 63.6 | 68.7 | 69.8 | 69.1 | 68.1 | 1.8 | 0.11 | 0.30 |
| MCH[3] (pg) | 19.8 | 21.9 | 21.3 | 20.8 | 20.9 | 0.6 | 0.10 | 0.46 |
| MCHC[4] (g/dL) | $31.2^{ab}$ | $31.9^{a}$ | $30.5^{bc}$ | $30.2^{c}$ | $30.6^{bc}$ | 0.3 | <0.01 | 0.40 |
| Neutrophils ($10^9$/L) | 3951 | 2758 | 3990 | 3535 | 3267 | 615 | 0.43 | 0.41 |
| Lymphocytes ($10^9$/L) | $3623^{b}$ | $4335^{ab}$ | $5201^{a}$ | $5310^{a}$ | $5302^{ab}$ | 485 | 0.07 | 0.20 |

[1]Values represent least-square means, n=10.
[2]Mean corpuscular volume.
[3]Mean corpuscular hemoglobin.
[4]Mean corpuscular hemoglobin concentration.
[a-c] Means within a row lacking a common superscript differ ($P < 0.05$).

**Table 11. Bacterial translocation was not affected by supplementing *B. longum*AH1206 to suckling piglets[1]**

| | *B. longum*AH1206 (CFU/d) | | | | | |
|---|---|---|---|---|---|---|
| % Positive Cultures | 0 | $10^9$ | $10^{11}$ | Standard Deviation | *P* | *Linear P* |
| Anaerobic Bacteria | | | | | | |
| Spleen | 20 | 0 | 10 | 42 | 1.00 | 0.96 |
| Liver | 20 | 11 | 0 | 42 | 1.00 | 1.00 |
| Mesenteric Lymph nodes | 20 | 0 | 10 | 42 | 0.73 | 0.98 |
| Aerobic Bacteria | | | | | | |
| Spleen | 10 | 0 | 0 | 32 | 1.00 | 0.97 |
| Liver | 20 | 0 | 10 | 42 | 1.00 | 0.96 |
| Mesenteric lymph nodes | 20 | 22 | 0 | 44 | 1.00 | 1.00 |

[1]Values represent least-square means, n=10.

**Table 12. Effects of supplementing *B. longum*AH1206 on intestinal morphology, disaccharidases and digesta pH of suckling piglets[1]**

| | | | *B. longum*AH1206 (CFU/d) | | | | | |
|---|---|---|---|---|---|---|---|---|
| | NB | SR | 0 | $10^9$ | $10^{11}$ | SEM | *P* | *Linear P* |
| Jejunum | | | | | | | | |
| Villi Height (μm) | 674[a] | 440[b] | 620[ab] | 681[a] | 770[a] | 71 | 0.03 | 0.22 |
| Villi Width (μm) | 115[c] | 124[cb] | 161[a] | 149[a] | 159[a] | 12 | 0.01 | 0.98 |
| Crypt Depth (μm) | 129 | 98 | 196 | 208 | 297 | 61 | 0.14 | 0.26 |
| Ileum | | | | | | | | |
| Villi Height (μm) | 624 | 457 | 507 | 607 | 528 | 52 | 0.13 | 1.00 |
| Villi Width (μm) | 88[c] | 113[b] | 127[ab] | 140[a] | 130[ab] | 8 | <0.01 | 1.00 |
| Crypt Depth (μm) | 98 | 103 | 100 | 129 | 108 | 11 | 0.26 | 0.71 |
| Maltase (mU/mg protein) | 48[b] | 50[b] | 132[a] | 93[ab] | 75[b] | 20 | 0.01 | 0.10 |
| Lactase (mU/mg protein) | 43.0[a] | 19.4[b] | 11.7[b] | 21.5[b] | 29.9[b] | 4.5 | <0.01 | 0.09 |
| Cecum pH | 6.13[b] | 6.50[a] | 5.97[b] | 6.13[b] | 5.93[b] | 0.121 | <0.01 | 0.20 |
| Colon pH | 6.49 | 6.4 | 6.17 | 6.39 | 6.36 | 0.078 | 0.06 | 0.06 |

[1]Values represent least-square means, n=10.
[a-c]Means within a row lacking a common superscript differ (P < 0.05).

**Table 13. Systemic and ileal immunologic changes due to supplementing *B. longum*AH1206 to suckling piglets[1]**

| | | | *B. longum*AH1206 (CFU/d) | | | | | |
|---|---|---|---|---|---|---|---|---|
| | NB | SR | 0 | $10^9$ | $10^{11}$ | SEM | *P* | *Linear P* |
| Serum | | | | | | | | |
| IgM (μg/mg serum protein) | 31.1[a] | 17.7[b] | 14.7[b] | 14.6[b] | 13.6[b] | 2.3 | <0.01 | 0.54 |
| IgG (μg/mg serum protein) | 415[a] | 127[b] | 149[b] | 146[b] | 127[b] | 21 | <0.01 | 0.09 |
| Ileal | | | | | | | | |
| IEL (IEL/100 enterocytes) | 7 | 14 | 13 | 11 | 13 | 2 | 0.22 | 0.53 |
| MPO (mole/g protein | 15.5 | 21.7 | 9.0 | 14.7 | 13.0 | 6.3 | 0.59 | 0.58 |
| TNFα (copy number/100ng cDNA) | 112 | 186 | 60 | 144 | 126 | 39 | 0.14 | 0.08 |
| IL-8 (copy number/100ng cDNA) | 1363 | 1619 | 1843 | 1946 | 2174 | 404 | 0.62 | 0.63 |
| IL-10 (copy number/100ng cDNA) | 227[b] | 620[a] | 178[b] | 223[b] | 300[b] | 72 | <0.01 | 0.01 |
| IL-1β (copy number/100ng cDNA) | 78 | 159 | 75 | 72 | 180 | 79 | 0.70 | 0.30 |

[1]Values represent least-square means, n=10.
[a-b]Means within a row lacking a common superscript differ (P < 0.05).

**Supplementary Tables and Figures**

**Supplementary Table 1. Basal Diet composition (provided by Milk Specialties, Dundee, IL).**

| Ingredient | Percentage |
|---|---|
| Na Caseinate | 11.25 |
| Delactosed Whey | 18.11 |
| Dicalcium Phosphate | 1.88 |
| Calcium Chloride | 0.33 |
| Vitamin E Premix | 0.04 |
| Mineral Premix | 0.50 |
| Vitamin Premix | 0.08 |
| Artificial Flavor | 0.03 |
| Potassium Sorbate | 0.45 |
| D,L Methionine | 0.49 |
| Whey | 28.59 |
| Whey Protein Concentrate | 18.15 |
| Edible Lard | 19.39 |
| Sodium Hexametaphosphate | 0.18 |
| Antioxidant | 0.01 |
| Flow Agent | 0.14 |
| Emulsifier | 0.22 |
| Lecithin | 0.19 |
| TOTAL | 100.00 |

**Supplementary Table 2. Relative weights[1] of organs and digesta pH from pigs fed formula contain probiotics versus sow-reared (SR) and newborn (NB) reference pigs[2].**

| | | | *B. breve*AH1205 (CFU/d) | | | | | |
|---|---|---|---|---|---|---|---|---|
| Organ | NB | SR | 0 | $10^9$ | $10^{11}$ | SEM | *P* | *Linear P* |
| Gallbladder | 0.055 | 0.076 | 0.087 | 0.084 | 0.088 | 0.011 | 0.20 | 0.84 |
| Liver | 3.22[a] | 2.30[b] | 3.13[b] | 3.13[b] | 3.16[b] | 0.16 | <0.01 | 0.90 |
| Kidney | 0.488[a] | 0.319[b] | 0.446[a] | 0.409[a] | 0.380[a] | 0.027 | <0.01 | 0.10 |
| Spleen | 0.175 | 0.184 | 0.18 | 0.178 | 0.165 | 0.012 | 0.78 | 0.27 |
| Pancreas | 0.111[b] | 0.102[b] | 0.167[a] | 0.155[a] | 0.159[a] | 0.011 | <0.01 | 0.64 |
| Thymus | 0.099 | 0.059 | 0.085 | 0.08 | 0.079 | 0.011 | 0.20 | 0.60 |
| Lung | 1.73[a] | 0.486[b] | 0.542[b] | 0.530[b] | 0.512[b] | 0.073 | <0.01 | 0.18 |
| Heart | 0.823[a] | 0.674[b] | 0.599[c] | 0.607[c] | 0.589[c] | 0.027 | <0.01 | 0.49 |
| Eye | 0.176[a] | 0.047[b] | 0.039[b] | 0.041[b] | 0.042[b] | 0.006 | <0.01 | 0.64 |
| Cecum | 0.054[c] | 0.085[b] | 0.131[a] | 0.111[a] | 0.111[a] | 0.007 | <0.01 | 0.05 |
| Colon | 0.411[b] | 0.435[b] | 0.542[a] | 0.556[a] | 0.574[a] | 0.029 | <0.01 | 0.40 |

[1]Organ weights are percentage of body weights.
[2]Values represent least-square means, n=10.
[a-c]Means within a row lacking a common superscript differ ($P < 0.05$).

**Supplementary Table 3. Feeding *B. breve* AH1205 did not affect organ histology[1].**

| | | *B. breve*AH1205 (CFU/d) | | | | |
|---|---|---|---|---|---|---|
| | SR | 0 | $10^9$ | $10^{11}$ | SEM | *P* |
| Liver[2] | 3.19 | 2.77 | 2.69 | 2.92 | 0.08 | 0.35 |
| Spleen[3] | 3.20 | 3.10 | 3.10 | 3.00 | 0.03 | 0.57 |
| Thymus[4] | 0.00 | 1.00 | 1.00 | 1.00 | 0.37 | 1.00 |
| Pancreas[4] | 1.00 | 1.11 | 1.00 | 1.00 | 0.04 | 0.24 |
| Kidney[4] | 1.19 | 1.08 | 1.18 | 1.03 | 0.09 | 0.53 |
| Heart[4] | 0.99 | 1.09 | 1.29 | 1.09 | 0.09 | 0.15 |
| | | | Mesenteric lymph nodes | | | |
| Cellularity[3] | 3.69[a] | 3.11[b] | 3.09[b] | 2.79[b] | 0.04 | <0.01 |
| Follicles[5] | 1.43 | 1.90 | 1.53 | 1.91 | 0.15 | 0.39 |

[1]Values represent least-square means, n=10.
[2]Scoring included: 0; no vacuolization, 1; minimal irregular clear vacuoles, zonal, 2; mild irregular clear vacuolization, sublobularto diffuse, 3; moderate vacuolization, 4; marked vacuolization, 5; marked vacuolization with cell distention.
[3]The scoring scheme used was : 0- absence of lymphocytes, 1-depletion of lymphocytes,very low numbers of lymphocytes, 2 -low numbers of lymphocytes to an abnormal extent> 30% and < 60% lymphocytes cover surface area, 3- moderate cellularity > 60% < 80% of surface area covered with lymphocytes, 4- abundant cellularity >80% surface area covered by lymphocytes, node size within normal limits, 5->90% of surface area covered with lymphocytes and node enlarged, 6- lymphoma.
[4]Organs without lesions received a score of 1 while those with lesions were graded as a 2. Scores were averaged.
[5]Follicular activity-0; absence of active follicles, 1; 1-3 active follicles per node section evaluated, 2;3-6 active follicles per node section evaluated, 3;6-9 active follicles per node section evaluated, 4;9-12 active follicles per node section evaluated. Scoring for the spleen was: 0; absence of lymphocytes, 1; marked depletion of lymphocytes, 2; mild depletion of lymphocytes, 3; lymphocytes within normal limits, 4; lymphocytes within normal limits, but with periarteriolar lymphoid sheath (PALS) in opposition, 5; Lymphocyte hyperplasia, 6; lymphoma.
[a-c]Means within a row lacking a common superscript differ ($P < 0.05$).

**Supplementary Table 4. Whole blood hematology of pigs fed formula containing incremental *B. breve*AH1205 versus sow-reared (SR) and newborn (NB) pigs[1].**

| | | | *B. breve*AH1205 (CFU/d) | | | | | |
|---|---|---|---|---|---|---|---|---|
| | NB | SR | 0 | $10^9$ | $10^{11}$ | SEM | *P* | *Linear P* |
| Complete Blood Count | | | | | | | | |
| Hemoglobin (g/dL) | 8.14[b] | 11.72[a] | 12.53[a] | 11.89[a] | 12.40[a] | 0.34 | <0.01 | 0.31 |
| Hematocrit (%) | 25.9[b] | 37.6[a] | 40.7[a] | 38.3[a] | 39.9[a] | 1.2 | <0.01 | 0.26 |
| White Blood Count ($10^3$/μL) | 5.98[b] | 10.00[a] | 11.78[a] | 12.86[a] | 11.09[a] | 1.16 | <0.01 | 0.56 |
| Red Blood Count ($10^6$/μL) | 3.91[b] | 6.61[a] | 6.46[a] | 6.12[a] | 6.43[a] | 0.22 | <0.01 | 0.41 |
| MCV[2] (fL) | 67 | 61.1 | 63.3 | 62.7 | 62.2 | 1.5 | 0.07 | 0.62 |
| MCH[3] (pg) | 20.9[a] | 19.0[b] | 19.4[b] | 19.5[b] | 19.4[b] | 0.3 | 0.01 | 0.93 |
| MCHC[4] (g/dL) | 32 | 31.2 | 30.8 | 31.1 | 31.2 | 0.5 | 0.53 | 0.29 |
| Platelet Count ($10^3$/μL) | 279[b] | 563[a] | 466[a] | 470[a] | 423[a] | 41 | <0.01 | 0.59 |
| Neutrophils ($10^9$/L) | 61.3[a] | 66.1[a] | 43.4[b] | 40.5[b] | 46.5[b] | 3.1 | <0.01 | 0.42 |
| Lymphocytes ($10^9$/L) | 33.9[a] | 28.7[a] | 48.3[b] | 53.5[b] | 46.4[b] | 3 | <0.01 | 0.28 |
| Urinalysis | | | | | | | | |
| pH | 5.61[b] | 6.02[b] | 7.51[a] | 7.81[a] | 7.71[a] | 0.34 | <0.01 | 0.31 |
| Specific Gravity | 1.03 | 1.01 | 1.07 | 1.02 | 1.01 | 0.04 | 0.56 | 0.52 |

[1]Values represent least-square means, n=10. [2]Mean corpuscular volume. [3]Mean corpuscular hemoglobin. [4]Mean corpuscular hemoglobin concentration. [a-b] Means within a row lacking a common superscript differ (P < 0.05).

**Supplementary Table 5. Relative weights[1] of organs and digesta pH from pigs fed formula contain probiotics versus sow-reared (SR) and newborn (NB) reference pigs[2].**

| | | | *B. longum*AH1206 (CFU/d) | | | | | |
|---|---|---|---|---|---|---|---|---|
| | NB | SR | 0 | $10^9$ | $10^{11}$ | SEM | *P* | *Linear P* |
| Liver | 3.02[b] | 2.50[c] | 3.57[a] | 3.58[a] | 3.63[a] | 0.116 | <0.01 | 0.91 |
| Kidney | 0.418[a] | 0.298[b] | 0.419[a] | 0.413[a] | 0.413[a] | 0.015 | <0.01 | 0.71 |
| Spleen | 0.146[b] | 0.210[a] | 0.186[a] | 0.194[a] | 0.201[a] | 0.01 | 0.00 | 0.43 |
| Gallbladder | 0.114 | 0.041 | 0.078 | 0.082 | 0.131 | 0.04 | 0.37 | 0.91 |
| Heart | 0.733[a] | 0.604[b] | 0.673[b] | 0.611[b] | 0.608[b] | 0.023 | 0.00 | 0.33 |
| Pancreas | 0.129[b] | 0.132[b] | 0.196[a] | 0.195[a] | 0.186[a] | 0.012 | <0.01 | 0.70 |
| Brain | 1.926[a] | 0.608[b] | 0.604[b] | 0.583[b] | 0.595[b] | 0.057 | <0.01 | 0.11 |
| Left Eye | 0.144 | 0.044 | 0.042 | 0.043 | 0.044 | 0.006 | 0.22 | 0.57 |
| Left Lung | 0.567[a] | 0.497[b] | 0.452[c] | 0.460[bc] | 0.483[bc] | 0.016 | <0.01 | 0.97 |
| Left Thymus | 0.045[b] | 0.083[a] | 0.059[b] | 0.061[b] | 0.052[b] | 0.006 | 0.00 | 0.89 |
| Empty Cecum | 0.071[b] | 0.098[a] | 0.114[a] | 0.115[a] | 0.111[a] | 0.008 | 0.00 | 0.74 |
| Empty Colon | 0.512[b] | 0.499[b] | 0.783[a] | 0.746[a] | 0.817[a] | 0.044 | <0.01 | 0.65 |

[1]Organ weights are percentage of body weights.
[2]Values represent least-square means, n=10.
[a-c]Means within a row lacking a common superscript differ ($P < 0.05$).

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## SUMMARY

The current definition of a prebiotic is "a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microbiota that confers benefits upon host well-being and health" (1). Very few feed additives have been identified as true prebiotics. Probiotics have been defined as "living microorganisms that, on ingestion in sufficient numbers, exert health benefits beyond basic nutrition" (2). Probiotic microorganisms delivered via food or dairy products are mainly members of *Lactobacillus* and *Bifidobacteria* genus. Prebiotic and probiotics are similar in that they exert their effects via changes in the intestinal microbiota. Prebiotics change the microbiota via the introduction of a substrate, while probiotics change the microbiota by sheer numbers. The three major activities prebiotic and probiotics are thought to participate in are 1) changing the microbiota; 2) fermentation producing organic acids; 3) producing a health benefit to their host.

Stabilized rice bran (SRB) and polydextrose (PDX) were investigated as prebiotics. SRB was found to increase colonic bifidobacteria in weaning pigs. Bifidobacteria was increased, but fermenative products were not different than control pigs. Although the full prebiotic criteria was not meet, SRB improved feed efficiency and tended to increase colonic bifidobacteria. PDX fulfilled the prebiotic criteria of fermentation by intestinal microflora by increasing intestinal lactobacillus, increasing the fermentative product lactic acid and decreasing pH in suckling swine. Changes in intestinal cytokine message was also observed

with PDX supplementation. The implications of changing cytokine message on health and wellbeing was not identified. Both SRB and PDX fulfilled partial criteria of a prebiotic.

Two novel probiotic strains, *B. breve* AH1205 (BB) and *B. longum* AH1206 (BL), were investigated on the health, growth and development of neonatal pigs as a surrogate for human infants. BB was shown to be viable in the cecum, while the viability of BL could not be concluded. BL increased the expression of the cytokines TNFα and IL-10. The increased expression of these cytokines indicate an improvement in the maturation of the intestinal immune system to overcome the Th2 biases at birth and decrease the risk to bacterial infection. Feeding BB and BL in infant formula may change the microbiota of formula-fed infants to be more similar to that of breast-fed infants. Feeding the novel *Bifidobacterium* strains BB and BL in neonatal diets is safe and causes differential intestinal effects, but specific effects on long term health and well being were not investigated.

The future direction of research into the pre- and probiotic characteristics of SRB, PDX, BB and BL must include investigations into long term health effects and synergistic effects of feeding the prebiotics and probiotics together. All feed additives have been shown to meet some of the criteria of the biotic definition via changes in the intestinal microbiotia. How these changes in the intestinal microbiota affect health still need to be eluded. Effectiveness of the probiotics may be improved with the addition of a substrate in the form of a supplemented prebiotic. To create stable, long term changes in the intestinal microbiota of young animals and the neonate, a strategy of feeding prebiotics and probiotics together may offer more substantial changes than that observed when feeding the additives singularly.

SRB improved feed efficiency in weaning swine and tended to increase colonic bifidobacteria. The prebiotic PDX was also observed to be a safe feed additive in the suckling swine neonate model and changed the intestinal microbiotia via increased lactobacillus with increasing lactobacillus, a fermentative by-product. The novel probiotics BB and BL were demonstrated to be safe feed additives in the suckling swine neonate model and changes to the intestinal microbiotia via increases in beneficial Bifidobacteria. Although the additives were not shown to improve health, future research is warranted based on these observations of prebiotic and probiotic characteristics of SRB, PDX, BB and BL.

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