

The Safety and Adequacy of Galactooligosaccharides and Fructooligosaccharides in Infant Pig Formula

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Thesis submitted to the faculty of The Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree
of

Master of Science
In
Animal and Poultry Sciences

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July 29, 2014
Blacksburg, VA

Keywords: oligosaccharides, pigs, growth, microbiota
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ABSTRACT

Breast milk remains the optimum vehicle to deliver high quality nutrients, including oligosaccharides, in quantities sufficient to sustain normal growth, however, it is currently unknown whether the addition of prebiotics to infant formula would alter neonate growth and development. The objective of this study was to determine the effect of Galactooligosaccharides (GOS) and Fructooligosaccharides (FOS) supplementation in nursery pig diets on growth and efficiency of food utilization. Forty-eight 4-day old crossbred pigs ($1.628 \pm .037$ Kg BW) were randomly assigned to 1 of 8 diets: 1) milk-based formula containing Type 2 GOS and Type 1 FOS (4 + 1 g/L); 2) soy-based formula containing Type 1 FOS (3g/L); 3) milk-based formula with no prebiotic added; 4) milk-based formula containing Type 2 GOS only (5g/L); 5) milk-based formula containing Type 1 GOS only (5g/L); 6) milk-based formula containing Type 2 GOS and Type 2 FOS (4 + 1 g/L); 7) milk-based formula containing Type 1 GOS and Type 1 FOS (4 + 1 g/L); 8) soy-based formula with no prebiotic added. Diets were isonitrogenous, isocaloric and fed at 250 mL/kg body for a 2-week period. At sacrifice, blood and tissue samples were collected for analysis. A diet by time interaction ($P < 0.001$) indicated a smaller rate of accretion in bone mineral content for soy-based diets. Total bacteria and lactobacillus were significantly affected by treatment ($P < 0.001$). In conclusion, the addition of GOS and FOS to formula does not appear to alter growth however, the gut microbiota was significantly modified.

Keywords: oligosaccharides, pigs, growth, microbiota

ACKNOWLEDGEMENTS

Several people contributed to the completion of the research described in this thesis.
Descriptions of their contributions are included below.

Robert P. Rhoads – Ph.D. (Department of Animal and Poultry Sciences, Virginia Tech). Primary advisor and committee chair. Performed animal surgeries. Actively involved in implementation of thesis research project. Provided feedback and guidance throughout duration of project.

Samer W. El-Kadi – Ph.D. (Department of Animal and Poultry Sciences, Virginia Tech) Committee member. Performed animal surgeries. Actively involved in implementation of thesis research project. Provided feedback and guidance throughout duration of the project.

Monica A. Ponder – Ph.D. (Department of Food Science and Technology, Virginia Tech) Committee member. Actively involved in execution of gut microbiota analysis. Provided feedback and guidance throughout duration of project.

Patricia Williams – (Department of Animal and Poultry Sciences, Virginia Tech) Laboratory specialist. Assisted with DEXA scans, ordering of supplies, usage and maintenance of all animal and surgery rooms.

Diane Bourne – (Department of Food Science and Technology, Virginia Tech) Lab research specialist. Assisted with all aerobic and anaerobic bacteria culturing.

Guehao Xie – Fellow graduate student in Dr. Rhoads lab. Assisted with various aspects of the animal trial. Performed animal care and assisted with sample collection.

Lidan Zhao – Postdoctoral scholar in Dr. Rhoads lab. Assisted with various aspects of the animal trial. Performed animal care and assisted with sample collection.

Haibo Zhu – Fellow graduate student in Dr. El-Kadi's lab. Assisted with various aspects of the animal trial. Performed animal care and assisted with sample collection.

Courtney Klotz – M.S. (Food Science and Technology). Assisted with bacteria culturing and gut microbiota analysis.

Kirsten Seelenbinder – Fellow graduate student. Assisted with animal surgeries and sample collection.

Undergraduates:

Sara Seeba (Virginia Tech)
Alumbria Gates (Virginia Tech)
Rosalie Petrone (Virginia Tech)
Kalynn Harbw (Virginia Tech)

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List of Abbreviations

ACC – Acetyl-CoA carboxylase

ADG – Average daily gain

AST(GOT) – Aspartate aminotransferase (glutamic oxaloacetic transaminase)

BMC – Bone mineral content

BW – Body weight

CK – Creatine Kinase

CV – Coefficient of variation

DEXA – Dual energy x-ray absorptiometry

EDTA – Ethylenediaminetetraacetic acid

ELISA – Enzyme-linked immunosorbent assay

FAS – Fatty acid synthase

FOS – Fructooligosaccharides

G-6-PDH – Glucose 6-phosphate dehydrogenase

GGT – Gamma-glutamyl transferase

GI – Gastrointestinal

GIT – Gastrointestinal tract

GOS – Galactooligosaccharides

H&E – Hematoxylin and eosinstain

HDL – High density lipoprotein

IL-6 - Interleukin 6

IL-10 – Interleukin 10

lcFOS – Long-chain fructooligosaccharides

LDH – Lactate dehydrogenase

LPL – Lipoprotein lipase

ME – Malic enzyme

NK cell – Natural killer cell

PCR – Polymerase chain reaction

rRNA – Ribosomal ribonucleic acid

scFOS – Short-chain fructooligosaccharides

scGOS – Short-chain galactooligosaccharides

TDMAC – Tridodecyl methyl ammonium chloride

TNF α – Tumor necrosis factor α

VLDL – Very low-density lipoprotein

CHAPTER I

REVIEW OF LITERATURE

Neonatal nutrition

Breast milk is known to be the most advantageous source of nutrition for growing infants, especially for the first six months of life. Breast-feeding is beneficial not only to the health of the infant but also for the connection between mother and child. However, many factors, such as poor milk volume, poor breast-feeding technique, nipple and breast problems, as well as the emotional compromise of the mother (Callen et al., 2005) can make breast-feeding impossible or impractical and therefore the use of formula becomes necessary. The ability not only to establish breast milk production but also to maintain adequate production in order to meet the nutritional requirements of the infant is a concern for many mothers (Heon et al., 2014). For some mothers, being able to express breast milk without the need for suckling can be difficult (Flaherman and Lee, 2013) while others face the ultimate challenge of insufficient breast milk production, especially mothers of preterm infants (Hill et al., 2005). Mothers who face these challenges often have feelings of separation, stress or anxiety (Chatterton et al., 2000; Zanardo et al., 2011).

While some mothers are unable to breast-feed due to the aforementioned challenges, other mothers simply choose not to breast-feed. According to the Center for Disease Control United States Breastfeeding Report Card for 2014, "Breastfeeding rates continue to rise in the United States, yet breastfeeding did not continue for as long as recommended. Of the infants born in 2011, 49% were breastfeeding at 6 months and 27% at 12 months" (Centers for Disease Control and

Prevention [CDC], 2014). This decision by mothers is of vital importance given that health outcomes are enhanced in breastfed infants compared to infants who receive formula (Froh et al., 2014; CDC, 2014; American Academy of Pediatrics, 2012)

Differences in health between breast-fed and formula fed infants

It is well known that differences in health exist between breast-fed and formula-fed infants. In addition to impacting growth by meeting the nutritional needs of the infant, the components found in human breast milk stimulate cognitive development through healthy neural growth (Deoni et al., 2013), as well as influencing the microbes present within the gut (Harmsen et al., 2000) which have a major role in the overall health of the infant.

Breast fed infants have a gut microbiota with a larger abundance of bifidobacteria (Harmsen et al., 2000) compared to formula-fed infants that typically have more diverse gut microbes and higher levels of clostridia species (Azad et al., 2013) and bacteroides (Penders et al., 2006). Bifidobacteria are extremely beneficial to an infant because they thrive on the components in human milk oligosaccharides. The majority of the health benefits associated with breast-feeding over formula-feeding can be attributed to the differences in the species of bacteria present within in the infant gut.

Breast-feeding has been linked to lower rates of pneumonia, otitis media, bacteremia, meningitis (Beaudry et al., 1995), gastrointestinal infections (Duijts et al., 2010), and respiratory infections (Bachrach et al., 2003; Chantry et al., 2006). Breast-feeding has also shown correlation to fewer occurrences of acute lymphoblastic leukemia and acute myeloblastic leukemia (Kwan et al., 2004) as well

as fewer post neonatal infant deaths (Chen et al., 2004) than in formula-fed infants. Infants who are breast-fed show a lower risk for type 2 diabetes later in life (Owen et al., 2006) as well a lower risk for necrotizing enterocolitis (Sisk et al., 2007). Breast milk fed exclusively for the first few months of life also reduces the occurrence of atopic dermatitis in children at high risk of developing the condition (Gdalevich et al., 2001).

Despite the known health advantages associated with breast-feeding, many infants are still formula-fed. To ensure that formula fed infants grow and develop normally, it is extremely important that the formula consumed resembles human breast milk as closely as possible. Therefore, the discovery of potential ingredients that mimic the natural components of breast milk is crucial. This remains a concern of high priority that drives infant formula manufacturers to find ingredients that parallel the important components of breast milk and can then be used to supplement infant formulas.

Feed additives in infant formula

Prebiotics

Prebiotics are non-digestible food components that benefit the host mainly through alterations of the gut microbiota (Monaco et al, 2011). In order to be classified as a prebiotic, a food ingredient must not be digested within the host's upper digestive tract, reaching the large intestine, where it is available for fermentation by the resident microbiota of the large intestine (Macfarlane et al., 2008). Ideally, prebiotic ingredients will serve as growth substrates for commensal colonic bacteria (Gibson & Roberfroid, 1995). Several food ingredients, such as

certain carbohydrates (oligo- and polysaccharides), peptides, proteins and lipids can be classified as potential prebiotics based on these properties (Gibson & Roberfroid, 1995). While all of these ingredients meet some of the qualifications, currently, only nondigestible carbohydrates, such as oligosaccharides, fulfill all of the requirements of a prebiotic (Gibson & Roberfroid, 1995; de Vrese & Schrezenmeir, 2008). While commercially used prebiotic formulations lack the complexity in structure of the oligosaccharides found in human milk, they differ in composition based on whether they are derived from lactose or plants (Kim et al., 2013).

There is great potential in pre- and probiotics for the improvement of infant formula supplementation that allows for optimal growth and development in insufficient circumstances (Jacobi & Odle, 2012). However, given that prebiotics modify the function of the gut by influencing the bacteria already commensal to the large intestine, prebiotics are more useful and effective at altering the gut microbiota compared to probiotics (Macfarlane et al., 2008). Probiotics affect the host through beneficially improving the balance of the intestinal microbiota through a live microbial food supplement (Collins and Gibson, 1999).

Prebiotics consisting of galactose polymers (galactooligosaccharides) and fructose polymers (fructooligosaccharides) are often supplementary in many foods including infant formulas (Underwood et al., 2014). According to Macfarlane and colleagues, “prebiotics have been used in infant formulas in Japan for over 20 years, and have been available in Europe for the last 5 years” (Macfarlane et al., 2008) due to the beneficial effects they confer on their host.

Oligosaccharides
Human milk oligosaccharides

Human milk is comprised of protein, fats, carbohydrates, which are calculated as lactose, mineral constituents in the form of ash, as well as oligosaccharides (Jenness, 1979). During infancy in mammals, oligosaccharides in milk serve as natural sources of a prebiotic due to a variety of factors, such as escaping digestion in the upper digestive tract, and reaching the large intestine where it is known to influence the microbiota of the gastrointestinal tract (Boehm & Stahl, 2007). Human milk oligosaccharides are prominent among the functional components of human breast milk (Intanon et al., 2014). They represent the third component of breast milk, following lactose and lipids (Coppa et al., 2006; Williams et al., 2014) with concentrations of $\sim 15\text{g/L}^{-1}$ (Kim et al., 2013). However, this amount will steadily decrease in the first few months after birth (Macfarlane et al., 2008).

There are approximately 200 molecular species of oligosaccharides smaller than 20 carbohydrate units known to be present in human milk (Ninonuevo et al., 2006; Herfel et al., 2011; Rudloff & Kunz, 2012; Williams et al., 2014). Neutral oligosaccharides make up a larger proportion of milk (1%) than acidic oligosaccharides (0-1%) (Macfarlane et al., 2008). The prebiotic effect of human milk can be mainly credited to the oligosaccharide component (Boehm & Stahl, 2007). Due to this prebiotic effect, it is speculated that human milk oligosaccharides are significant in the maintenance and development of the immune system and gastrointestinal tract of breast-fed infants (Williams et al., 2014).

Human milk oligosaccharides have a variety of structure and are comprised of five monosaccharides: D-glucose, D-galactose, L-fucose, N-acetylglucosamine, and N-acetylneuramic acid (Macfarlane et al., 2008; Kim et al., 2013; Goehring et al.,

2014). They are synthesized in the breast beginning at the reducing end with lactose, which is then repetitively attached by β 1-3 or β 1-4 glycosidic linkages to galactose and N-acetylglucosamine to make up the various core molecule structures (Boehm & Stahl, 2007; Kim et al., 2013). More variety in structure occurs when neutral oligosaccharides (fucose) or acidic oligosaccharides (N-acetylneuramic acid/sialic acid) are added to the core molecule through α -glycosidic linkages (Boehm & Stahl, 2007; Kim et al., 2013). The prebiotic effect of human milk oligosaccharides can be attributed to the lack of human enzymes with the capability of hydrolyzing the β -glycosidic linkages, which allows the β -glycosidically bound galactose to escape digestion in the upper GIT and arrive at the lower GIT (Boehm & Stahl, 2007; Kim et al., 2013).

Domestic animal milk oligosaccharides

Oligosaccharides are present in much lower concentrations in the milk of domestic animal species compared to that of human breast milk. Domestic animal milk oligosaccharides lack the complexity of the oligosaccharides in human milk due to differences in their composition. Compared to the 200 molecular species found in human milk oligosaccharides, porcine milk oligosaccharides only have 29 identified molecular species (Tao et al., 2010). Similar to human milk, domestic animal milk contains both neutral and acidic oligosaccharides where sialic acid is predominant in the acidic fraction (Boehm and Stahl, 2007), specifically in porcine milk where more than 50% of the oligosaccharides are sialylated (Tao et al., 2010). Linkages of N-acetylglucosamine or galactose are dominant in the neutral portion of

oligosaccharides in animal milk compared to the few linkages to fucose (Boehm and Stahl, 2007).

Natural sources of oligosaccharides identical to those found in human milk are difficult to find due to the complexity of their structure and therefore other sources must be considered (Boehm and Stahl, 2007). These sources, while different in structure must have biological functions similar to those found in human milk, especially the prebiotic effect (Boehm and Stahl, 2007). Mixtures of Galactooligosaccharides and Fructooligosaccharides have demonstrated an ability to mimic this prebiotic effect (Moro et al., 2007).

Galactooligosaccharides and Fructooligosaccharides

Galactooligosaccharides (GOS) are nondigestible carbohydrates synthesized from either the transgalactosylation of lactose by β -galactosidases (Intanon et al., 2014) or soya beans (Sangwan et al., 2011). The GOS synthesized from lactose found in cow's milk is comparable to the naturally occurring galactose-based human milk oligosaccharides, which makes them useful primarily in infant formulas (Sangwan et al., 2011). The addition of GOS only as well as mixtures of GOS and other prebiotics at doses of 2 to 8g/L of formula and has shown to be a safe addition to infant formula (Monaco et al., 2011).

The main health benefit associated with GOS is their ability to increase numbers of bifidobacteria as well as lactobacillus (Ben et al., 2008; Macfarlane et al., 2008; Fanaro et al., 2009; Davis et al., 2010). Given that GOS have been shown to modulate the gut microbiota, mainly by increasing the numbers of bifidobacteria, it

has been used in pediatric nutrition as a prebiotic supplement with growing evidence to support its use in infant formula (Monaco et al., 2011).

There are currently several different forms of commercially available GOS that are used in infant formula. One form is known as Vivinal GOS, which can come as a syrup containing GOS, glucose, lactose, and galactose, or in powder form containing protein or no protein (Tzortzis, 2009). Bimuno GOS is another commercially available type of GOS made by Clasado Ltd. that comes in either powder or syrup form and is produced from enzymes of *Bifidobacterium bifidum* (Tzortzis, 2009).

Fructooligosaccharides (FOS) are oligosaccharides derived from plants such as chicory, garlic, banana, onions, asparagus, wheat artichoke, etc. (Sabeter-Molina et al., 2009). FOS can also be derived from β -D-fructofuranosidase or fructosyltransferase enzyme activity on sucrose and inulin (Bali et al., 2012). Chemically, the structures of FOS are formed from a glucose molecule bound to two, three, or four molecules of fructose (Rivero-Urgell and Santamaria-Orleans, 2001) bound by β 2-1 osidic linkages which mammalian enzymes do not hydrolyze (Rumessen et al., 1990). These linkages allow FOS to escape digestion in the upper intestine and be unaffected by pancreatic and intestinal enzymes in the small intestine (Rivero-Urgell and Santamaria-Orleans, 2001).

Similarly to GOS, FOS are also known for their prebiotic effect on the gut. FOS have been shown to increase numbers of *bifidobacterium* in the gut, especially when mixed with GOS (Boehm et al., 2005; Haarman and Knol, 2002). *Bifidobacteria* are able to utilize FOS by hydrolysis of the β -1,2 glycosidic bonds by p-fructosidase, which provides fructose as a substrate for the hexose fermentation pathway

executed by solely bifidobacteria (Bornet et al., 2002). Due to their prebiotic effect on the intestinal microflora, FOS are currently included in food products, as well as infant formulas (Sabeter-Molina et al., 2009). Several types of FOS are commercially available to use as dietary supplements. One type used is Orafti FOS which is derived from chicory inulin (Liong and Shah, 2005; Semjonovs et al., 2007). Another type of FOS is Nutraflora FOS, which is naturally synthesized from sucrose and is added to infant formulas as well as other food products (Kaplan and Hutkins, 2000).

Swine as a model for neonatal nutrition studies

Pigs are commonly used as an animal model for a variety of human studies. More specifically, piglets are considered an optimal model in pediatric nutritional research due to their remarkable similarities in gastrointestinal development, anatomy, physiology, and metabolism to human infants (Herfel et al. 2011; Guilloteau et al., 2010). Similar physical size, as well as analogous digestive tracts make the piglet an ideal model for the human infant.

While piglets are similar in physical size to human infants, their growth rate from birth to six weeks is more rapid, although both piglets and humans experience increased metabolic rates after birth. The significance of this rapid growth is that nutritional deficiencies that can occur in both pigs and humans will appear more rapidly (Flamm, 2013). However, this is a potential drawback of piglets as a model given that adjustments are needed to compensate for the accelerated growth of the piglets in order to make them comparable to the human infant.

Neonatal piglets have been shown to be efficient at the deposition of ingested amino acids into proteins, as well as having similar responses to feeding as humans and have therefore been used in muscle growth and metabolism studies (El-Kadi et al., 2012). Piglets have also shown similarities in the pathways of lipid metabolism, the development of the intestine, as well as in the digestion and absorption of fat, which makes them useful in lipid nutrition studies in the human infant (Corl et al., 2008).

Due primarily to the fact that humans and pigs are both omnivores, the gastrointestinal tracts are physiologically similar and both species rely on microorganisms present in the gut for modification of ingested ingredients. The microorganisms in the pig gastrointestinal tract have shown high similarity of bacterial phylotypes (>97%) to species identified from the human gut (Leser et al., 2002). Consequently, these similarities make the pig a more advantageous model to use in human infant nutritional studies compared to other animal models such as mice and rats (Guilloteau et al., 2010).

Neontal Growth

Protein synthesis

In the neonatal stage, the growth rate is faster than in any other period of postnatal life with skeletal muscle contributing to the bulk of the increase in mass (Davis and Fiorotto, 2009). Features of growth during the neonatal stage are high protein turnover and deposition rates (El-Kadi et al., 2012). During growth, the rate of protein synthesis is determined primarily by the availability of amino acids and ATP for the synthetic processes (Munro, 1970).

It has been shown that in the neonatal period of the pig, skeletal muscle protein synthesis is more sensitive to feeding (Davis et al., 1996). This response is mainly mediated by insulin, which promotes translation initiation by stimulating the uptake of amino acids, in the skeletal muscle; in contrast to the liver, where protein synthesis is stimulated more by the concentration of amino acids (Davis et al., 1998). While almost all tissues show an increase in protein synthesis in response to feeding, the response in skeletal muscles containing fast-twitch glycolytic fibers is mediated separately by an increase in both amino acids and insulin after a meal (Escobar et al., 2005). The feed-induced increase of protein synthesis in the pig has been shown to be consistent with that of human newborns (Denne et al., 1991).

It is also well established that growth performance in neonatal pigs can increase substantially when they are being fed a manufactured liquid diet (Harrell et al., 1993; Oliver et al., 2002). Although, whether energy source affects protein accretion in the young pig remains unclear (Oliver and Miles, 2010).

Lipid Accretion

In infant nutrition, fat is extremely important, not only as a source of essential fatty acids, but also as a concentrated source of energy in the diet, especially in human milk (Innis, 1993). With regard to food intake and the deposition of fat, pigs and humans share several phenotypic and physiological similarities (Kim et al., 2004). Specifically, when studying lipid nutrition, the piglet is comparable to the human infant in both the digestion and absorption of fat, intestinal development, and also numerous pathways involved in lipid metabolism (Corl et al., 2008).

There are several different factors involved in lipid metabolism in adipose tissue. Lipid degradation, transport of fatty acids, *de novo* fatty acid synthesis, triacylglycerol synthesis as well as the rate of uptake are all processes that determine the deposition of fat (Zhao et al., 2010). The rates at which fatty acids are synthesized can be affected by changes in the activity of lipogenic enzymes such as fatty acid synthase (FAS), malic enzyme (ME), acetyl-CoA carboxylase (ACC), and glucose 6-phosphate dehydrogenase (G-6-PDH) (Zhao et al., 2010).

Immune system

Immunity against invading organisms is provided by the innate immune system through physical barriers such as the skin and mucous membranes, and cell-mediated barriers, which include inflammatory cells, natural killer cells, dendritic cells, phagocytic cells, as well as soluble mediators such as cytokines (Schley and Field, 2002). Therefore, in order to determine the effects of prebiotics such as GOS and FOS on the immune system, several immunological parameters need to be assessed.

Susceptibility of the neonatal pig to stress and infection provide conditions that favor the secretion of tumor necrosis factor α (TNF α) (Ramsay et al., 2013). The TNF α gene encodes an important proinflammatory cytokine that is involved in immunity, inflammation, as well as cellular organization (Biswas et al., 2014). TNF α is produced by macrophages as well as others types of cells when activated, these include B cells, T cells, and natural killer (NK) cells (Pradines-Figueres and Raetz, 1992). TNF α activates macrophages, neutrophils, and monocytes, and enhances other proinflammatory cytokines (Ying et al., 1991).

Another important parameter of immune response is natural killer cell activity. Natural killer cells are populations of lymphocytes that participate in innate immunity and early defense against a range of infections through protective responses (Biron et al., 1999). Natural killer cells have the capability to produce cytokines important in immune regulation, as well as the ability to attack malignant body cells as well as spontaneously pathogen-infected cells (Gerner et al., 2009). These cells are an important component of the response to an infectious disease by an animal's immune system, which makes NK cell activity a practical indicator of changes in an animal's immunological status (Knoblock and Canning, 1992).

Gut Microbiota

Microbial ecology of the swine gut

The investigation of the abundance and diversity of organisms present in addition to their activities determined *in vitro* are important for the study of gastrointestinal microbial ecology (Zoetendal et al., 2004). A metabolically active and numerically dense complex community of microbes is present within the gastrointestinal tract (GIT) of monogastric animals, which include pigs and humans (Macfarlane and Macfarlane, 2007).

The microbiota within the pig GIT is a combination of gram-positive bacteria, such as obligate anaerobes Clostridium, Ruminococcus, and Peptostreptococcus, facultative anaerobe Escherichia, and aerotolerant Streptococcus; but also gram-negative bacteria such as Bacteroides, Selenomas, Butyrivibrio, Fusobacterium, and Prevotella which are obligate anaerobes (Rist et al., 2013). The top six most abundant groups of bacteria derived from the metagenomes of swine were

Clostridiales, unclassified *Firmicutes*, *Spirochaetales*, *Bacteroides*, *Lactobacillales*, and unclassified gammaproteobacteria (Lamendella et al., 2011). The densities of bacterial species commonly increase from the proximal parts of the GIT to the distal parts while both the quantity and proportion of species vary along the digestive tract (Gaskins, 2000). A radial distribution of microbes also exists within each section of the gut in addition to the proximal to distal gradient found along the GIT (Rist et al., 2013).

Development of microbiota from gestation to adulthood

It has been previously understood that the human fetus is born immunologically immature with a sterile intestine due to the fact that it is developing in a sterile environment, (Rautava et al., 2012). However, current evidence has shown the DNA of nonpathogenic bacteria, specifically *bifidobacterium* and *lactobacillus* in the human placenta, which demonstrates that there is microbial contact in the fetomaternal interface (Satokari et al., 2009). Other evidence has revealed a presence of microbial DNA in the meconium of preterm infants, which implies a prenatal microbial origin (Mshvildadze et al., 2010).

After probable colonization in utero, the mode of delivery is another factor that influences the initial colonization of the newborn gastrointestinal tract. Babies that are born by vaginal delivery are exposed to the commensal bacteria of the mother's intestinal and vaginal microbiota (Hansen et al., 2014). In contrast an acquisition of bacterial groups similar to those found in skin is seen in infants born by Cesarean section (C-section) as well as a delay in colonization (Dominguez-Bello et al., 2010). Several studies have reported lower numbers of *bifidobacterium* and

bacteroides in children born by C-section compared to delivery through the vagina (Grolund et al., 1999; Jakobsson et al., 2014). Despite normal variations in the gut microbiota, the differences in the microbiota associated with the mode of delivery in infants have been identified in children up to 7 years of age (Salminen et al., 2004). The stepwise process of initial microbial colonization seen in humans is similar to that of the piglet given that the GIT of newborn piglets is rapidly colonized after contact with the vaginal fluids, feces of the sow, as well as the environment. The gut microbiota present in the neonate will begin to diversify and become similar to that of adults post weaning (Kurokawa et al., 2007).

The feeding of the neonate, breast-milk or formula, is an important extrinsic factor for the timing and quality of the colonization of the intestine given that the development of the intestinal microbiota is one of the major differences seen between breast-fed and formula-fed infants (Harmsen et al., 2000). Changes in an animal's or human's diet can have significant effects on the microbiota within the GIT which could impact the overall health of the host.

Role of diet changes on gut microbiota

Various factors such as diet and age can influence the composition of the gut microbiota (Li et al., 2012). It is becoming well recognized that relatively small changes in food consumption modify the species that make up the microbiota, as well as many of its physiological traits (Macfarlane et al., 2008). When fermentable oligosaccharides, such as human milk oligosaccharides and prebiotics are present, they allow for the capture of energy from the carbohydrates that would otherwise

escape digestive processes by aiding in the establishment of commensal bacteria (Schell et al., 2002).

It is well known that differences in the composition of the gut microbiota exist in response to an infant's feeding (Gibson et al., 1995). It is known that the development of the intestinal flora, which is important for the maturation of the intestinal immune system as well as for protection against harmful microorganisms, is a major difference between breast-fed and formula-fed infants (Harmsen et al., 2000). Therefore, there is interest in increasing the activities and numbers of bacterial groups with health-promoting properties (such as *Bifidobacterium* and *Lactobacillus*) (Gibson et al., 1995).

Modulation of the intestinal microbiota and shifting the balance of bacteria in favor of more beneficial microorganisms such as species of *bifidobacteria* and *lactobacilli*, have been strongly linked with dietary supplementation of prebiotics (Beohm et al., 2003). Given their ability to help maintain healthy mucosal surfaces of the GIT as well as the capacity to inhibit pathogenic bacteria, *bifidobacteria* are considered beneficial commensal bacteria (Ismail and Hooper, 2005). Several clinical trials have demonstrated effects on the intestinal microbiota by supplementation with a mixture of galactooligosaccharides and fructooligosaccharides, that shifted the counts of fecal bifidobacteria in formula-fed infants towards that of breast-fed infants (Boehm and Stahl, 2007). Studies investigating a range of blends and concentrations of galactooligosaccharides and fructooligosaccharides have shown similar results with respect to an increase in the numbers of *bifidobacteria* and *lactobacilli* in term infants compared to infants that were fed unplemented formulas (Nakamura et al., 2008).

Microbial analysis techniques

A dramatic increase in the application of approaches based on the sequence diversity of the 16S ribosomal RNA (rRNA) gene have been made during the past decade in order explore the diversity of bacterial communities in the mammalian GI tract as well as a variety of other ecosystems (Vaughan et al., 2000). The 16S rRNA sequence relationships dominate our molecular phylogenetic view of the microbial world, and the standard tool used to study microbial communities is the Ribosomal Database Project which contains a wealth of sequence information accumulated for 16S rRNA genes from thousands of organisms (Hill et al., 2002). Findings from culture-based methods have recently been supplemented with molecular ecology techniques that enable quantification and characterization of the gut microbiota while also predicting phylogenetic relationships based on the 16S rRNA gene (Zoetendal et al., 2004).

Polymerase chain reaction (PCR) that targets the 16S rRNA gene is used to identify individual members of microbial communities. A more recently applied quantitative PCR method is the real-time PCR approach, which has been applied successfully to characterize samples from the human and newborn pig GI tract as well as the rumen (Zoetendal et al., 2000). Molecular approaches based on PCR provide powerful tools for revealing the true phylogenetic diversity of microorganisms within environmental samples despite the introduction of different types of bias (Pryde et al., 1999).

Conclusion and Future Directions

In summary, it is well known that breast milk is the most advantageous vehicle to deliver sufficient quantities of high quality nutrients in order to sustain normal growth, but a variety of factors can make breast-feeding insufficient or impractical. As a result, infant formula manufacturers constantly strive to produce formulas that safely mimic the natural components of human breast milk through the addition of new ingredients, such as oligosaccharides.

The addition of oligosaccharides to infant formula is intended to imitate the effects of naturally occurring prebiotics in breast milk. Several investigators have speculated that the difference in intestinal microbiota between breast-and formula-fed infants contributes to the functional benefits that breast-feeding has over formula-feeding. While the current study measured parameters related to growth, immune response, as well as the microbiota present within the neonatal porcine gut, more evidence is needed to support the beneficial effects of GOS and FOS in infant formula.

Given that the addition of GOS and FOS to infant formula have been shown to be safe and not detrimental to the infant, in the future, it would be beneficial to assess these health parameters in a situation where there is an immune challenge. An immune challenge experiment, especially a challenge impacting the gastrointestinal tract, could possibly further demonstrate the true potential of oligosaccharides, specifically GOS and FOS to benefit the health of the infant. Another future direction dealing with the gut microbiota specifically would be to investigate the effect of GOS and FOS on the ability of the intestinal flora to compete with other pathogens known to target the gastrointestinal tract.

Chapter II

Effects of galactooligosaccharides and fructooligosaccharides on neonatal growth, blood metabolites, and immune response

ABSTRACT

Breast milk remains the optimum method of delivery for high quality nutrients, including oligosaccharides, in quantities sufficient to sustain normal growth, however, it is currently unknown whether the addition of prebiotics to infant formula would change the development and growth of the neonate. The objective of this study was to determine the effect of Galactooligosaccharides (GOS) and Fructooligosaccharides (FOS) supplementation in nursery pig diets on efficiency of food utilization and growth. Forty-eight 4-day old crossbred pigs ($1.628 \pm .037$ Kg BW) were randomly assigned to 1 of 8 diets: 1) milk-based formula containing Type 2 GOS and Type 1 FOS (4 + 1 g/L); 2) soy-based formula containing Type 1 FOS (3g/L); 3) milk-based formula with no prebiotic added; 4) milk-based formula containing Type 2 GOS only (5g/L); 5) milk-based formula containing Type 1 GOS only (5g/L); 6) milk-based formula containing Type 2 GOS and Type 2 FOS (4 + 1 g/L); 7) milk-based formula containing Type 1 GOS and Type 1 FOS (4 + 1 g/L); 8) soy-based formula with no prebiotic added. Diets were isonitrogenous, isocaloric and fed at 250 mL/kg body weight every 4 hrs for a 2-week period. Jugular and carotid blood vessels were catheterized prior to study. Body weight was measured every 3rd day. Body composition was measured prior to and at the end of the study using dual-energy X-ray absorptiometry (DXA). On the day of sacrifice, blood samples were taken before feeding for LDH, phagocytosis assays, and cytokine analysis. Additional blood samples were taken

immediately before feeding and 60 min after feeding. At sacrifice select organs and skeletal muscles were dissected and weighed. Body weight increased over time for all treatments. Plasma urea nitrogen and glucose concentrations increased relative to feeding ($P < 0.001$). Bone mineral content increased for all treatments during the study and a diet by time interaction ($P < 0.001$) indicated a smaller rate of accretion in bone mineral content for soy-based diets compared to milk-based diets. In conclusion, the addition of GOS and FOS to formula does not appear to alter growth however; the milk-based formulas were superior to the soy-based formulas in bone mineral content accretion.

Introduction

While it is known that breast-milk is the ideal source of nutrition for an infant, many factors such as breast milk insufficiency, poor milk volume, poor-breast feeding technique, as well as the mother's decision not to breast feed, make the use of infant formula necessary. Although breast-feeding rates are currently increasing in the United States, at 42%, the numbers remain low (Centers for Disease Control and Prevention, 2014). The decision or need to formula feed is extremely crucial given that breast-fed infants have enhanced health outcomes compared to formula-fed infants (Froh et al., 2014; American Academy of Pediatrics, 2012).

The differences in the health of breast-fed infants compared to formula-fed infants remains an area of high priority for infant formula manufacturers today. This stems from the fact that infant formula must promote normal growth and development as it may serve as the sole source of nutrition for the infant's first few

months of life (Flamm, 2013). Therefore, it is necessary to discover new ingredients that have the ability to imitate the components found naturally in breast milk.

Promising results have been obtained from recent work conducted in supplemental pre-and probiotics in the neonatal piglets (Jacobi and Odle, 2012). With new safety and efficacy data emerging and a regulatory climate becoming more favorable, the number of prebiotic-containing infant formulas is expected to grow, however, only a few of these supplemented formulas have been marketed (Kullen et al., 2005).

Oligosaccharides added to infant formula from available sources are intended to imitate the prebiotic effect of human milk given that currently analogues of human milk oligosaccharides are not available (Boehm et al., 2003). Galactooligosaccharides (GOS) and fructooligosaccharides (FOS) have been shown to exert a prebiotic effect on the gut (Boehm et al., 2005; Haarman and Knol, 2002) and have been used as a supplement in pediatric nutrition with mounting evidence to support their use in infant formula (Monaco et al., 2011; Sabeter - Molina et al., 2009).

Currently there is not enough evidence to state that supplementation of preterm infant formula with prebiotics alters growth and clinical outcomes in preterm infants (Mugambi et al., 2012). Therefore, this study examines the effect of galactooligosaccharides and fructooligosaccharides on neonatal pig growth, blood plasma metabolites, phagocytosis, natural killer cell activity and cytokines.

Materials and Methods

Animals and experimental design:

Forty-eight 2-day old female crossbred pigs were acquired from Murphy Brown facilities. Piglets were acclimated to their environment for 24h and housed individually and kept on a standard light cycle (12h light/dark) at 80-85 degrees Fahrenheit. At 5 days of age, pigs ($1.628 \pm .037$ Kg BW) were randomly assigned to one of eight diets: 1) milk-based formula containing Bimuno GOS and Vivinal FOS (4 + 1 g/L); 2) soy-based formula containing Orafti (3g/L); 3) milk-based formula with no prebiotic added; 4) milk-based formula containing Bimuno GOS only (5g/L); 5) milk-based formula containing Vivinal GOS only (5g/L); 6) milk-based formula containing Bimuno GOS and Nutraflora FOS (4 + 1 g/L); 7) milk-based formula containing Vivinal GOS and Orafti FOS (4 + 1 g/L); 8) soy-based formula with no prebiotic added (Table 2.1). Diets were isonitrogenous, isocaloric and fed at 250mL/kg body weight every 4 hours (refer to Table 2.2 for diet composition) for a 2-week period. Body weight was measured every two days and amount of formula was adjusted accordingly. The experimental protocol involving animal use (13-001-APSC) was approved by the Virginia Tech Institutional Animal Care and Use Committee (IACUC).

Jugular and carotid catheterization

At 4 days of age catheters were implanted into the jugular vein and carotid artery. Piglets were fasted prior to surgery and anesthetized. Isoflurane dosage varied during surgery. Catheters were made out of tygon tubing (Saint-Gobain Performance Plastics Corporation) and the inside was coated with tridodecyl methyl ammonium chloride (TDMAC) heparin. Catheters then underwent cool gas sterilization at the Virginia Maryland Regional College of Veterinary Medicine. Prior to incision the ventral neck and dorsal interscapular areas were disinfected three

times with alternating 70% isopropanol and betadine scrub. Catheters were then inserted into the jugular vein and carotid artery. During surgery, catheters were filled with sterile heparinized saline (50 U/ml). Catheters were secured in place with sutures. The catheters were then exited through a blunt puncture in the skin of the neck and the incision was closed with black Vicryl. The wound was covered with Betadine ointment and alu-shield, dressed with sterile gauze and covered with a chest jacket. A dosage of 7.5mg/kg of body weight of Baytril (Valley Vet Supply, Marysville, Kansas) and 2.2mg/kg of body weight of Banamine (Valley Vet Supply, Marysville, Kansas) were administered on the day of surgery and for 3 days post surgery. Piglets were monitored for any signs of discomfort and rectal temperatures were taken at 2h, 4h, 6h, 12h and 24h post surgery. Catheters were flushed every other day throughout the duration of the study using heparinized saline.

Dual-energy X-ray absorptiometry

Initial and final body composition (Total body bone mineral content (BMC), non-bone lean tissue, and total body fat mass) analysis was performed using dual-energy X-ray absorptiometry (DEXA) body scanner in the whole infant mode. Piglets were fasted prior to DEXA and lightly anesthetized using isoflurane. DEXA scans were performed on the piglets at 3 days of age and day 13 of the experiment.

Blood sampling

On the day of sacrifice, blood samples were taken via catheters immediately before feeding (time 0) and 60 minutes after feeding (time 60). Blood samples were collected in both lithium heparin blood tubes and EDTA blood tubes. The blood

samples were kept on ice and then centrifuged at 2,000 x g at 4 degrees Celsius for 20 minutes. Blood plasma samples were sent to the Virginia-Maryland Regional College of Veterinary Medicine for blood chemistry analysis using the Beckman-Coulter AU-480 with ISE Chemistry System.

Tissue Sampling

Animals were euthanized using a captive bolt gun and exsanguinated on day 14 of the experiment. Muscle samples from the longissimus dorsi, semitendinosus, and soleus were dissected, weighed, and snap frozen in liquid nitrogen and then stored at -80 degrees Celsius. Select organs were also dissected and weighed. These organs included the stomach, liver, small intestine, and cecum. The intestinal samples were flushed with saline to remove digesta prior to weighing. Samples from the liver, ileum, and cecum were snap frozen in liquid nitrogen and then stored at -80 degrees Celsius.

Cytokine Analysis

Tumor necrosis factor α was measured using a Swine TNF α ELISA kit (Invitrogen Corporation, Frederick, MD.) with a measured inter-assay CV of 9 %. Samples and standards were run in duplicate. The analyses were run according to the protocol for each individual kit.

Natural killer cell and phagocytosis assays

On the last day of the experiment, a blood sample from each pig was collected in heparin tubes prior to feeding. Mononuclear cells required for natural killer (NK)

cell activity and phagocytosis assays were obtained by the separation from whole blood by Histopaque-1077 (Sigma-Aldrich, St. Louis, MO) according to the product information sheet. The cells were then used for the phagocytosis assay according to the protocol of the CytoSelect 96-well phagocytosis Assay (Zymosan, Colorimetric Format) from Cell Biolabs, Inc.

The LDH cytotoxicity assay used to determine NK cell activity is an indirect method of measuring the ability of mononuclear cells from blood to kill foreign cells. The attack on foreign cells by mononuclear cells damages the foreign cells' plasma membrane, which results in the release of LDH. Higher levels of LDH indicate stronger killing ability of the mononuclear cells. The foreign cells used for this assay were K-562 (ATCC CCL-243). In order to determine optimization, three ratios (mononuclear cells:K-562; 5:1, 10:1; 20:1) were used. Prior to running the assay, cell number of each ratio sample was determined by a TC10 automated cell counter (Bio-Rad Laboratories, Inc.). Lactate dehydrogenase activity was measured using the LDH Cytotoxicity Detection Kit (Clontech by Takara Bio Company). The optimal ratio (20:1) was then used for statistical analysis.

Data Analysis

All data were analyzed using the proc glimmix procedure of SAS 9.3 (SAS Institute, Cary, NC). Pig, treatment, type, and block were all included in the class statement. Comparisons were made within milk treatments, soy treatments, and all treatments. The statistical model $Y_i = \mu + D_i + e_i$ in which μ = overall mean, D_i = effect

of the i th diet, and e_i = error was used. Statistical significance was set at a P-value of less than 0.05.

Results

Body, organ, and muscle weight results

All pigs gained weight linearly throughout the experiment with an overall average daily gain (ADG) of approximately 0.05 kg/day (Figure 2.1). However, the rate of growth was not affected by treatment. The weights of the stomach liver small intestine, and cecum were not significantly affected by diet (Figures 2.5, 2.6, 2.7, and 2.8). Likewise, the weights of the longissimus dorsi semitendinosus and soleus muscles remained similar across all dietary treatments (Figures 2.2, 2.3, and 2.4).

Blood Metabolite results

Liver function

Albumin, globulin and total proteins concentrations decreased slightly after a meal (Figures 2.9, 2.20, and 2.26) and were not significantly affected by any of the experimental diets, which reflect normal liver and gallbladder function. Similarly, total, direct, and indirect serum bilirubins (Figures 2.28, 2.18 and 2.28) were similar across all dietary treatments indicating normal liver and hepatobiliary functions. In addition, there was no effect of dietary treatment on urea nitrogen although levels were slightly higher post meal across all treatments (Figure 2.29), which suggests normal hepatic urea production and kidney disposal. Gamma-glutamyl transferase concentration decreased post meal across all treatments (Figure 2.19) and was not

significantly different among the dietary treatments indicating that none of the dietary treatments had any effect on liver integrity (hepatocellular function).

Renal function

Anion gap was similar in all treatments compared to the control pigs for both milk and soy based diets indicating adequate electrolyte balance (Figure 2.10). Moreover, creatinine concentrations were similar in response to a meal across all experimental groups (Figure 2.17), which reflect adequate kidney clearance. Carbon dioxide concentrations decreased in all experimental groups, aside from treatment 2 where a modest increase was seen in response to a meal (Figure 2.16). However, there was no significant effect of treatment.

Metabolic function

Creatine kinase (CK) concentrations were not affected by the experimental diets indicating no signs of muscle injury (Figure 2.15). Blood glucose increased across all treatments (Figure 2.21) after a meal while cholesterol (Figure 2.14) decreased in all experimental groups including milk and soy based control diets. Glucose levels (Figure 2.21) across all treatments increased after feeding while cholesterol levels decreased (Figure 2.14). Serum calcium (Figure 2.12), chloride (Figure 2.13), magnesium (Figure 2.23), phosphorus (Figure 2.24), potassium (Figure 2.25) and sodium (Figure 2.27) remained relatively stable in all groups and were not affected by dietary treatments. This demonstrates no differences in electrolyte balance and no signs of metabolic acidosis and alkalosis.

While none of the blood metabolites measured were significantly affected by treatment, some were affected by feeding. Time relative to feeding was highly significant for carbon dioxide (Figure 2.16) magnesium (Figure 2.23), CK (Figure 2.15), protein total (Figure 2.26), albumin (Figure 2.9), potassium (Figure 2.25), phosphorus (Figure 2.24), globulin (Figure 2.20), cholesterol (Figure 2.14), glucose (Figure 2.21), urea nitrogen (Figure 2.29), and GGT (Figure 2.19) ($P < .01$). Calcium (Figure 2.12) and chloride concentrations (Figure 2.13) in the blood plasma were also affected by time of feeding, ($P < .05$). Anion Gap (Figure 2.10), indirect bilirubin (Figure 2.22), direct bilirubin (Figure 2.18), total bilirubin (Figure 2.28), sodium (Figure 2.27), AST (GOT) (Figure 2.11), and creatinine (Figure 2.17) were not affected by time of feeding.

Dual-energy X-ray absorptiometry results

The bone mineral content (BMC) (g) showed a strongly significant treatment by time interaction ($P < 0.0001$; Figure 2.30), where the pigs on the milk-based treatments showed a larger increase in BMC than the pigs on the soy-based treatments. Lean mass and percentage of lean increased over time (Figures 2.31 and 2.33) while fat mass and fat percentage decreased (Figures 2.32 and 2.34). Both lean and fat mass and percentage were significantly affected by time ($P < .0001$) but not significantly different across treatments indicating similar rates of protein synthesis and lipid breakdown among the experimental groups.

LDH, phagocytosis and Cytokine results

Natural killer cell ability was significantly different between pigs fed milk-based treatments and pigs fed soy-based treatments ($P < 0.05$) where the pigs fed the milk diets containing GOS only showed a slightly higher killing ability (Figure 2.35). Phagocytosis activity was unaffected by diet (Figure 2.36). Tumor necrosis factor (TNF α) was significantly affected by treatment ($P < 0.05$; Figure 2.37).

Discussion

It has been well established that GOS and FOS provide beneficial health effects and are safe to use when added to infant formula although they do not impact overall growth (Boehm et al., 2002; Monaco et al., 2011). Results from this study indicate that the weight gain of the animal, the amount of lean and the amount of fat were not significantly affected by the addition of GOS and FOS either alone or as a mixture. Our results are consistent with other studies in describing no significant effects on body weight gain (Boehm et al., 2002; Indrio et al., 2009). This may be due to the fact that oligosaccharides are non-digestible and only fermentable to gas and acids and therefore do not provide any nutritional value to the host. In conclusion GOS and FOS do not appear to alter pig growth.

Galactooligosaccharides and FOS did not significantly alter the plasma concentrations of metabolites and proteins measured. These results are consistent with other studies that reported no changes in serum total proteins or albumin (Bunout et al., 2002). Results from this study showed that GOS and FOS had no significant impact on glucose. This is consistent with another study testing GOS and inulin derived FOS that found no alterations in glucose absorption (van Dokkum et al., 1999). The addition of GOS and FOS did not have a significant impact on

creatinine similar to another study investigating the effects of lactulose, a source of GOS (Pereira et al., 2014). Although cholesterol levels decreased post feeding across all treatments, there were no significant effects in the supplemented diets compared to the controls. This finding parallels two studies testing the dietary effects of inulin, the source of the Orafti FOS used in the current study, on lipid profiles, which showed no significant improvement in cholesterol in the supplemented groups compared to the controls (Davidson et al. 1998). Given that GOS and FOS are nondigestible and mainly impact the gut microbiota, they do not appear to directly impact blood metabolites or blood proteins.

The results in this study demonstrated a significant difference in bone mineral content (BMC) between the milk-based diets and the soy-based diets. Although the majority of studies on calcium metabolism have been performed using rat models, experimental evidence indicates that prebiotics have a role in increasing the bioavailability of calcium (Macfarlane et al., 2008). Our results are consistent with studies performed using GOS as a prebiotic supplement that demonstrated a stimulated absorption of Ca at normal dietary concentrations (Chonan and Watnuki, 1995, 1996) as well as a reduction in the loss of calcium content and bone mass (Chonan et al., 2001). Studies using FOS as a prebiotic supplement have also shown increased Ca absorption (Brommage et al., 1993; Taguchi et al., 1995) as well as a dose-related increase in bone mineralization (Scholz-Ahrens et al., 1998). However, given that in the present study the milk-based treatments had higher levels of BMC compared to the soy-based treatments, there may be another factor at play. In mammals, it has been recognized that lactose enhances the absorption of calcium (Kwak et al., 2012). In the current study, the milk-based diets contained lactose

while the soy-based diets did not which could have contributed to the higher BMC in the milk-based diets. Thus, it is unclear from the results in the current study if the differences in BMC were a result of GOS and FOS or the presence of lactose.

The activity of the natural killer (NK) cells was significantly increased in the treatments containing GOS only with no significant difference between the different types of GOS. This effect is consistent with a study in mice where the addition of galactooligosaccharides alone significantly increased the percentage of NK cells in the spleen (Gopalakrishnan et al., 2012). These findings suggest that the addition of GOS alone impacts the innate immune system; however, the extent of this effect as well as the mechanisms by which it occurs are areas for further research.

Our results of no significant effect of the prebiotics GOS and FOS on phagocytic activity were similar to other studies testing dietary supplementation with prebiotics. A study testing the effects of inulin and oligofructose (IN/OF) on immune function found that supplementation of IN/OF resulted in minor changes of systemic immune functions such as decrease in phagocytic activity (Watzl et al., 2005) although, this study did not test the addition of GOS like in the current study. An experiment comparing the effects of prebiotics (PRO), prebiotics (PRE), and synbiotics (SYN), a combination of pre and probiotics, found that monocyte phagocytosis (percentage active cells and mean fluorescence intensity) was not affected by PRO, PRE or SYN (Roller et al., 2004). Given that phagocytosis activity was not significantly different between treatments; it appears that GOS and FOS do not have a strong influence on the immune response. However, the pigs in this study were not subjected to any type of immune challenge. Future studies involving an

immune challenge may be able to further elicit the direct effect of GOS and FOS on the immune system.

Table 2.1. Experimental milk replacer formulas containing Vivinal or Bimuno GOS and/or Nutraflora or Orafti FOS. ¹Dose per unit of liquid formula.

Treatment Group	Dose¹
01: milk-based formula containing Vivinal GOS and NutraFlora FOS	4 + 1 g/L
02: soy-based formula containing NutraFlora FOS	3g/L
03: milk-based formula no added prebiotic – Control for Milk	---
04: milk-based formula containing Vivinal GOS	5g/L
05: milk-based formula containing Bimuno GOS	5g/L
06: milk-based formula containing Vivinal GOS and Boneo Orafti FOS	4 + 1 g/L
07: milk-based formula containing Bimuno GOS and NutraFlora FOS	4 + 1 g/L
08: soy-based formula containing no added prebiotic- Soy Control	--

Table 2.2 Diet composition table. Formulas were prepared by Perrigo nutritionals and shipped with the GOS and FOS additives in powder form to the animal facility. Treatment 1: milk-based formula containing Vivinal GOS and NutraFlora FOS; 2: soy-based formula containing NutraFlora FOS; 3: milk-based formula no added prebiotic; 4: milk-based formula containing Vivinal GOS; 5: milk-based formula containing Bi2muno GOS; 6: milk-based formula containing Vivinal GOS and Boneo Orafti FOS; 7: milk-based formula containing Bi2muno GOS and NutraFlora FOS; 8: soy-based formula containing no added prebiotic.

Treatment Base	1 Milk	2 Soy	3 Milk	4 Milk	5 Milk	6 Milk	7 Milk	8 Soy
Ingredient (gm)								
Protein	53.5	53.6	53.5	53.5	53.5	53.5	53.5	53.6
Total Carbohydrate (Digestible)	38.8	38.4	38.9	38.8	38.7	38.8	38.8	38.2
Lactose	28.5	N/A	38.0	26.6	33.5	28.5	34.1	N/A
Malodextrin	6.8	36.3	0	8.5	0	6.8	0	37.3
Galactooligosaccharides (GOS)	4	0	0	5	5	4	4	0
Fructooligosaccharides (FOS)	1	3	0	0	0	1	1	0
Lutein	N/A	0.0003	N/A	N/A	N/A	N/A	N/A	N/A
Fat	12.6	12.7	12.6	12.6	12.6	12.6	12.6	12.7
Fiber	5	3	0	5	5	5	5	0
kcal								
Protein	214	215	214	214	214	214	214	215
Total Carbohydrate	155	154	155	155	155	155	155	153
Lactose	114	N/A	152	106	134	114	136	N/A
Fat	113	114	113	113	113	113	113	114
Total	482	482	482	482	482	482	482	392

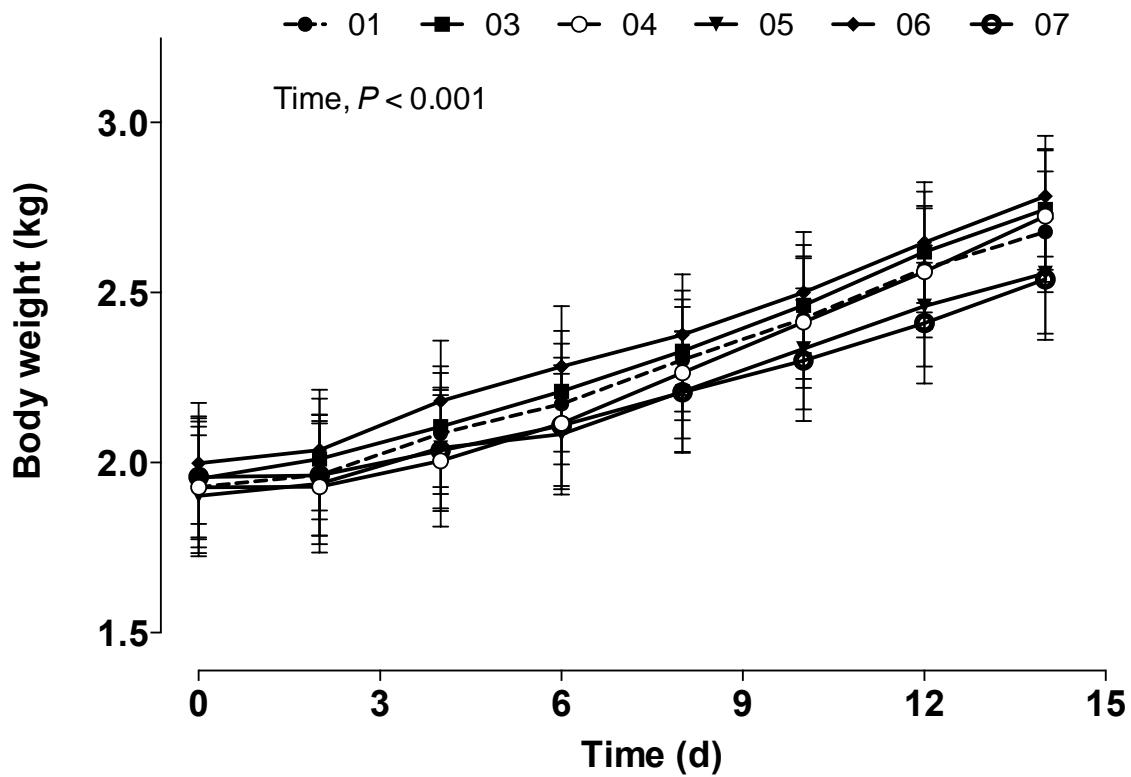


Figure 2.1. Effect of GOS and FOS dietary supplementation on body weight (kg) of pigs fed experimental milk replacer formulas. Piglets ($1.628 \pm .037$ Kg BW) were randomly assigned to one of eight diets ($n = 6/\text{diet}$): 1) milk-based formula containing Bimuno GOS and Vivinal FOS (4 + 1 g/L); 2) soy-based formula containing Orafti (3g/L); 3) milk-based formula with no prebiotic added; 4) milk-based formula containing Bimuno GOS only (5g/L); 5) milk-based formula containing Vivinal GOS only (5g/L); 6) milk-based formula containing Bimuno GOS and Nutraflora FOS (4 + 1 g/L); 7) milk-based formula containing Vivinal GOS and Orafti FOS (4 + 1 g/L); 8) soy-based formula with no prebiotic added. Diets were isonitrogenous, isocaloric and fed at 250mL/kg body weight every 4 hours for a 2-week period. Body weight was measured every two days and feed amount adjusted accordingly. Values represent least squared means \pm SEM and statistical significance set at $P < 0.05$.

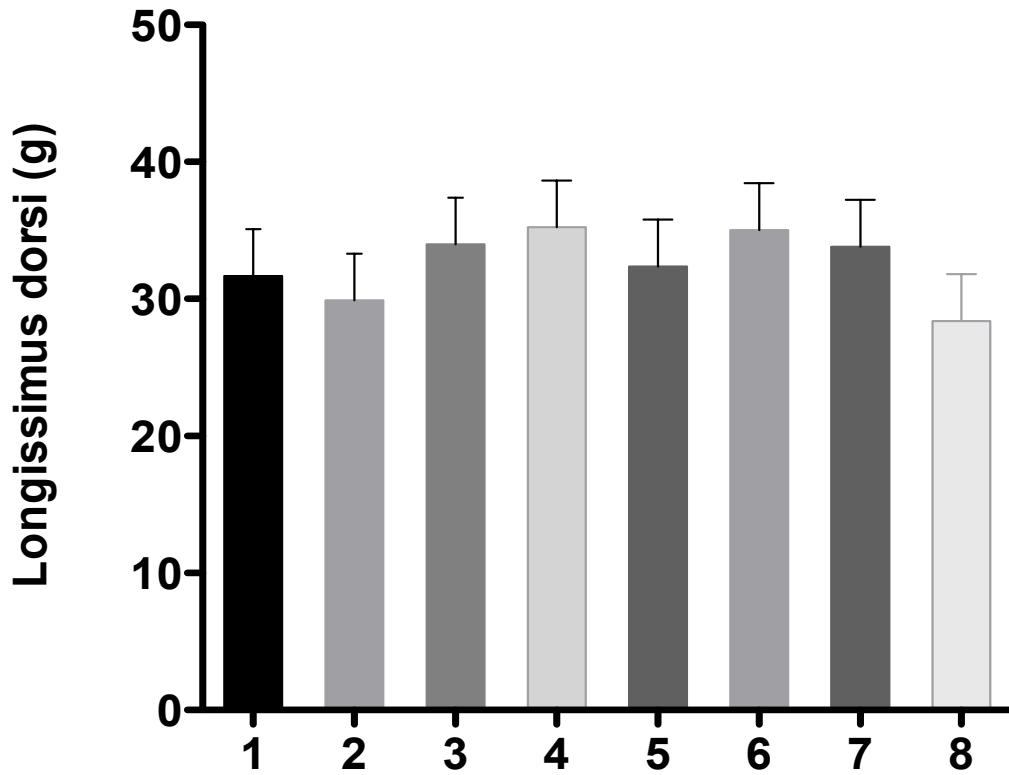


Figure 2.2. Effects of GOS and FOS dietary supplementation on longissimus dorsi muscle weights (g) of pigs fed experimental milk replacer formula. Piglets ($1.628 +/- .037$ Kg BW) were randomly assigned to one of eight diets ($n = 6/diet$): 1) milk-based formula containing Bimuno GOS and Vivinal FOS (4 + 1 g/L); 2) soy-based formula containing Orafti (3g/L); 3) milk-based formula with no prebiotic added; 4) milk-based formula containing Bimuno GOS only (5g/L); 5) milk-based formula containing Vivinal GOS only (5g/L); 6) milk-based formula containing Bimuno GOS and Nutraflora FOS (4 + 1 g/L); 7) milk-based formula containing Vivinal GOS and Orafti FOS (4 + 1 g/L); 8) soy-based formula with no prebiotic added. Diets were isonitrogenous, isocaloric and fed at 250mL/kg body weight every 4 hours for a 2-week period. Values represent least squared means \pm SEM and statistical significance set at $P < 0.05$.

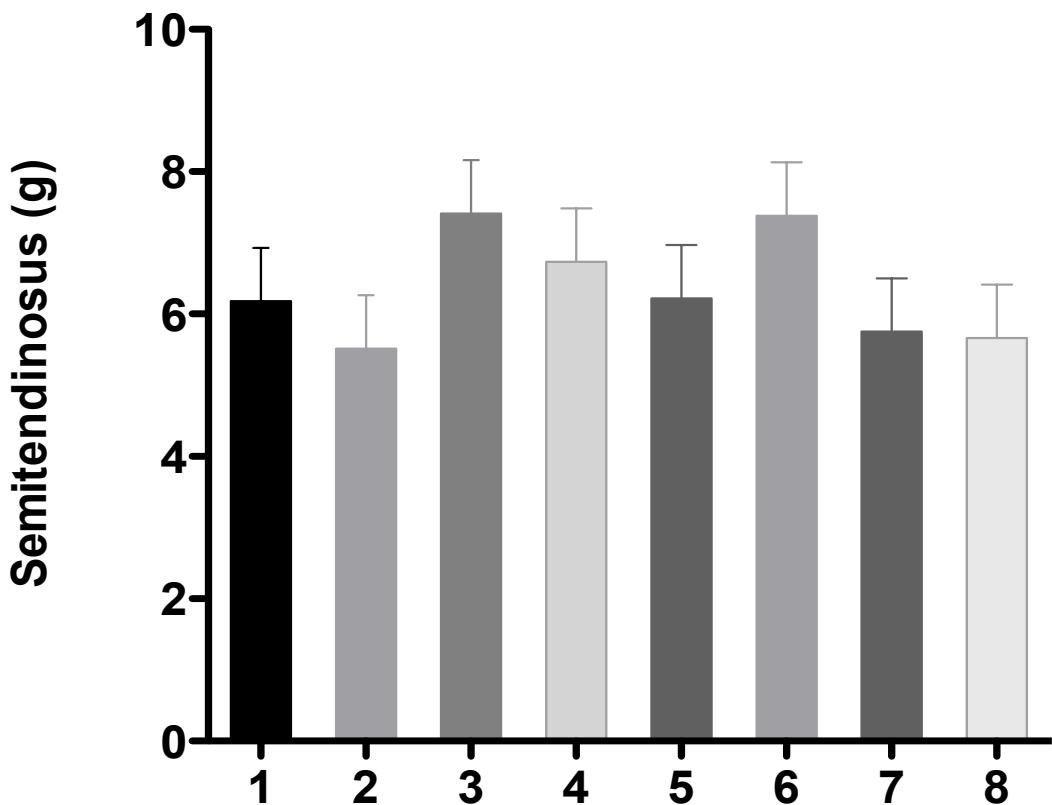


Figure 2.3. Effects of GOS and FOS dietary supplementation on semitendinosus muscle weights (g) of pigs fed experimental milk replacer formula. Piglets ($1.628 +/_{.037}$ Kg BW) were randomly assigned to one of eight diets ($n = 6/\text{diet}$): 1) milk-based formula containing Bimuno GOS and Vivinal FOS (4 + 1 g/L); 2) soy-based formula containing Orafti (3g/L); 3) milk-based formula with no prebiotic added; 4) milk-based formula containing Bimuno GOS only (5g/L); 5) milk-based formula containing Vivinal GOS only (5g/L); 6) milk-based formula containing Bimuno GOS and Nutraflora FOS (4 + 1 g/L); 7) milk-based formula containing Vivinal GOS and Orafti FOS (4 + 1 g/L); 8) soy-based formula with no prebiotic added. Diets were isonitrogenous, isocaloric and fed at 250mL/kg body weight every 4 hours for a 2 -week period. Values represent least squared means \pm SEM and statistical significance set at $P < 0.05$.

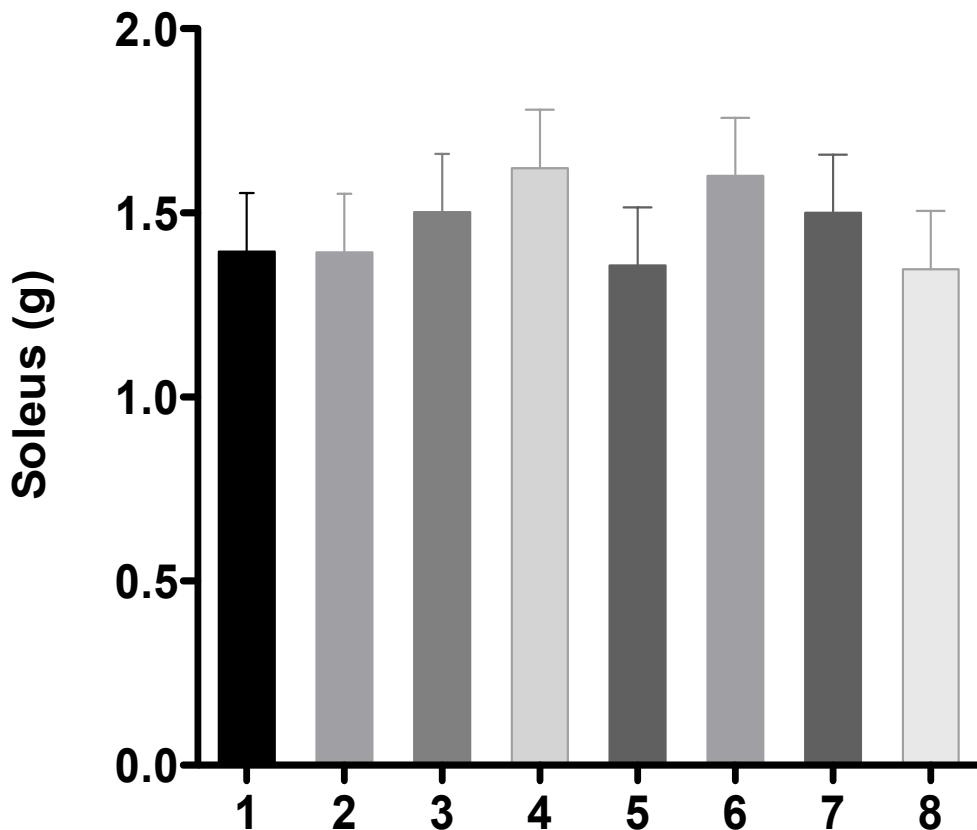


Figure 2.4. Effects of GOS and FOS dietary supplementation on soleus muscle weights (g) of pigs fed experimental milk replacer formula. Piglets ($1.628 +/_{.037}$ Kg BW) were randomly assigned to one of eight diets ($n = 6$ /diet): 1) milk-based formula containing Bimuno GOS and Vivinal FOS (4 + 1 g/L); 2) soy-based formula containing Orafti (3g/L); 3) milk-based formula with no prebiotic added; 4) milk-based formula containing Bimuno GOS only (5g/L); 5) milk-based formula containing Vivinal GOS only (5g/L); 6) milk-based formula containing Bimuno GOS and Nutraflora FOS (4 + 1 g/L); 7) milk-based formula containing Vivinal GOS and Orafti FOS (4 + 1 g/L); 8) soy-based formula with no prebiotic added. Diets were isonitrogenous, isocaloric and fed at 250mL/kg body weight every 4 hours for a 2 -week period. Values represent least squared means \pm SEM and statistical significance set at $P < 0.05$.

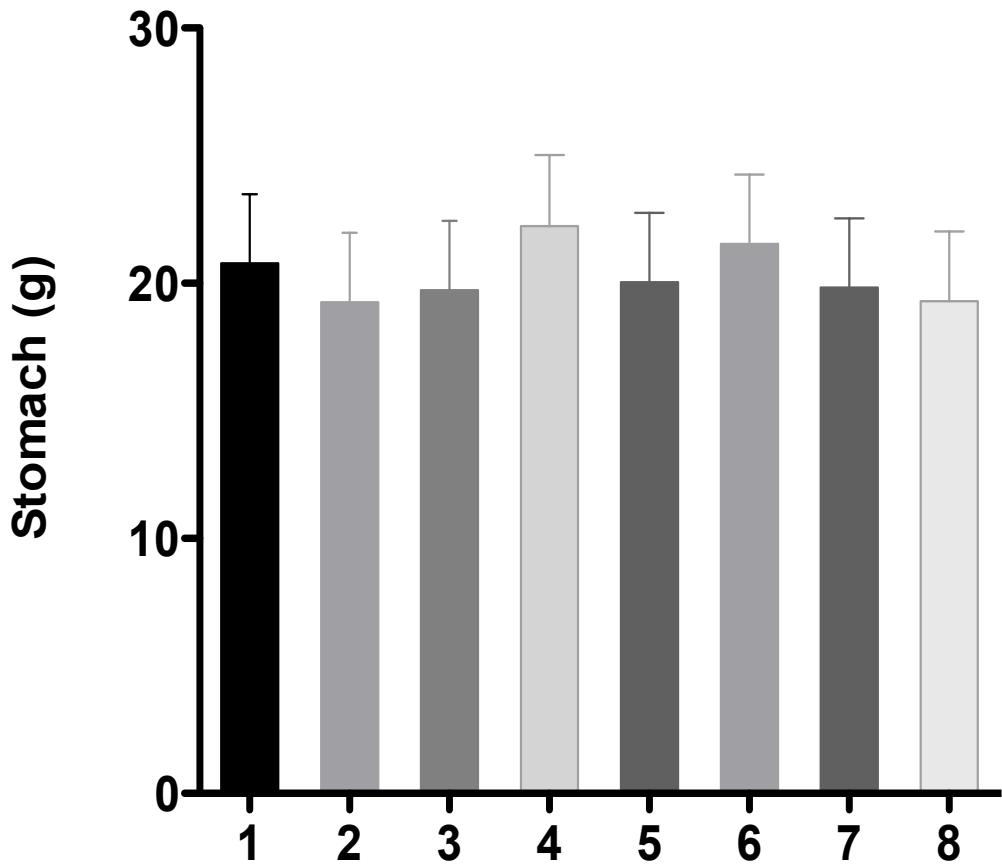


Figure 2.5. Effects of GOS and FOS dietary supplementation on stomach weights (g) of pigs fed experimental milk replacer formula. Piglets ($1.628 +/_{.037}$ Kg BW) were randomly assigned to one of eight diets ($n = 6/\text{diet}$): 1) milk-based formula containing Bimuno GOS and Vivinal FOS (4 + 1 g/L); 2) soy-based formula containing Orafti (3g/L); 3) milk-based formula with no prebiotic added; 4) milk-based formula containing Bimuno GOS only (5g/L); 5) milk-based formula containing Vivinal GOS only (5g/L); 6) milk-based formula containing Bimuno GOS and Nutraflora FOS (4 + 1 g/L); 7) milk-based formula containing Vivinal GOS and Orafti FOS (4 + 1 g/L); 8) soy-based formula with no prebiotic added. Diets were isonitrogenous, isocaloric and fed at 250mL/kg body weight every 4 hours for a 2 -week period. Values represent least squared means \pm SEM and statistical significance set at $P < 0.05$.

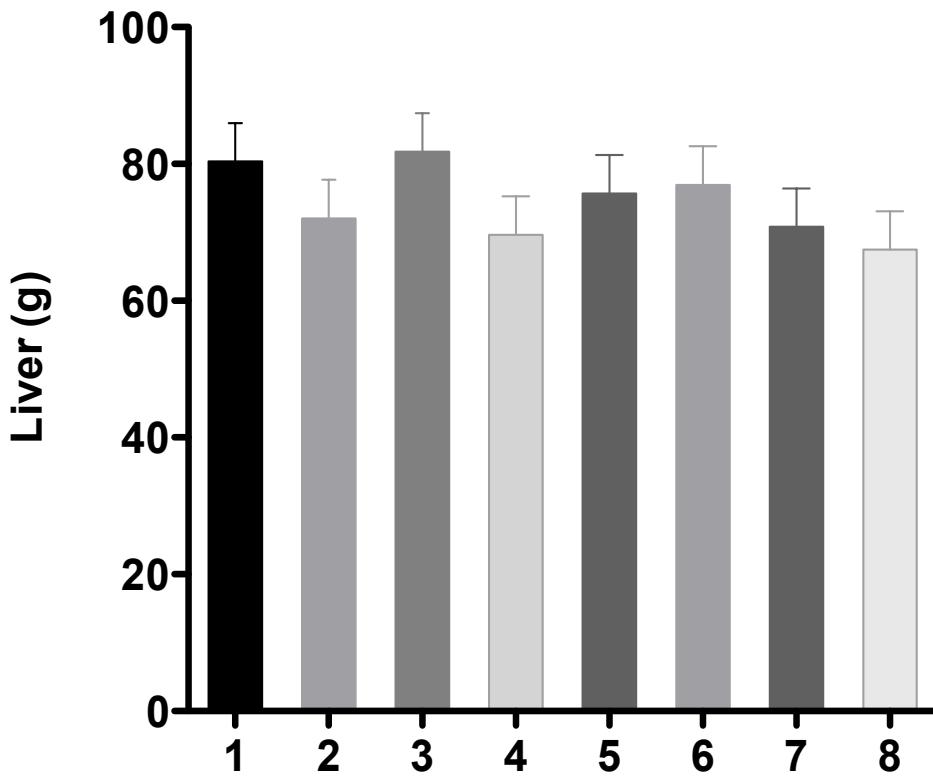


Figure 2.6. Effects of GOS and FOS dietary supplementation on liver weights (g) of pigs fed experimental milk replacer formula. Piglets ($1.628 +/_ .037$ Kg BW) were randomly assigned to one of eight diets (n = 6/diet): 1) milk-based formula containing Bimuno GOS and Vivinal FOS (4 + 1 g/L); 2) soy-based formula containing Orafti (3g/L); 3) milk-based formula with no prebiotic added; 4) milk-based formula containing Bimuno GOS only (5g/L); 5) milk-based formula containing Vivinal GOS only (5g/L); 6) milk-based formula containing Bimuno GOS and Nutraflora FOS (4 + 1 g/L); 7) milk-based formula containing Vivinal GOS and Orafti FOS (4 + 1 g/L); 8) soy-based formula with no prebiotic added. Diets were isonitrogenous, isocaloric and fed at 250 mL/kg body weight every 4 hours for a 2-week period. Values represent least squared means \pm SEM and statistical significance set at P<0.05.

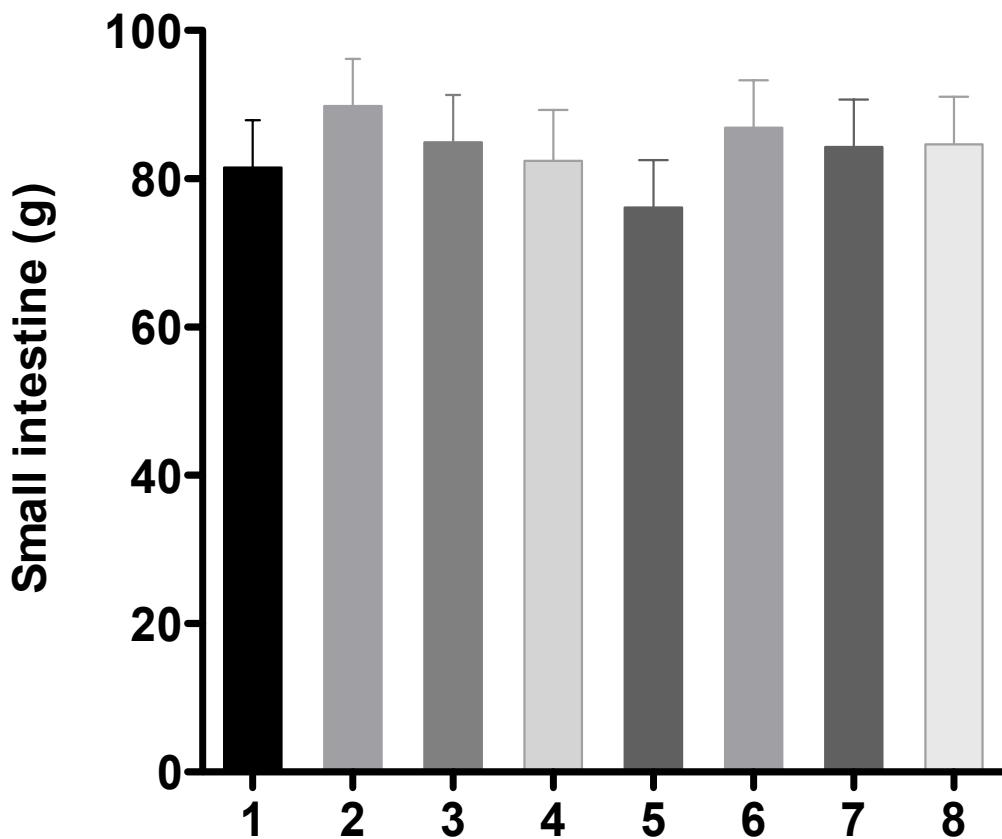


Figure 2.7. Effects of GOS and FOS dietary supplementation on small intestine weights (g) of pigs fed experimental milk replacer formula. Piglets ($1.628 +/_{.037}$ Kg BW) were randomly assigned to one of eight diets ($n = 6/\text{diet}$): 1) milk-based formula containing Bimuno GOS and Vivinal FOS (4 + 1 g/L); 2) soy-based formula containing Orafti (3g/L); 3) milk-based formula with no prebiotic added; 4) milk-based formula containing Bimuno GOS only (5g/L); 5) milk-based formula containing Vivinal GOS only (5g/L); 6) milk-based formula containing Bimuno GOS and Nutraflora FOS (4 + 1 g/L); 7) milk-based formula containing Vivinal GOS and Orafti FOS (4 + 1 g/L); 8) soy-based formula with no prebiotic added. Diets were isonitrogenous, isocaloric and fed at 250mL/kg body weight every 4 hours for a 2-week period. Values represent least squared means \pm SEM and statistical significance set at $P < 0.05$.

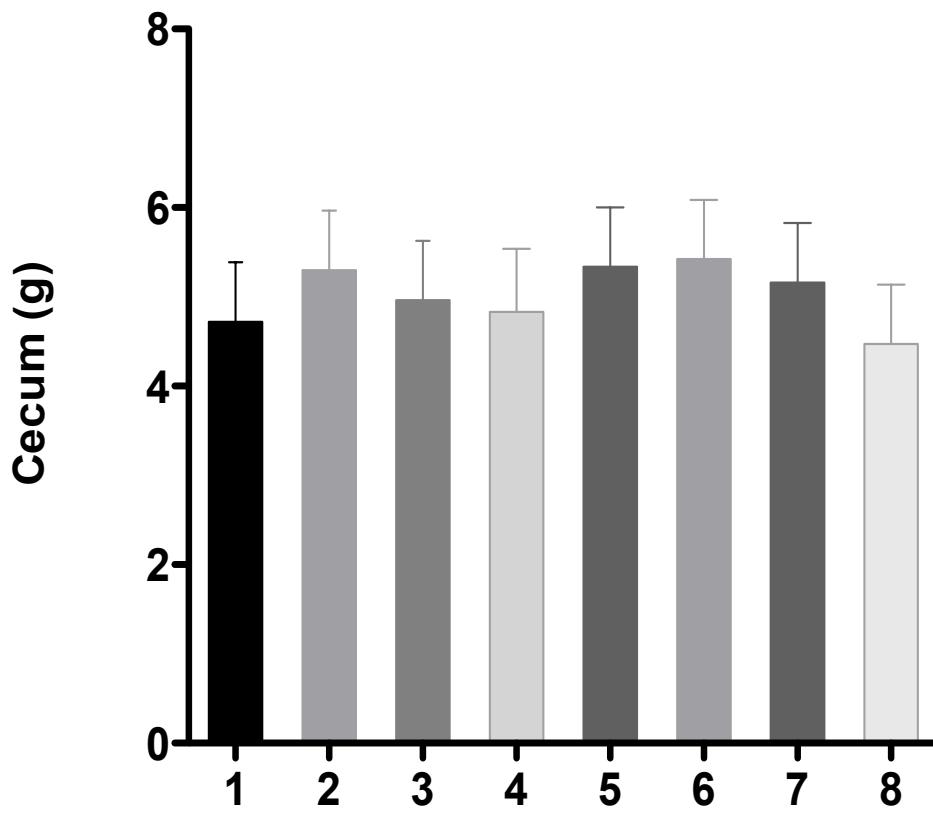


Figure 2.8. Effects of GOS and FOS dietary supplementation on cecum weights (g) of pigs fed experimental milk replacer formula. Piglets ($1.628 +/_{.037}$ Kg BW) were randomly assigned to one of eight diets ($n = 6$ /diet): 1) milk-based formula containing Bimuno GOS and Vivinal FOS (4 + 1 g/L); 2) soy-based formula containing Orafti (3g/L); 3) milk-based formula with no prebiotic added; 4) milk-based formula containing Bimuno GOS only(5g/L); 5) milk-based formula containing Vivinal GOS only (5g/L); 6) milk-based formula containing Bimuno GOS and Nutraflora FOS (4 + 1 g/L); 7) milk-based formula containing Vivinal GOS and Orafti FOS (4 + 1 g/L);8) soy-based formula with no prebiotic added. Diets were isonitrogenous, isocaloric and fed at 250mL/kg body weight every 4 hours for a 2-week period. Values represent least squared means \pm SEM and statistical significance set at $P < 0.05$.

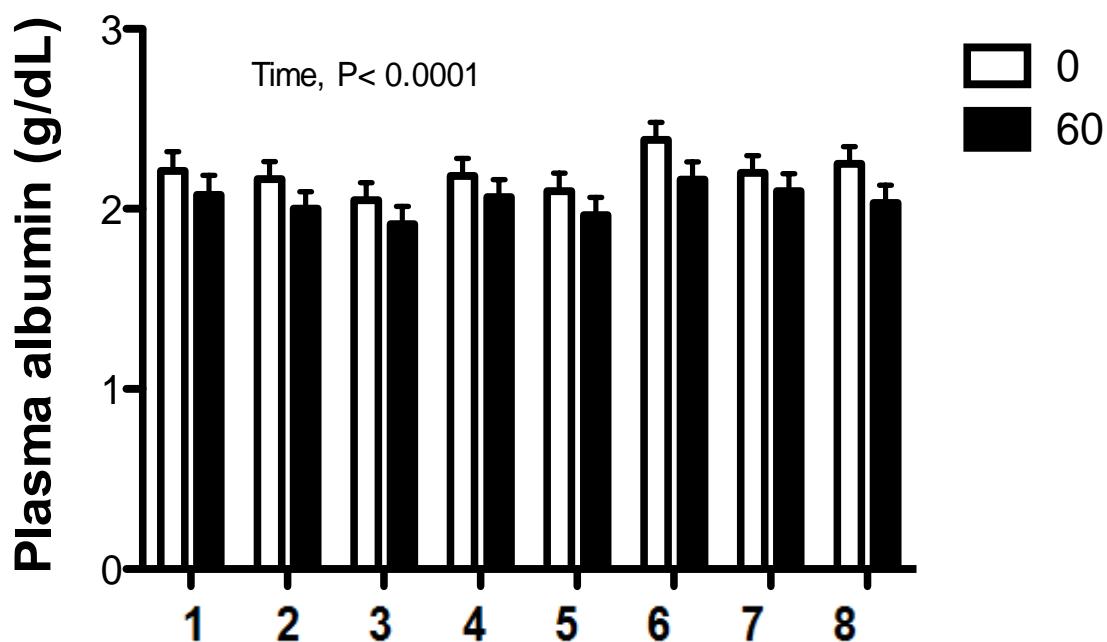


Figure 2.9. Effects of GOS and FOS dietary supplementation on plasma albumin (g/dL) levels of pigs fed experimental milk replacer formula. Piglets ($1.628 +/_{.037}$ Kg BW) were randomly assigned to one of eight diets ($n = 6/\text{diet}$): 1) milk-based formula containing Bimuno GOS and Vivinal FOS (4 + 1 g/L); 2) soy-based formula containing Orafti (3g/L); 3) milk-based formula with no prebiotic added; 4) milk-based formula containing Bimuno GOS only (5g/L); 5) milk-based formula containing Vivinal GOS only (5g/L); 6) milk-based formula containing Bimuno GOS and Nutraflora FOS (4 + 1 g/L); 7) milk-based formula containing Vivinal GOS and Orafti FOS (4 + 1 g/L); 8) soy-based formula with no prebiotic added. Diets were isonitrogenous, isocaloric and fed at 250mL/kg body weight every 4 hours for a 2-week period. Blood samples were taken before feeding (time 0) and an hour after feeding (time 60) on the day of sacrifice. Values represent least squared means \pm SEM and statistical significance set at $P < 0.05$.

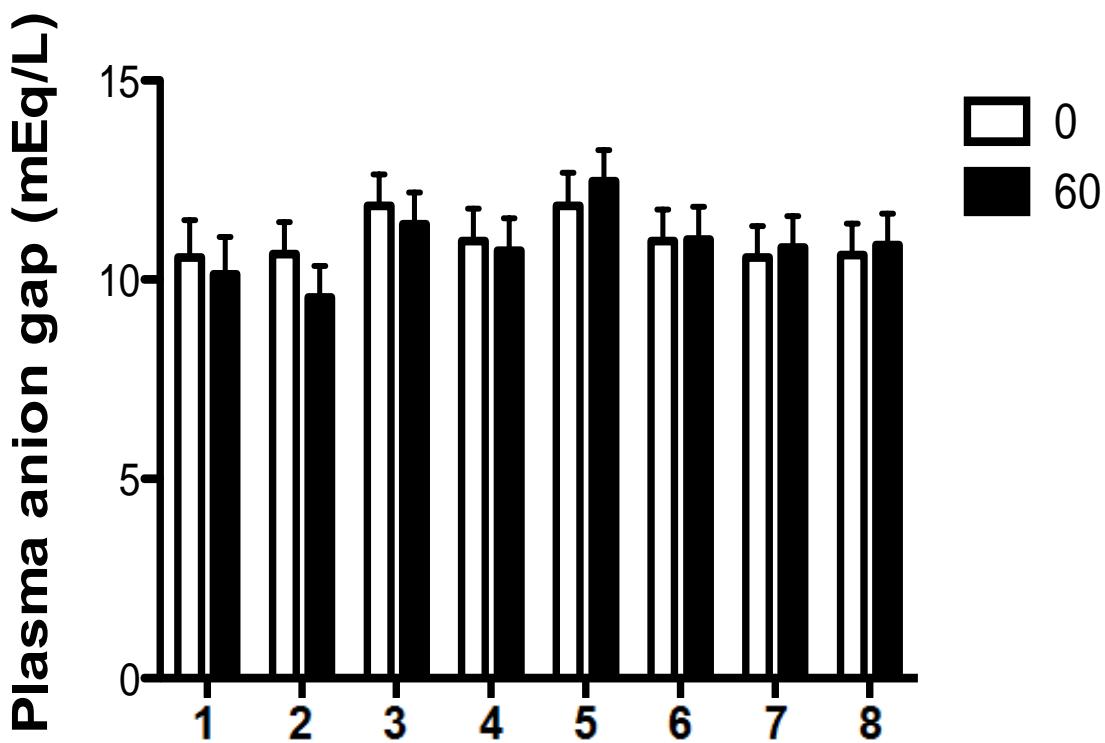


Figure 2.10. Effects of GOS and FOS dietary supplementation on plasma anion gap (mEq/L) of pigs fed experimental milk replacer formula. Piglets ($1.628 +/_{.037}$ Kg BW) were randomly assigned to one of eight diets ($n = 6/\text{diet}$): 1) milk-based formula containing Bimuno GOS and Vivinal FOS (4 + 1 g/L); 2) soy-based formula containing Orafti (3g/L); 3) milk-based formula with no prebiotic added; 4) milk-based formula containing Bimuno GOS only (5g/L); 5) milk-based formula containing Vivinal GOS only (5g/L); 6) milk-based formula containing Bimuno GOS and Nutraflora FOS (4 + 1 g/L); 7) milk-based formula containing Vivinal GOS and Orafti FOS (4 + 1 g/L); 8) soy-based formula with no prebiotic added. Diets were isonitrogenous, isocaloric and fed at 250mL/kg body weight every 4 hours for a 2-week period. Blood samples were taken before feeding (time 0) and an hour after feeding (time 60) on the day of sacrifice. Values represent least squared means \pm SEM and statistical significance set at $P < 0.05$.

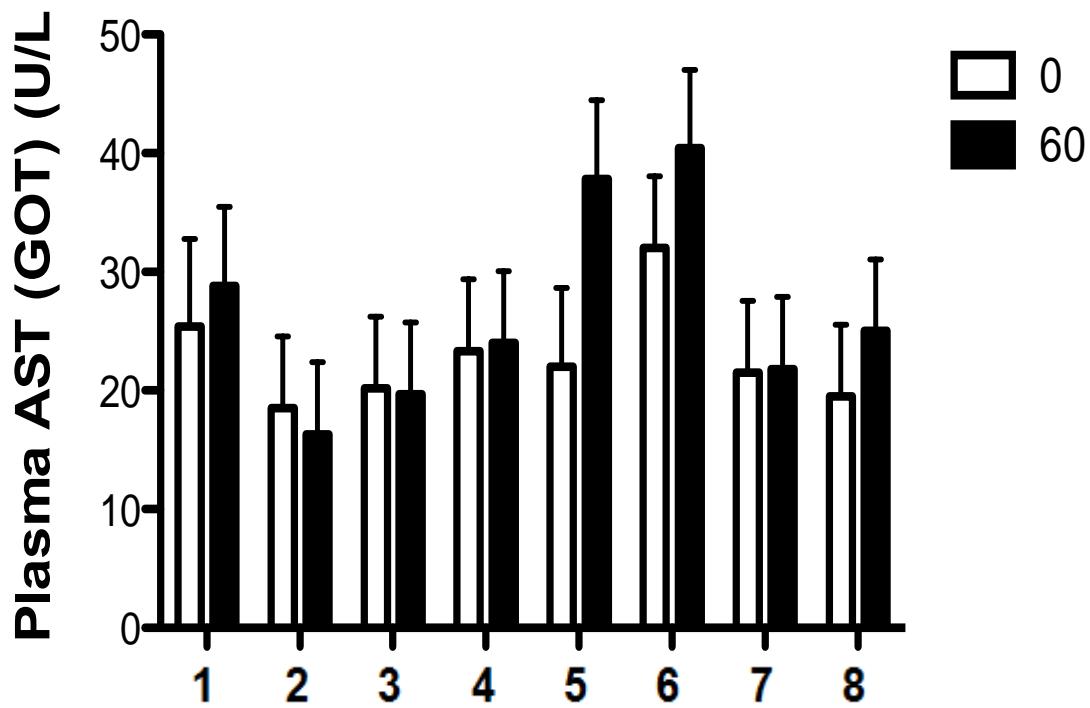


Figure 2.11. Effects of GOS and FOS dietary supplementation on plasma AST (GOT) (U/L) of pigs fed experimental milk replacer formula. Piglets ($1.628 +/_{.037}$ Kg BW) were randomly assigned to one of eight diets ($n = 6/\text{diet}$): 1) milk-based formula containing Bimuno GOS and Vivinal FOS (4 + 1 g/L); 2) soy-based formula containing Orafti (3g/L); 3) milk-based formula with no prebiotic added; 4) milk-based formula containing Bimuno GOS only (5g/L); 5) milk-based formula containing Vivinal GOS only (5g/L); 6) milk-based formula containing Bimuno GOS and Nutraflora FOS (4 + 1 g/L); 7) milk-based formula containing Vivinal GOS and Orafti FOS (4 + 1 g/L); 8) soy-based formula with no prebiotic added. Diets were isonitrogenous, isocaloric and fed at 250mL/kg body weight every 4 hours for a 2 -week period. Blood samples were taken before feeding (time 0) and an hour after feeding (time 60) on the day of sacrifice. Values represent least squared means \pm SEM and statistical significance set at $P < 0.05$.

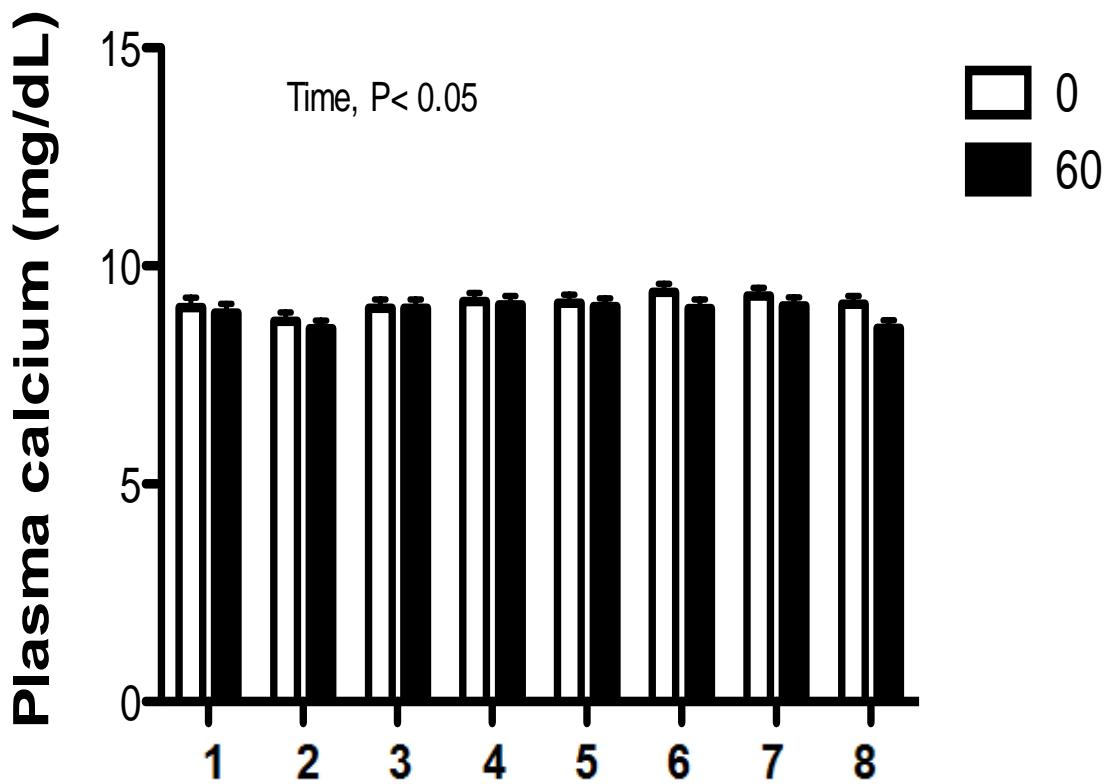


Figure 2.12. Effects of GOS and FOS dietary supplementation on plasma calcium (mg/dL) of pigs fed experimental milk replacer formula. Piglets ($1.628 +/_{.037}$ Kg BW) were randomly assigned to one of eight diets ($n = 6/\text{diet}$): 1) milk-based formula containing Bimuno GOS and Vivinal FOS (4 + 1 g/L); 2) soy-based formula containing Orafti (3g/L); 3) milk-based formula with no prebiotic added; 4) milk-based formula containing Bimuno GOS only (5g/L); 5) milk-based formula containing Vivinal GOS only (5g/L); 6) milk-based formula containing Bimuno GOS and Nutraflora FOS (4 + 1 g/L); 7) milk-based formula containing Vivinal GOS and Orafti FOS (4 + 1 g/L); 8) soy-based formula with no prebiotic added. Diets were isonitrogenous, isocaloric and fed at 250mL/kg body weight every 4 hours for a 2 -week period. Blood samples were taken before feeding (time 0) and an hour after feeding (time 60) on the day of sacrifice. Values represent least squared means \pm SEM and statistical significance set at $P < 0.05$.

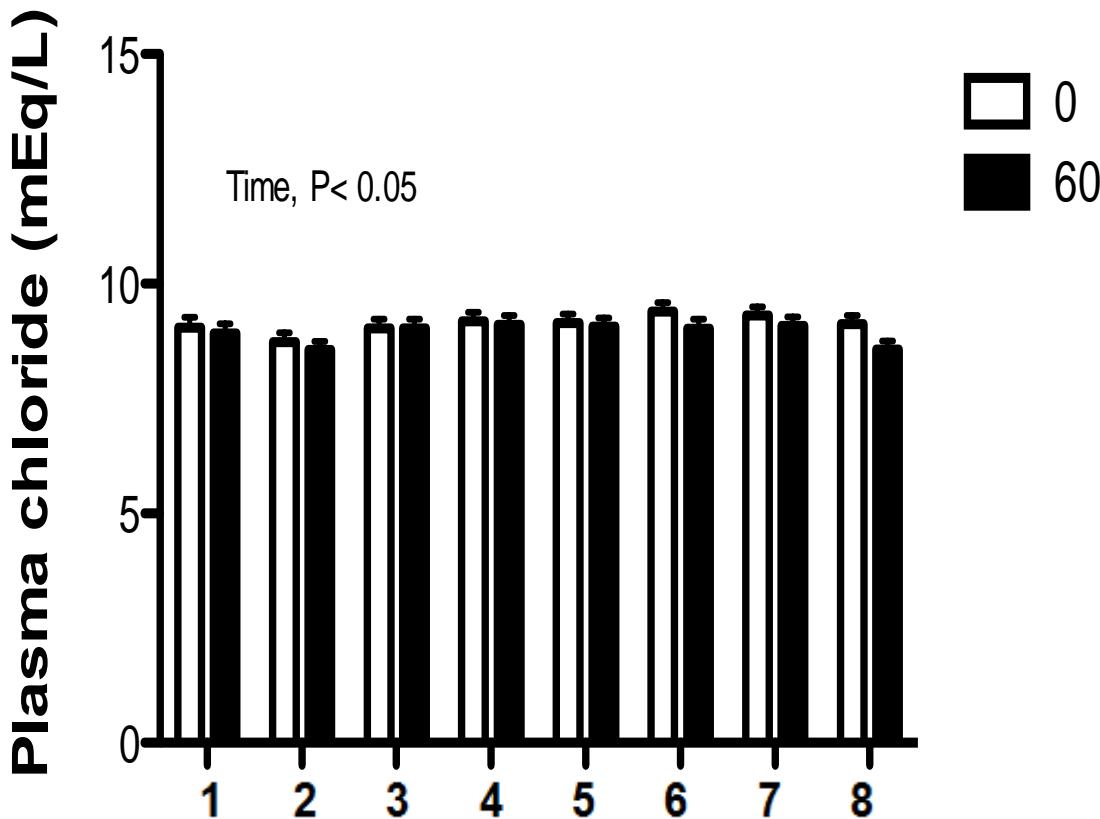


Figure 2.13. Effects of GOS and FOS dietary supplementation on plasma chloride (mEq/L) of pigs fed experimental milk replacer formula. Piglets ($1.628 \pm .037$ Kg BW) were randomly assigned to one of eight diets ($n = 6/\text{diet}$): 1) milk-based formula containing Bimuno GOS and Vivinal FOS (4 + 1 g/L); 2) soy-based formula containing Orafti (3g/L); 3) milk-based formula with no prebiotic added; 4) milk-based formula containing Bimuno GOS only (5g/L); 5) milk-based formula containing Vivinal GOS only (5g/L); 6) milk-based formula containing Bimuno GOS and Nutraflora FOS (4 + 1 g/L); 7) milk-based formula containing Vivinal GOS and Orafti FOS (4 + 1 g/L); 8) soy-based formula with no prebiotic added. Diets were isonitrogenous, isocaloric and fed at 250mL/kg body weight every 4 hours for a 2-week period. Blood samples were taken before feeding (time 0) and an hour after feeding (time 60) on the day of sacrifice. Values represent least squared means \pm SEM and statistical significance set at $P < 0.05$.

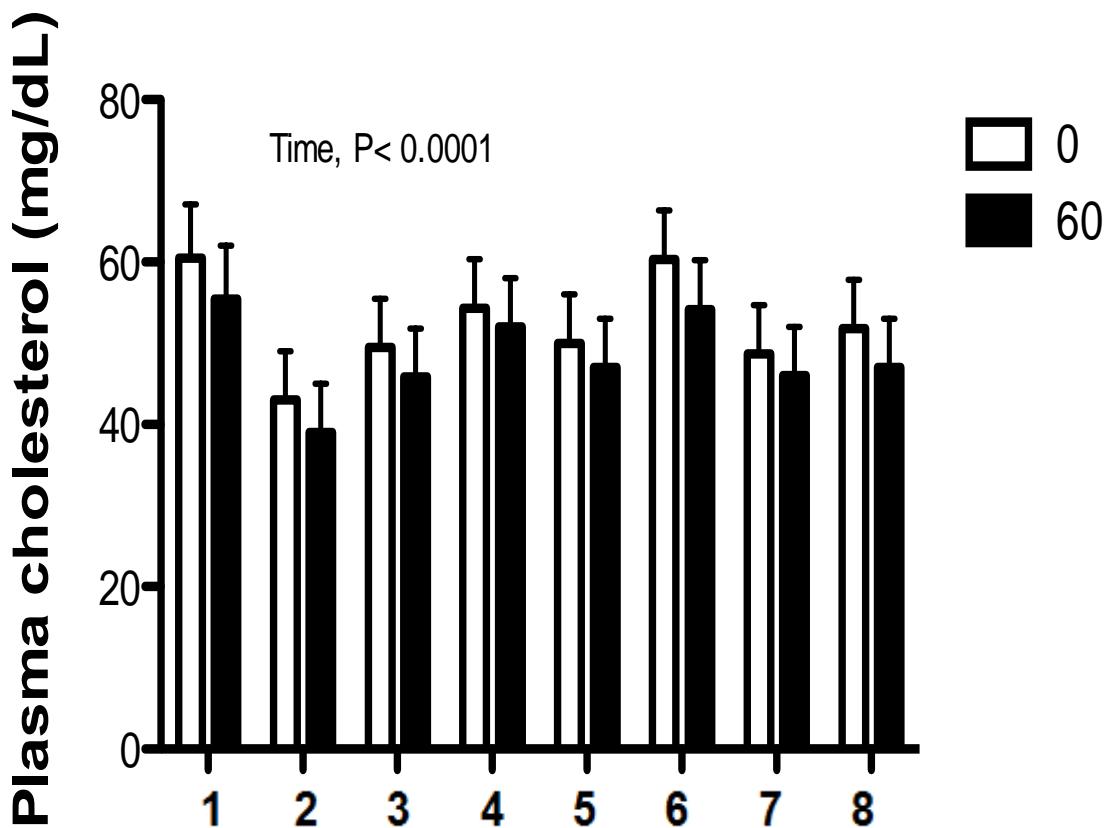


Figure 2.14. Effects of GOS and FOS dietary supplementation on plasma cholesterol (mg/dL) of pigs fed experimental milk replacer formula. Piglets ($1.628 +/_{.037}$ Kg BW) were randomly assigned to one of eight diets ($n = 6/\text{diet}$): 1) milk-based formula containing Bimuno GOS and Vivinal FOS (4 + 1 g/L); 2) soy-based formula containing Orafti (3g/L); 3) milk-based formula with no prebiotic added; 4) milk-based formula containing Bimuno GOS only (5g/L); 5) milk-based formula containing Vivinal GOS only (5g/L); 6) milk-based formula containing Bimuno GOS and Nutraflora FOS (4 + 1 g/L); 7) milk-based formula containing Vivinal GOS and Orafti FOS (4 + 1 g/L); 8) soy-based formula with no prebiotic added. Diets were isonitrogenous, isocaloric and fed at 250mL/kg body weight every 4 hours for a 2-week period. Blood samples were taken before feeding (time 0) and an hour after feeding (time 60) on the day of sacrifice. Values represent least squared means \pm SEM and statistical significance set at $P < 0.05$.

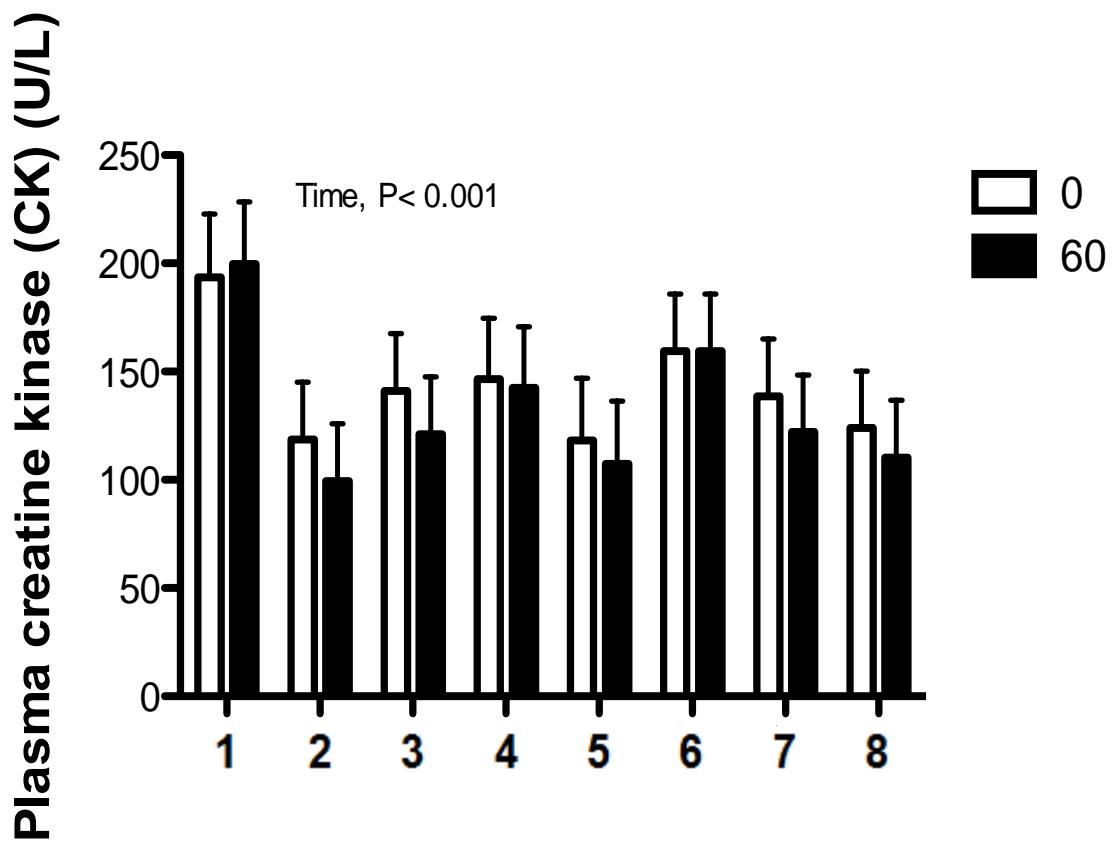


Figure 2.15. Effects of GOS and FOS dietary supplementation on plasma creatine kinase (CK) (U/L) of pigs fed experimental milk replacer formula. Piglets ($1.628 +/_{.037}$ Kg BW) were randomly assigned to one of eight diets ($n = 6/diet$): 1) milk-based formula containing Bimuno GOS and Vivinal FOS (4 + 1 g/L); 2) soy-based formula containing Orafti (3g/L); 3) milk-based formula with no prebiotic added; 4) milk-based formula containing Bimuno GOS only (5g/L); 5) milk-based formula containing Vivinal GOS only (5g/L); 6) milk-based formula containing Bimuno GOS and Nutraflora FOS (4 + 1 g/L); 7) milk-based formula containing Vivinal GOS and Orafti FOS (4 + 1 g/L); 8) soy-based formula with no prebiotic added. Diets were isonitrogenous, isocaloric and fed at 250mL/kg body weight every 4 hours for a 2-week period. Blood samples were taken before feeding (time 0) and an hour after feeding (time 60) on the day of sacrifice. Values represent least squared means \pm SEM and statistical significance set at $P < 0.05$.

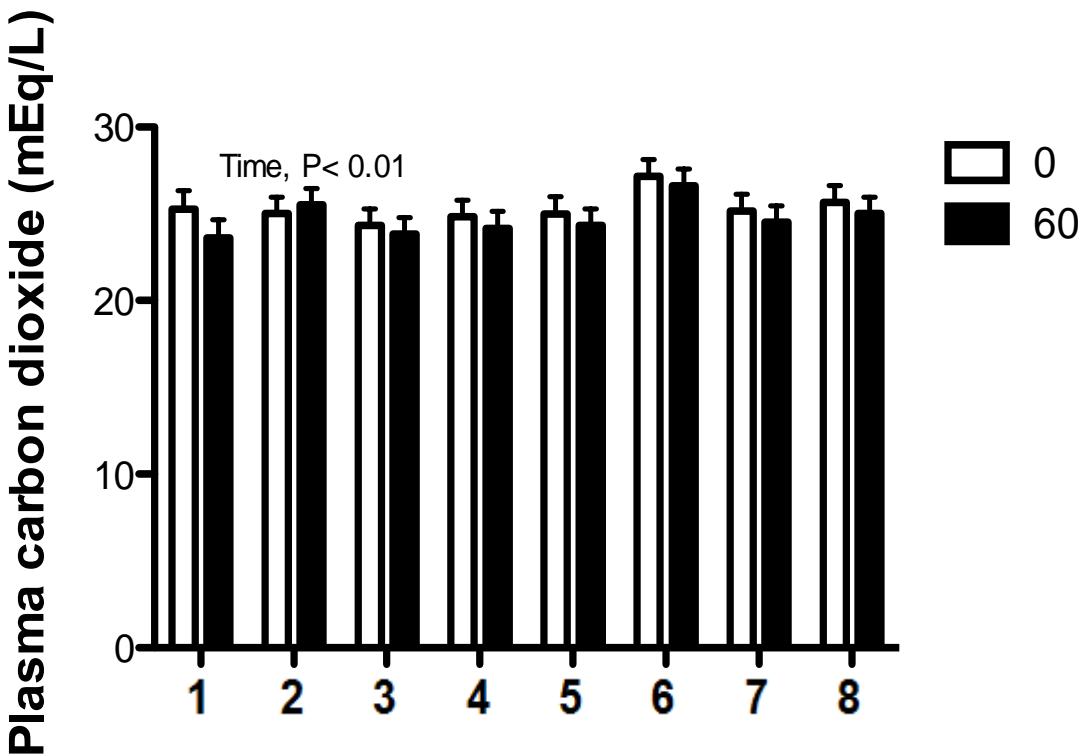


Figure 2.16. Effects of GOS and FOS dietary supplementation on plasma carbon dioxide (mEq/L) of pigs fed experimental milk replacer formula. Piglets ($1.628 +/_{.037}$ Kg BW) were randomly assigned to one of eight diets ($n = 6/\text{diet}$): 1) milk-based formula containing Bimuno GOS and Vivinal FOS (4 + 1 g/L); 2) soy-based formula containing Orafti (3g/L); 3) milk-based formula with no prebiotic added; 4) milk-based formula containing Bimuno GOS only(5g/L); 5) milk-based formula containing Vivinal GOS only (5g/L); 6) milk-based formula containing Bimuno GOS and Nutraflora FOS (4 + 1 g/L); 7) milk-based formula containing Vivinal GOS and Orafti FOS (4 + 1 g/L); 8) soy-based formula with no prebiotic added. Diets were isonitrogenous, isocaloric and fed at 250mL/kg body weight every 4 hours for a 2 -week period. Blood samples were taken before feeding (time 0) and an hour after feeding (time 60) on the day of sacrifice. Values represent least squared means \pm SEM and statistical significance set at $P < 0.05$.

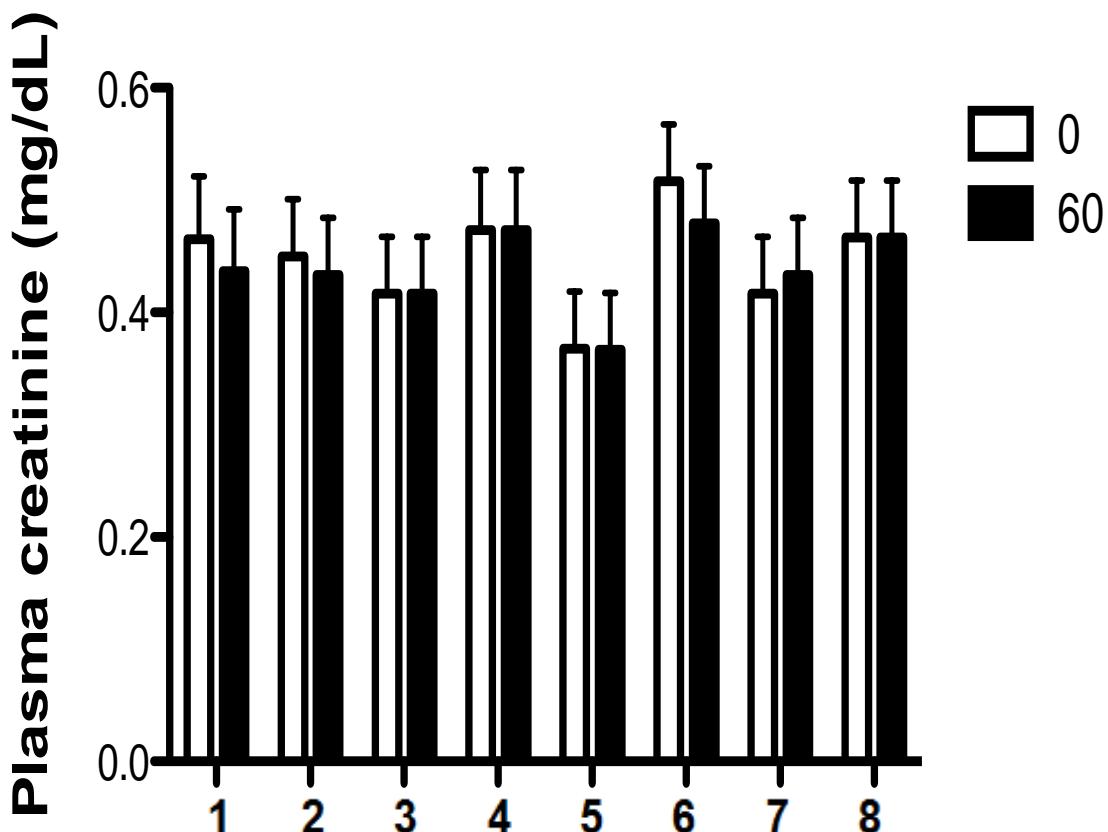


Figure 2.17. Effects of GOS and FOS dietary supplementation on plasma creatinine (mg/dL) of pigs fed experimental milk replacer formula. Piglets ($1.628 +/_{.037}$ Kg BW) were randomly assigned to one of eight diets ($n = 6/\text{diet}$): 1) milk-based formula containing Bimuno GOS and Vivinal FOS (4 + 1 g/L); 2) soy-based formula containing Orafti (3g/L); 3) milk-based formula with no prebiotic added; 4) milk-based formula containing Bimuno GOS only (5g/L); 5) milk-based formula containing Vivinal GOS only (5g/L); 6) milk-based formula containing Bimuno GOS and Nutraflora FOS (4 + 1 g/L); 7) milk-based formula containing Vivinal GOS and Orafti FOS (4 + 1 g/L); 8) soy-based formula with no prebiotic added. Diets were isonitrogenous, isocaloric and fed at 250mL/kg body weight every 4 hours for a 2-week period. Blood samples were taken before feeding (time 0) and an hour after feeding (time 60) on the day of sacrifice. Values represent least squared means \pm SEM and statistical significance set at $P < 0.05$.

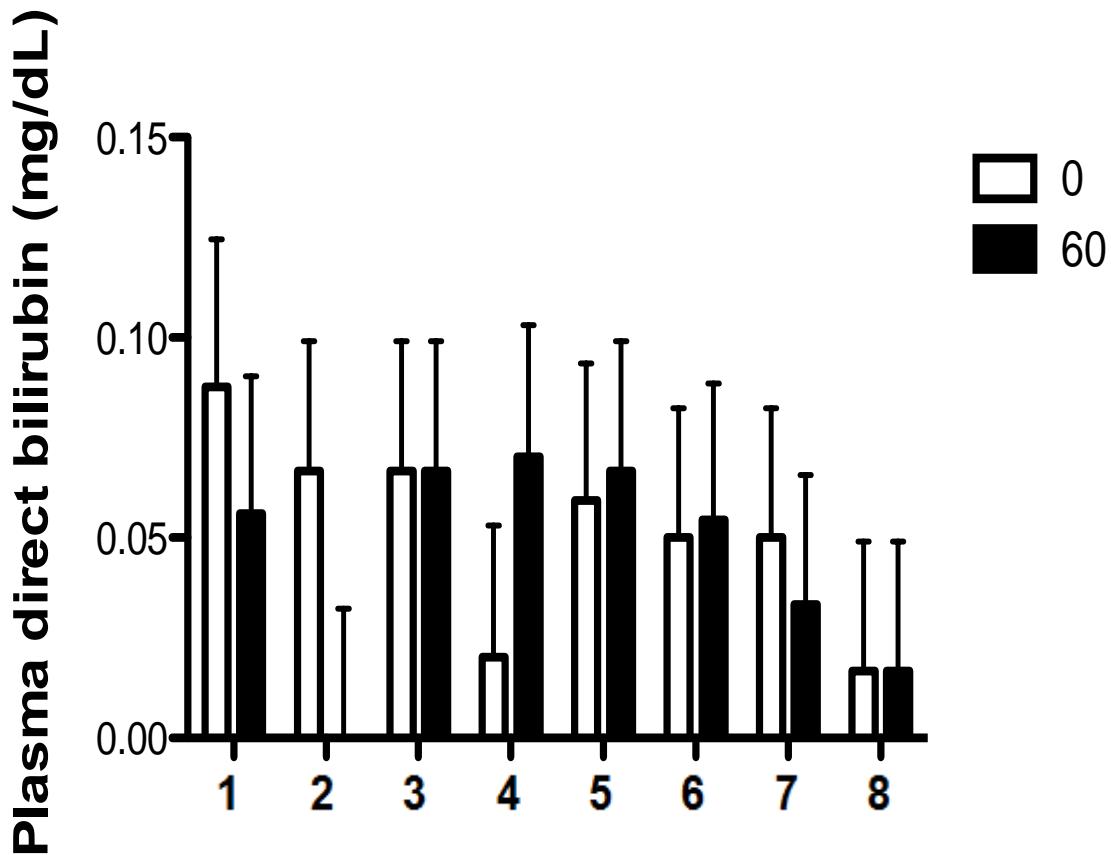


Figure 2.18. Effects of GOS and FOS dietary supplementation on plasma direct bilirubin (mg/dL) of pigs fed experimental milk replacer formula. Piglets ($1.628 +/_{.037}$ Kg BW) were randomly assigned to one of eight diets ($n = 6/\text{diet}$): 1) milk-based formula containing Bimuno GOS and Vivinal FOS (4 + 1 g/L); 2) soy-based formula containing Orafti (3g/L); 3) milk-based formula with no prebiotic added; 4) milk-based formula containing Bimuno GOS only (5g/L); 5) milk-based formula containing Vivinal GOS only (5g/L); 6) milk-based formula containing Bimuno GOS and Nutraflora FOS (4 + 1 g/L); 7) milk-based formula containing Vivinal GOS and Orafti FOS (4 + 1 g/L); 8) soy-based formula with no prebiotic added. Diets were isonitrogenous, isocaloric and fed at 250mL/kg body weight every 4 hours for a 2-week period. Blood samples were taken before feeding (time 0) and an hour after feeding (time 60) on the day of sacrifice. Values represent least squared means \pm SEM and statistical significance set at $P < 0.05$.

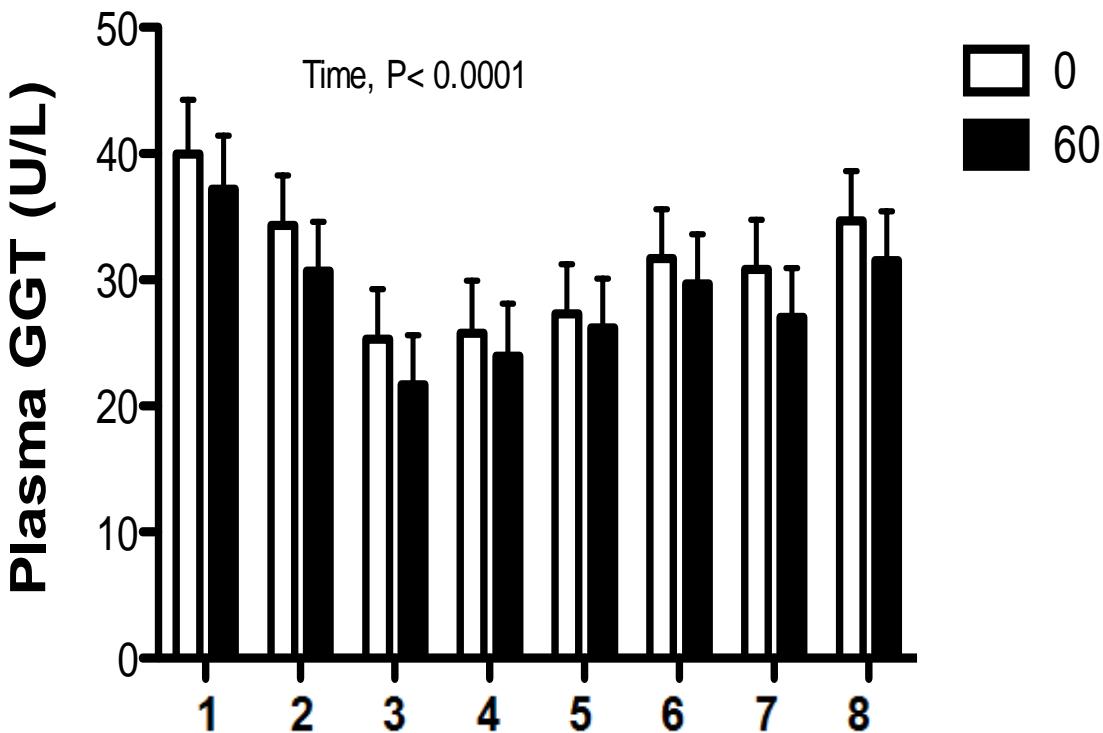


Figure 2.19. Effects of GOS and FOS dietary supplementation on plasma GGT (U/L) of pigs fed experimental milk replacer formula. Piglets ($1.628 +/_{.037}$ Kg BW) were randomly assigned to one of eight diets ($n = 6/\text{diet}$): 1) milk-based formula containing Bimuno GOS and Vivinal FOS (4 + 1 g/L); 2) soy-based formula containing Orafti (3g/L); 3) milk-based formula with no prebiotic added; 4) milk-based formula containing Bimuno GOS only (5g/L); 5) milk-based formula containing Vivinal GOS only (5g/L); 6) milk-based formula containing Bimuno GOS and Nutraflora FOS (4 + 1 g/L); 7) milk-based formula containing Vivinal GOS and Orafti FOS(4 + 1 g/L); 8) soy-based formula with no prebiotic added. Diets were isonitrogenous, isocaloric and fed at 250 mL/kg body weight every 4 hours for a 2-week period. Blood samples were taken before feeding (time 0) and an hour after feeding (time 60) on the day of sacrifice. Values represent least squared means \pm SEM and statistical significance set at $P < 0.05$.

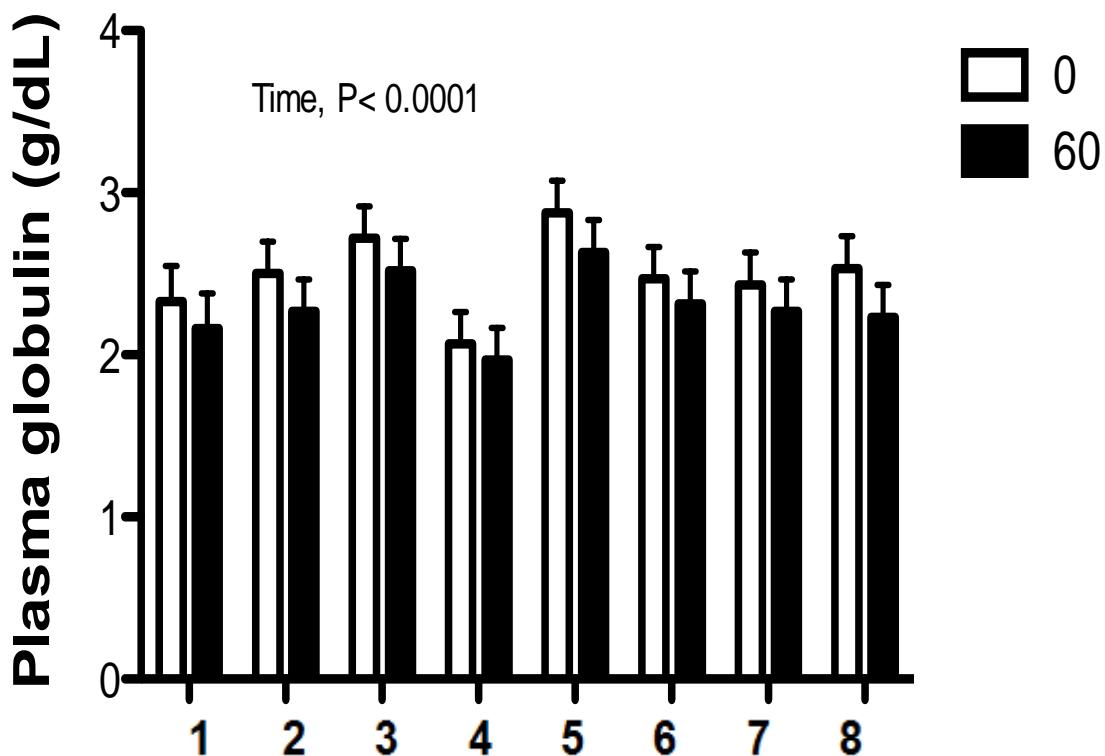


Figure 2.20. Effects of GOS and FOS dietary supplementation on plasma globulin (g/dL) of pigs fed experimental milk replacer formula. Piglets ($1.628 +/_{.037}$ Kg BW) were randomly assigned to one of eight diets ($n = 6/\text{diet}$): 1) milk-based formula containing Bimuno GOS and Vivinal FOS (4 + 1 g/L); 2) soy-based formula containing Orafti (3g/L); 3) milk-based formula with no prebiotic added; 4) milk-based formula containing Bimuno GOS only (5g/L); 5) milk-based formula containing Vivinal GOS only (5g/L); 6) milk-based formula containing Bimuno GOS and Nutraflora FOS (4 + 1 g/L); 7) milk-based formula containing Vivinal GOS and Orafti FOS(4 + 1 g/L); 8) soy-based formula with no prebiotic added. Diets were isonitrogenous, isocaloric and fed at 250mL/kg body weight every 4 hours for a 2-week period. Blood samples were taken before feeding (time 0) and an hour after feeding (time 60) on the day of sacrifice. Values represent least squared means \pm SEM and statistical significance set at $P < 0.05$.

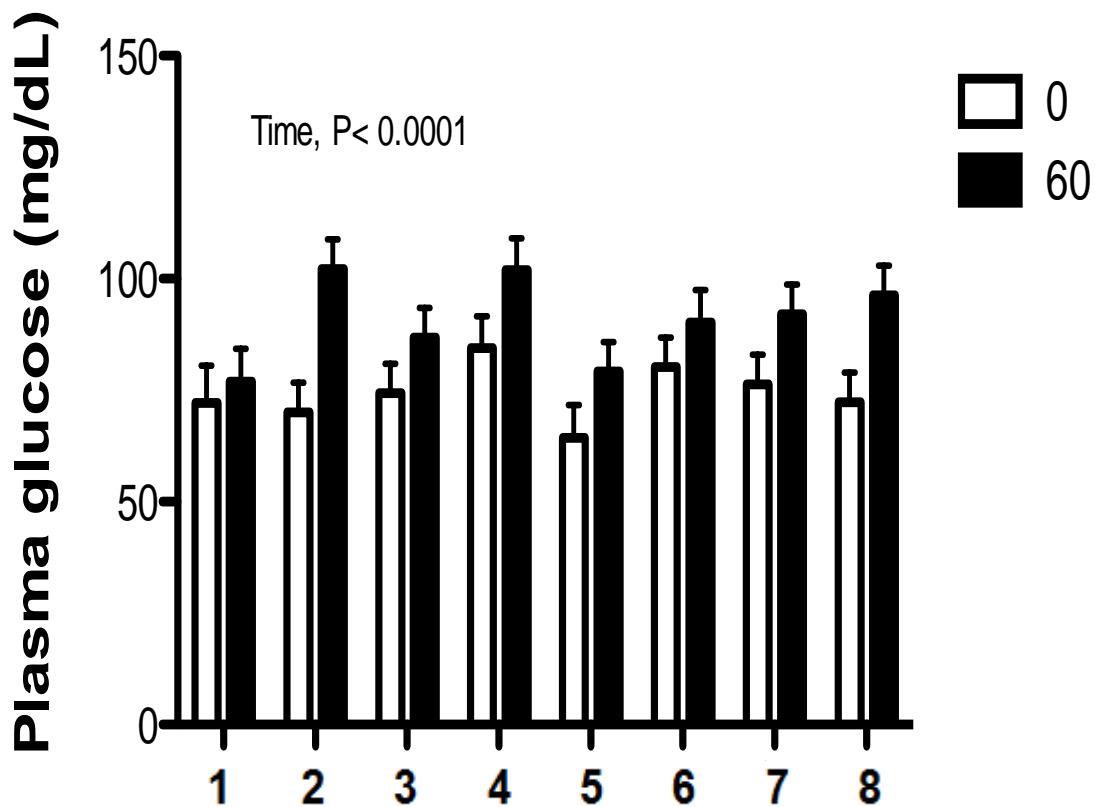


Figure 2.21. Effects of GOS and FOS dietary supplementation on plasma glucose (mg/dL) of pigs fed experimental milk replacer formula. Piglets ($1.628 +/_{.037}$ Kg BW) were randomly assigned to one of eight diets ($n = 6/\text{diet}$): 1) milk-based formula containing Bimuno GOS and Vivinal FOS (4 + 1 g/L); 2) soy-based formula containing Orafti (3g/L); 3) milk-based formula with no prebiotic added; 4) milk-based formula containing Bimuno GOS only (5g/L); 5) milk-based formula containing Vivinal GOS only (5g/L); 6) milk-based formula containing Bimuno GOS and Nutraflora FOS (4 + 1 g/L); 7) milk-based formula containing Vivinal GOS and Orafti FOS (4 + 1 g/L); 8) soy-based formula with no prebiotic added. Diets were isonitrogenous, isocaloric and fed at 250mL/kg body weight every 4 hours for a 2-week period. Blood samples were taken before feeding (time 0) and an hour after feeding (time 60) on the day of sacrifice. Values represent least squared means \pm SEM and statistical significance set at $P < 0.05$.

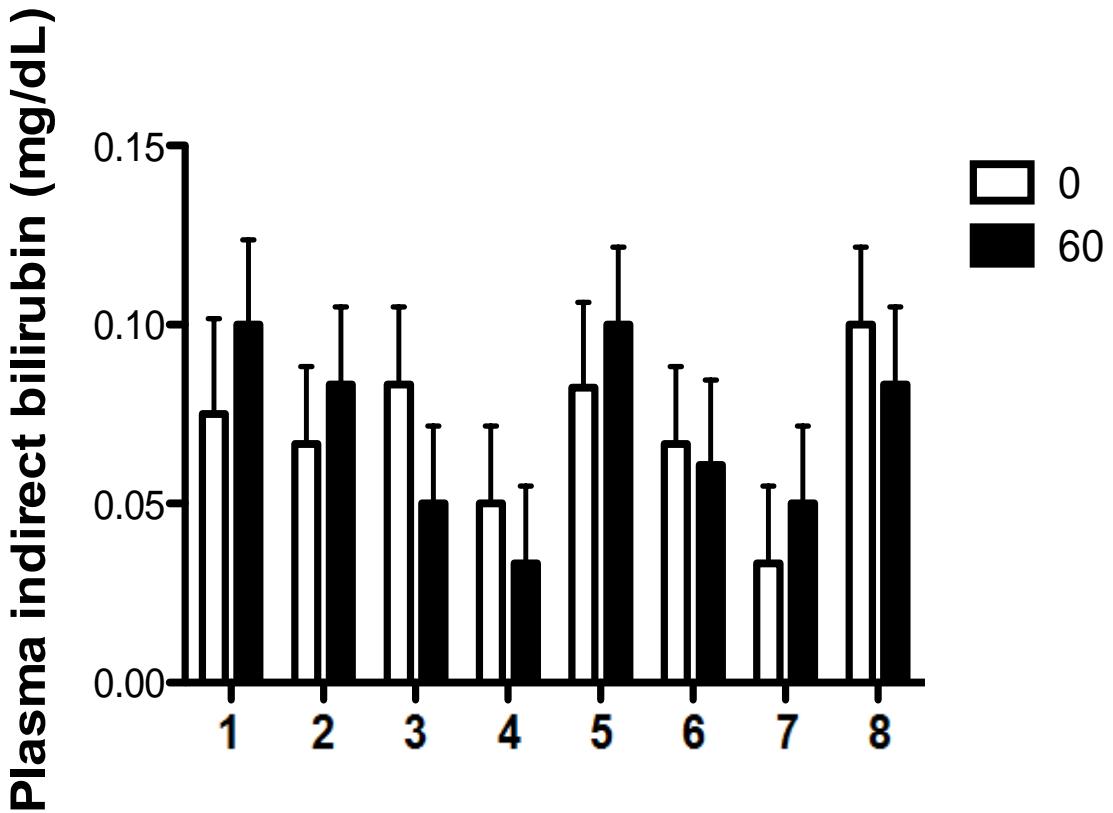


Figure 2.22. Effects of GOS and FOS dietary supplementation on plasma indirect bilirubin (mg/dL) of pigs fed experimental milk replacer formula. Piglets ($1.628 +/_{.037}$ Kg BW) were randomly assigned to one of eight diets ($n = 6$ /diet): 1) milk-based formula containing Bimuno GOS and Vivinal FOS (4 + 1 g/L); 2) soy-based formula containing Orafti (3g/L); 3) milk-based formula with no prebiotic added; 4) milk-based formula containing Bimuno GOS only (5g/L); 5) milk-based formula containing Vivinal GOS only (5g/L); 6) milk-based formula containing Bimuno GOS and Nutraflora FOS (4 + 1 g/L); 7) milk-based formula containing Vivinal GOS and Orafti FOS (4 + 1 g/L); 8) soy-based formula with no prebiotic added. Diets were isonitrogenous, isocaloric and fed at 250mL/kg body weight every 4 hours for a 2-week period. Blood samples were taken before feeding (time 0) and an hour after feeding (time 60) on the day of sacrifice. Values represent least squared means \pm SEM and statistical significance set at $P < 0.05$.

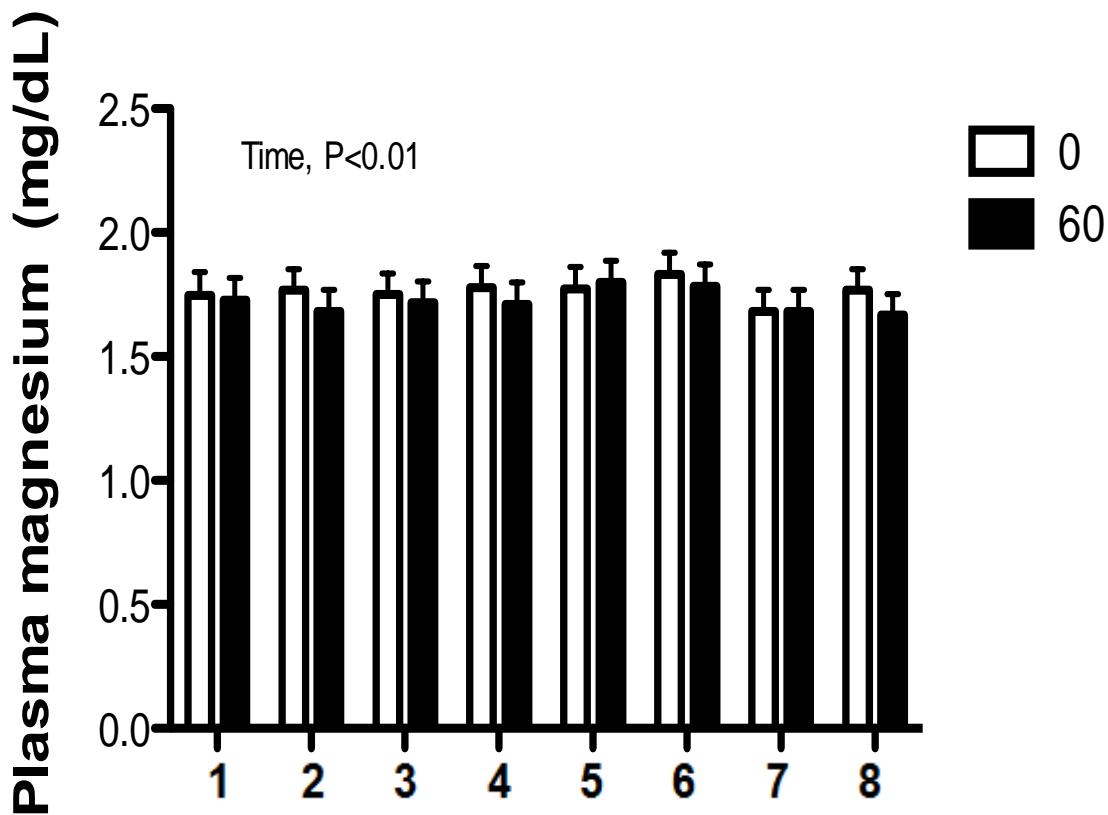


Figure 2.23. Effects of GOS and FOS dietary supplementation on plasma magnesium (mg/dL) of pigs fed experimental milk replacer formula. Piglets ($1.628 \pm .037$ Kg BW) were randomly assigned to one of eight diets ($n = 6$ /diet): 1) milk-based formula containing Bimuno GOS and Vivinal FOS (4 + 1 g/L); 2) soy-based formula containing Orafti (3g/L); 3) milk-based formula with no prebiotic added; 4) milk-based formula containing Bimuno GOS only (5g/L); 5) milk-based formula containing Vivinal GOS only (5g/L); 6) milk-based formula containing Bimuno GOS and Nutraflora FOS (4 + 1 g/L); 7) milk-based formula containing Vivinal GOS and Orafti FOS (4 + 1 g/L); 8) soy-based formula with no prebiotic added. Diets were isonitrogenous, isocaloric and fed at 250mL/kg body weight every 4 hours for a 2-week period. Blood samples were taken before feeding (time 0) and an hour after feeding (time 60) on the day of sacrifice. Values represent least squared means \pm SEM and statistical significance set at $P < 0.05$.

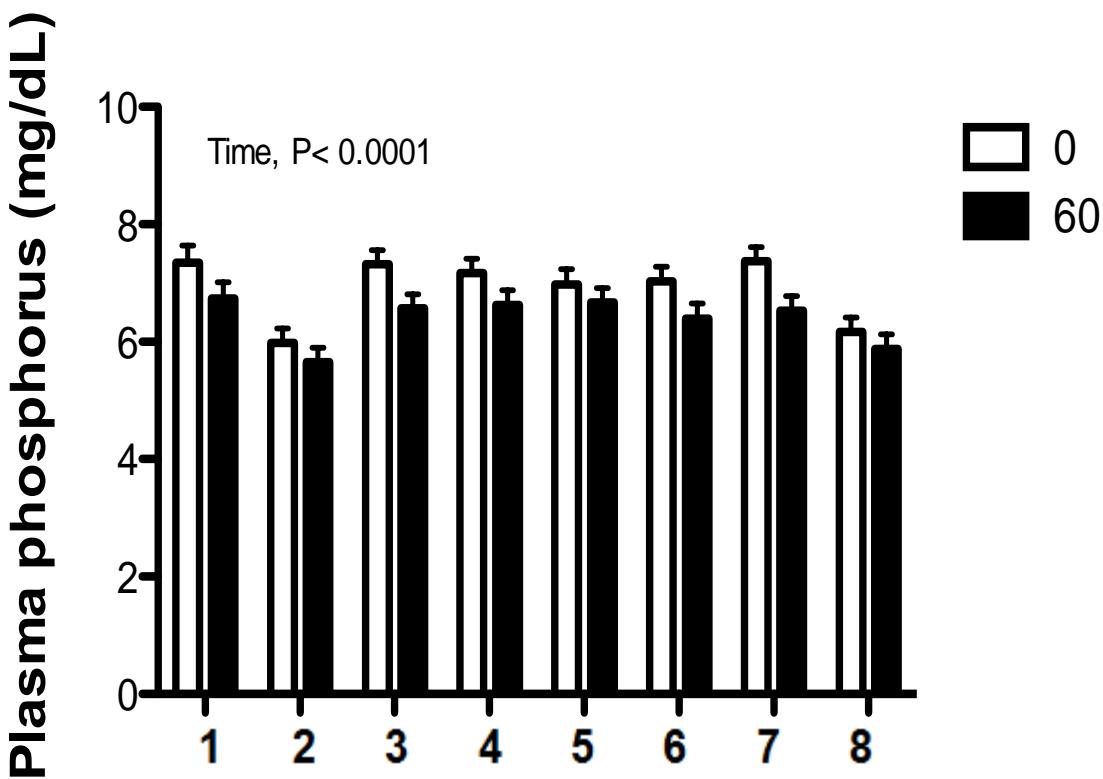


Figure 2.24. Effects of GOS and FOS dietary supplementation on plasma phosphorus (mg/dL) of pigs fed experimental milk replacer formula. Piglets ($1.628 +/_{.037}$ Kg BW) were randomly assigned to one of eight diets ($n = 6/\text{diet}$): 1) milk-based formula containing Bimuno GOS and Vivinal FOS (4 + 1 g/L); 2) soy-based formula containing Orafti (3g/L); 3) milk-based formula with no prebiotic added; 4) milk-based formula containing Bimuno GOS only (5g/L); 5) milk-based formula containing Vivinal GOS only (5g/L); 6) milk-based formula containing Bimuno GOS and Nutraflora FOS (4 + 1 g/L); 7) milk-based formula containing Vivinal GOS and Orafti FOS (4 + 1 g/L); 8) soy-based formula with no prebiotic added. Diets were isonitrogenous, isocaloric and fed at 250mL/kg body weight every 4 hours for a 2-week period. Blood samples were taken before feeding (time 0) and an hour after feeding (time 60) on the day of sacrifice. Values represent least squared means \pm SEM and statistical significance set at $P < 0.05$.

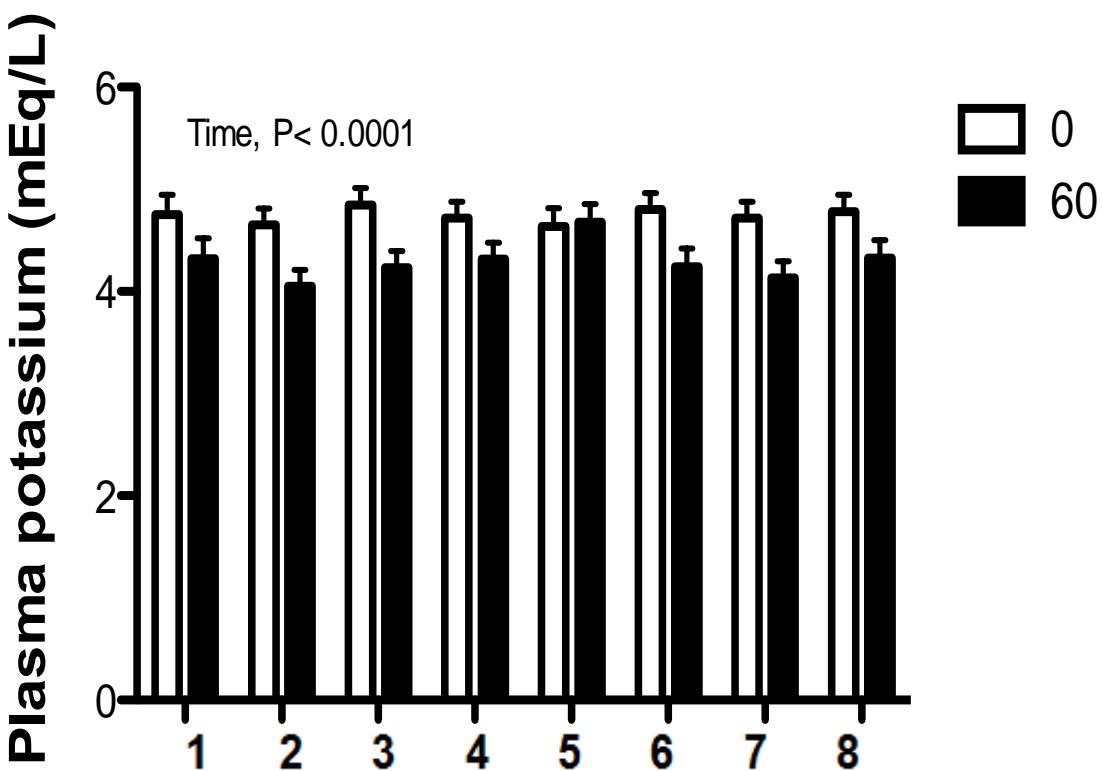


Figure 2.25. Effects of GOS and FOS dietary supplementation on plasma potassium (mEq/L) of pigs fed experimental milk replacer formula. Piglets ($1.628 +/_{.037}$ Kg BW) were randomly assigned to one of eight diets ($n = 6/\text{diet}$): 1) milk-based formula containing Bimuno GOS and Vivinal FOS (4 + 1 g/L); 2) soy-based formula containing Orafti (3g/L); 3) milk-based formula with no prebiotic added; 4) milk-based formula containing Bimuno GOS only (5g/L); 5) milk-based formula containing Vivinal GOS only (5g/L); 6) milk-based formula containing Bimuno GOS and Nutraflora FOS (4 + 1 g/L); 7) milk-based formula containing Vivinal GOS and Orafti FOS (4 + 1 g/L); 8) soy-based formula with no prebiotic added. Diets were isonitrogenous, isocaloric and fed at 250mL/kg body weight every 4 hours for a 2-week period. Blood samples were taken before feeding (time 0) and an hour after feeding (time 60) on the day of sacrifice. Values represent least squared means \pm SEM and statistical significance set at $P < 0.05$.

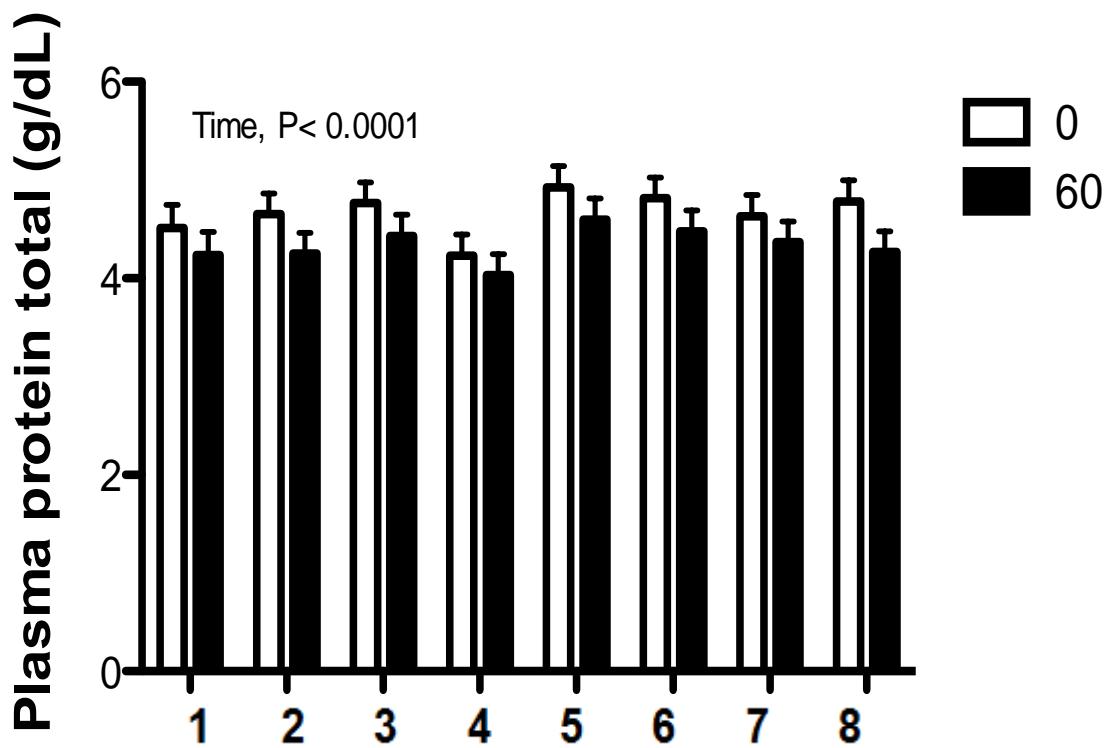


Figure 2.26. Effects of GOS and FOS dietary supplementation on plasma protein total (g/dL) of pigs fed experimental milk replacer formula. Piglets ($1.628 +/_{.037}$ Kg BW) were randomly assigned to one of eight diets ($n = 6/\text{diet}$): 1) milk-based formula containing Bimuno GOS and Vivinal FOS (4 + 1 g/L); 2) soy-based formula containing Orafti (3g/L); 3) milk-based formula with no prebiotic added; 4) milk-based formula containing Bimuno GOS only (5g/L); 5) milk-based formula containing Vivinal GOS only (5g/L); 6) milk-based formula containing Bimuno GOS and Nutraflora FOS (4 + 1 g/L); 7) milk-based formula containing Vivinal GOS and Orafti FOS (4 + 1 g/L); 8) soy-based formula with no prebiotic added. Diets were isonitrogenous, isocaloric and fed at 250mL/kg body weight every 4 hours for a 2-week period. Blood samples were taken before feeding (time 0) and an hour after feeding (time 60) on the day of sacrifice. Values represent least squared means \pm SEM and statistical significance set at $P < 0.05$.



Figure 2.27. Effects of GOS and FOS dietary supplementation on plasma sodium (mEq/L) of pigs fed experimental milk replacer formula. Piglets ($1.628 \pm .037$ Kg BW) were randomly assigned to one of eight diets ($n = 6/\text{diet}$): 1) milk-based formula containing Bimuno GOS and Vivinal FOS (4 + 1 g/L); 2) soy-based formula containing Orafti (3g/L); 3) milk-based formula with no prebiotic added; 4) milk-based formula containing Bimuno GOS only (5g/L); 5) milk-based formula containing Vivinal GOS only (5g/L); 6) milk-based formula containing Bimuno GOS and Nutraflora FOS (4 + 1 g/L); 7) milk-based formula containing Vivinal GOS and Orafti FOS (4 + 1 g/L); 8) soy-based formula with no prebiotic added. Diets were isonitrogenous, isocaloric and fed at 250mL/kg body weight every 4 hours for a 2-week period. Blood samples were taken before feeding (time 0) and an hour after feeding (time 60) on the day of sacrifice. Values represent least squared means \pm SEM and statistical significance set at $P < 0.05$.

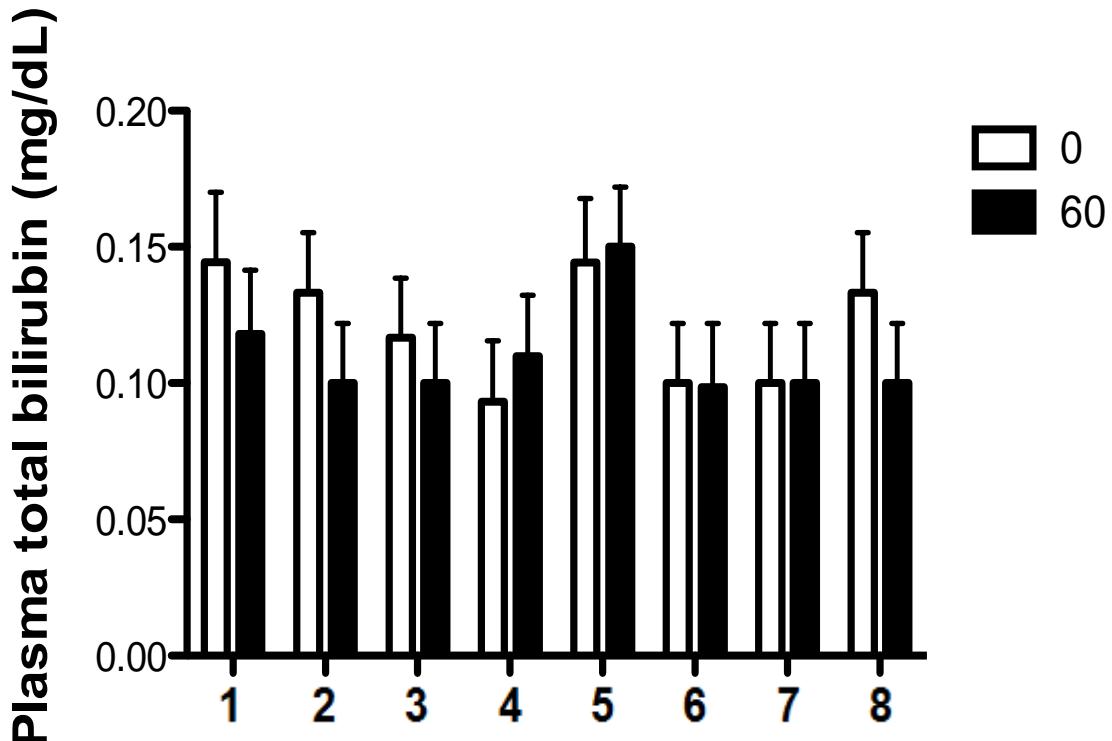


Figure 2.28. Effects of GOS and FOS dietary supplementation on plasma total bilirubin (mg/dL) of pigs fed experimental milk replacer formula. Piglets ($1.628 +/_{.037}$ Kg BW) were randomly assigned to one of eight diets ($n = 6$ /diet): 1) milk-based formula containing Bimuno GOS and Vivinal FOS (4 + 1 g/L); 2) soy-based formula containing Orafti (3g/L); 3) milk-based formula with no prebiotic added; 4) milk-based formula containing Bimuno GOS only(5g/L); 5) milk-based formula containing Vivinal GOS only (5g/L); 6) milk-based formula containing Bimuno GOS and Nutraflora FOS (4 + 1 g/L); 7) milk-based formula containing Vivinal GOS and Orafti FOS (4 + 1 g/L);8) soy-based formula with no prebiotic added. Diets were isonitrogenous, isocaloric and fed at 250mL/kg body weight every 4 hours for a 2 -week period. Blood samples were taken before feeding (time 0) and an hour after feeding (time 60) on the day of sacrifice. Values represent least squared means \pm SEM and statistical significance set at $P < 0.05$.

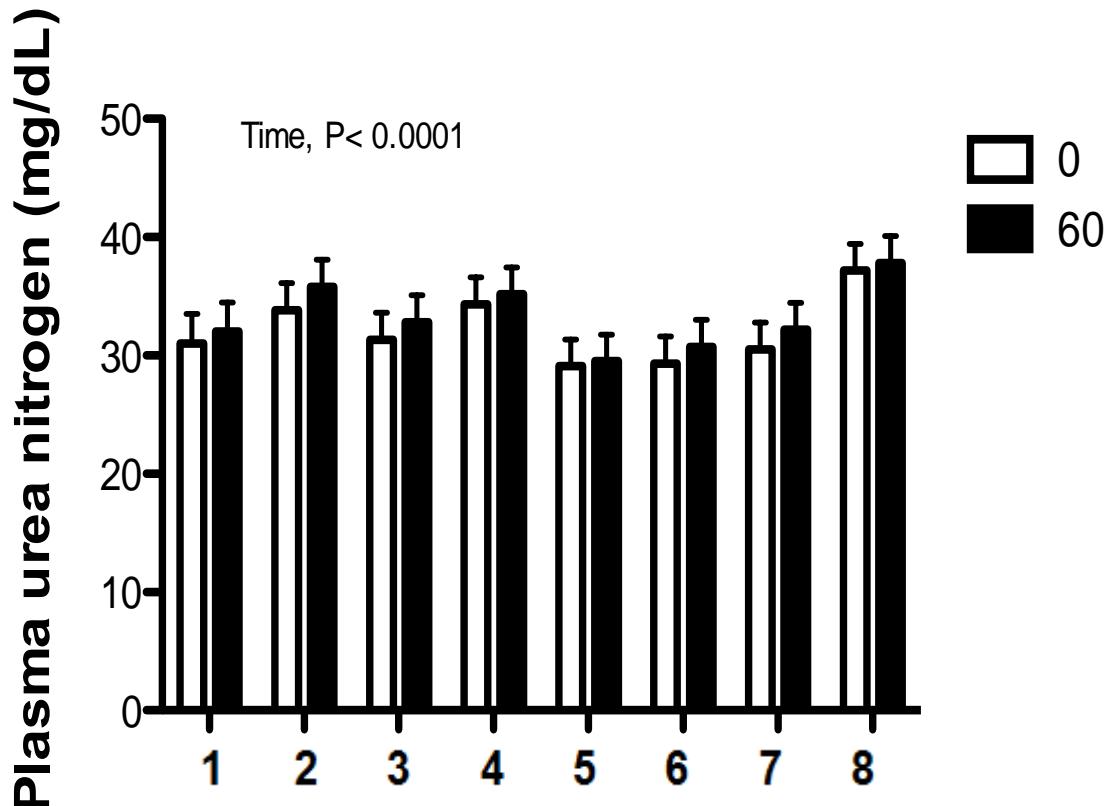


Figure 2.29. Effects of GOS and FOS dietary supplementation on plasma urea nitrogen (mg/dL) of pigs fed experimental milk replacer formula. Piglets ($1.628 +/_{.037}$ Kg BW) were randomly assigned to one of eight diets ($n = 6/\text{diet}$): 1) milk-based formula containing Bimuno GOS and Vivinal FOS (4 + 1 g/L); 2) soy-based formula containing Orafti (3g/L); 3) milk-based formula with no prebiotic added; 4) milk-based formula containing Bimuno GOS only (5g/L); 5) milk-based formula containing Vivinal GOS only (5g/L); 6) milk-based formula containing Bimuno GOS and Nutraflora FOS (4 + 1 g/L); 7) milk-based formula containing Vivinal GOS and Orafti FOS (4 + 1 g/L); 8) soy-based formula with no prebiotic added. Diets were isonitrogenous, isocaloric and fed at 250mL/kg body weight every 4 hours for a 2-week period. Blood samples were taken before feeding (time 0) and an hour after feeding (time 60) on the day of sacrifice. Values represent least squared means \pm SEM and statistical significance set at $P < 0.05$.

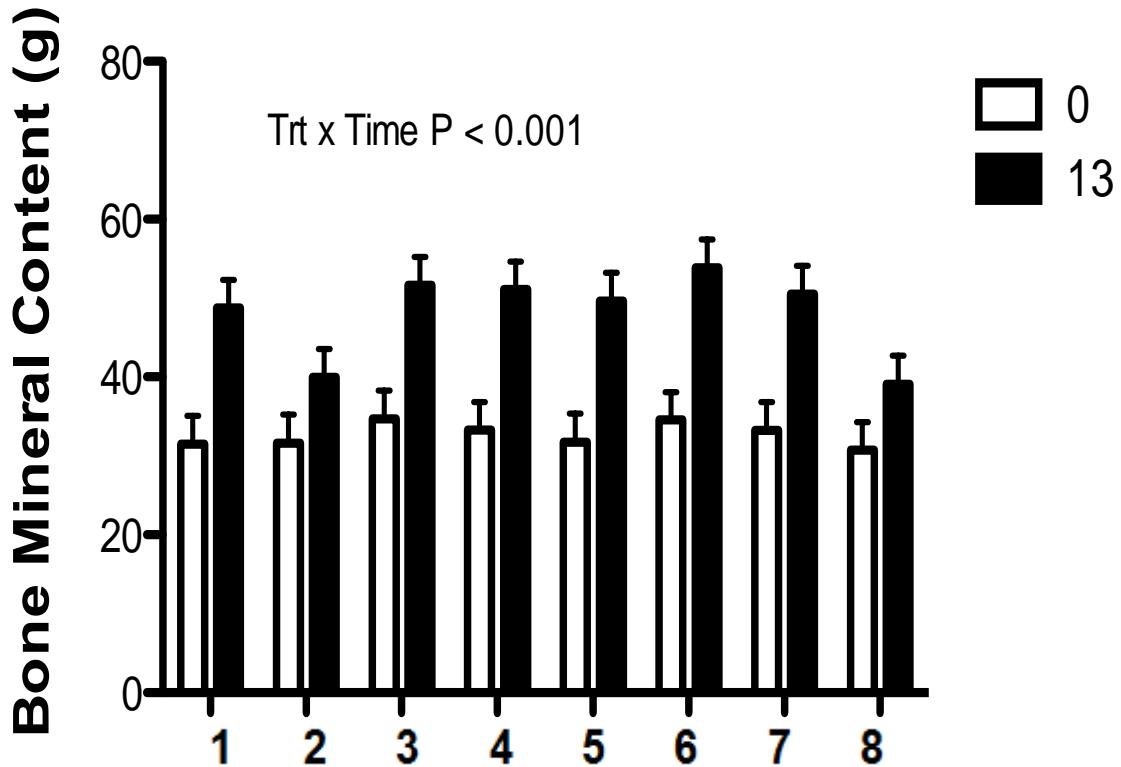


Figure 2.30. Effects of GOS and FOS dietary supplementation on bone mineral content (BMC) (g) of pigs fed experimental milk replacer formula. Piglets ($1.628 +/_{.037}$ Kg BW) were randomly assigned to one of eight diets ($n = 6/diet$): 1) milk-based formula containing Bimuno GOS and Vivinal FOS (4 + 1 g/L); 2) soy-based formula containing Orafti (3g/L); 3) milk-based formula with no prebiotic added; 4) milk-based formula containing Bimuno GOS only (5g/L); 5) milk-based formula containing Vivinal GOS only (5g/L); 6) milk-based formula containing Bimuno GOS and Nutraflora FOS (4 + 1 g/L); 7) milk-based formula containing Vivinal GOS and Orafti FOS (4 + 1 g/L); 8) soy-based formula with no prebiotic added. Diets were isonitrogenous, isocaloric and fed at 250mL/kg body weight every 4 hours for a 2-week period. Dual energy X-ray absorptiometry (DEXA) scans were performed before study (day 0) and at the end of study (day 13). Values represent least squared means \pm SEM and statistical significance set at $P < 0.05$.

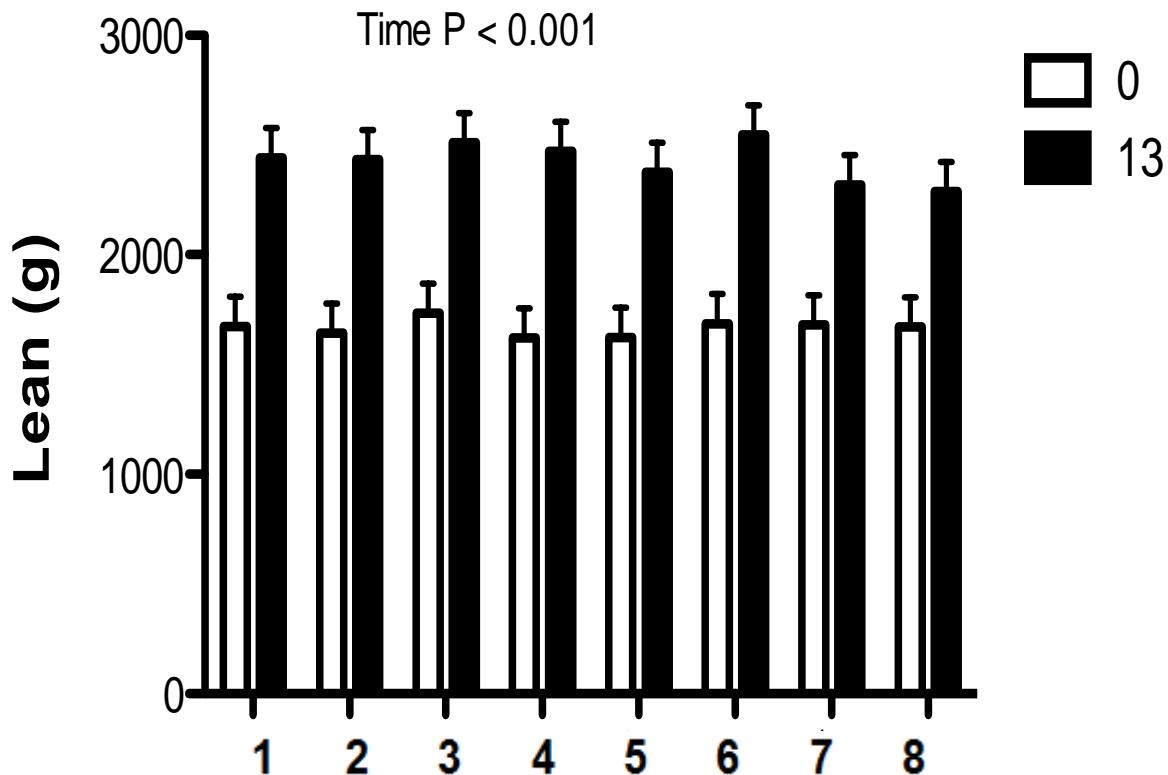


Figure 2.31. Effects of GOS and FOS dietary supplementation on lean (g) of pigs fed experimental milk replacer formula. Piglets ($1.628 +/_{.037}$ Kg BW) were randomly assigned to one of eight diets (n = 6/diet): 1) milk-based formula containing Bimuno GOS and Vivinal FOS (4 + 1 g/L); 2) soy-based formula containing Orafti (3g/L); 3) milk-based formula with no prebiotic added; 4) milk-based formula containing Bimuno GOS only (5g/L); 5) milk-based formula containing Vivinal GOS only (5g/L); 6) milk-based formula containing Bimuno GOS and Nutraflora FOS (4 + 1 g/L); 7) milk-based formula containing Vivinal GOS and Orafti FOS (4 + 1 g/L); 8) soy-based formula with no prebiotic added. Diets were isonitrogenous, isocaloric and fed at 250mL/kg body weight every 4 hours for a 2-week period. Dual energy X-ray absorptiometry (DEXA) scans were performed before study (day 0) and at the end of study (day 13). Values represent least squared means \pm SEM and statistical significance set at P < 0.05.

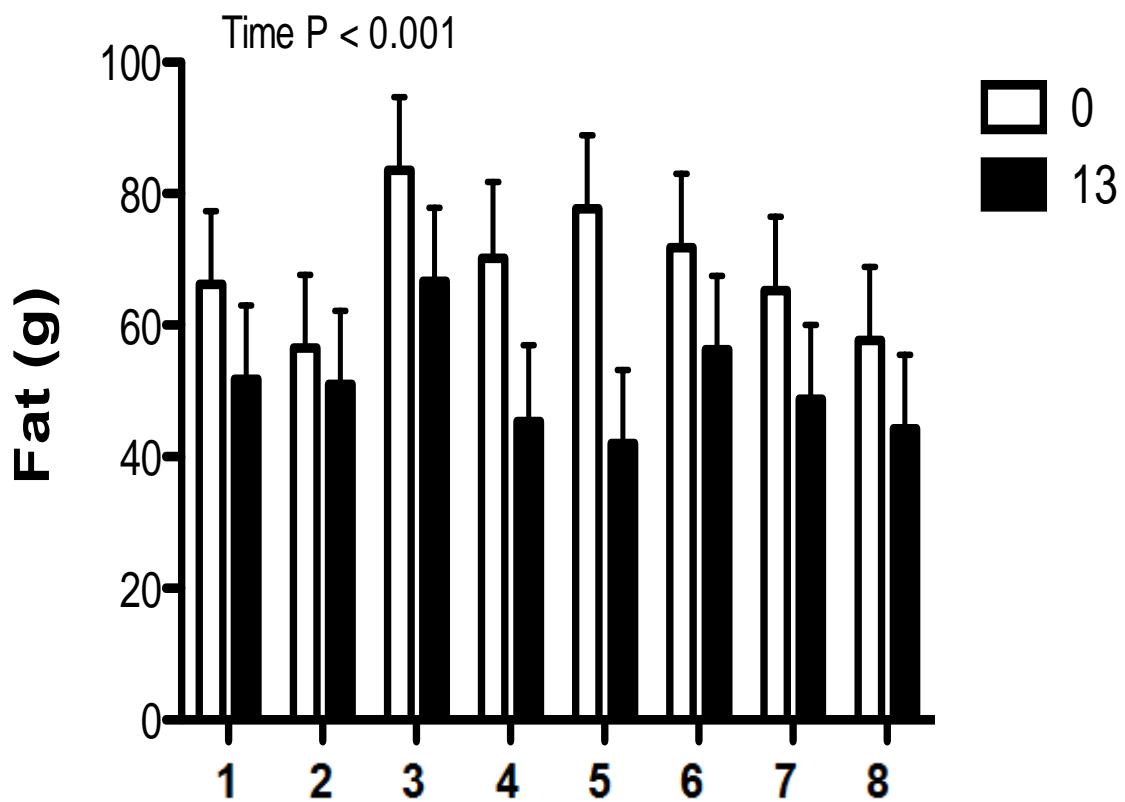


Figure 2.32. Effects of GOS and FOS dietary supplementation on fat (g) of pigs fed experimental milk replacer formula. Piglets ($1.628 +/-.037$ Kg BW) were randomly assigned to one of eight diets ($n = 6/\text{diet}$): 1) milk-based formula containing Bimuno GOS and Vivinal FOS (4 + 1 g/L); 2) soy-based formula containing Orafti (3g/L); 3) milk-based formula with no prebiotic added; 4) milk-based formula containing Bimuno GOS only (5g/L); 5) milk-based formula containing Vivinal GOS only (5g/L); 6) milk-based formula containing Bimuno GOS and Nutraflora FOS (4 + 1 g/L); 7) milk-based formula containing Vivinal GOS and Orafti FOS (4 + 1 g/L); 8) soy-based formula with no prebiotic added. Diets were isonitrogenous, isocaloric and fed at 250mL/kg body weight every 4 hours for a 2-week period. Dual energy X-ray absorptiometry (DEXA) scans were performed before study (day 0) and at the end of study (day 13). Values represent least squared means \pm SEM and statistical significance set at $P < 0.05$.

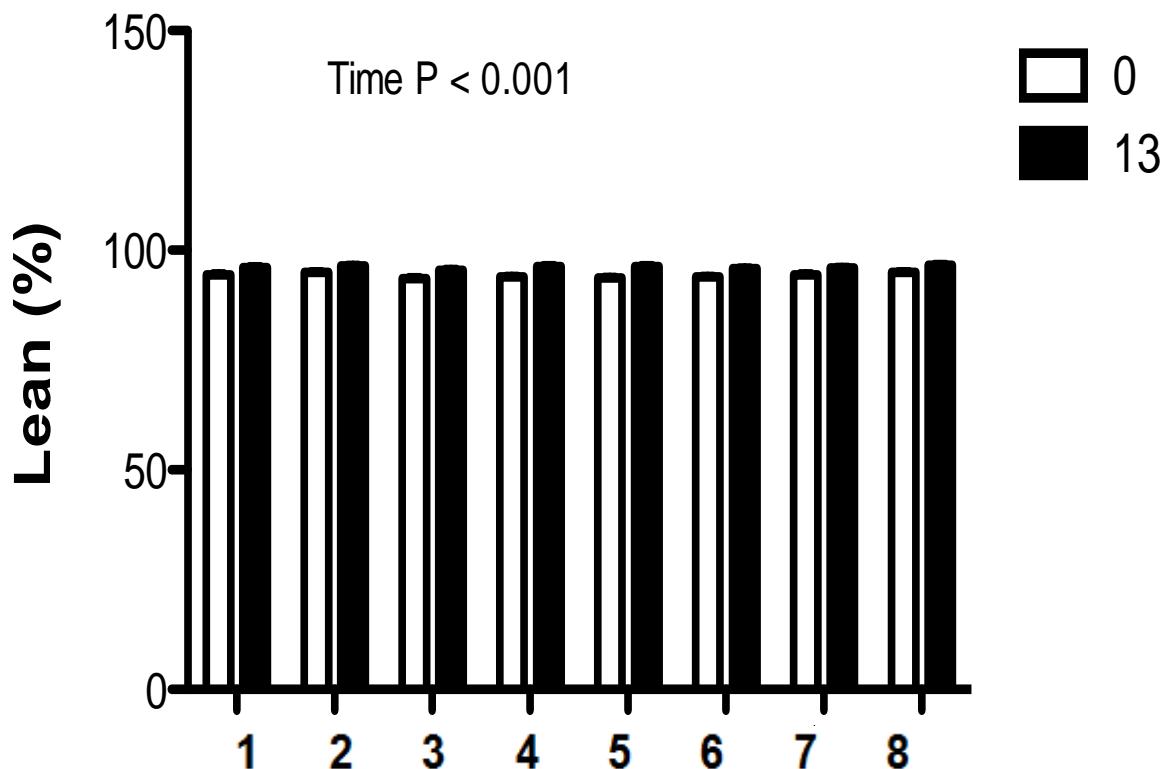


Figure 2.33. Effects of GOS and FOS dietary supplementation on percentage of lean (%) of pigs fed experimental milk replacer formula. Piglets ($1.628 \pm .037$ Kg BW) were randomly assigned to one of eight diets (n = 6/diet): 1) milk-based formula containing Bimuno GOS and Vivinal FOS (4 + 1 g/L); 2) soy-based formula containing Orafti (3g/L); 3) milk-based formula with no prebiotic added; 4) milk-based formula containing Bimuno GOS only (5g/L); 5) milk-based formula containing Vivinal GOS only (5g/L); 6) milk-based formula containing Bimuno GOS and Nutraflora FOS (4 + 1 g/L); 7) milk-based formula containing Vivinal GOS and Orafti FOS (4 + 1 g/L); 8) soy-based formula with no prebiotic added. Diets were isonitrogenous, isocaloric and fed at 250mL/kg body weight every 4 hours for a 2-week period. Dual energy X-ray absorptiometry (DEXA) scans were performed before study (day 0) and at the end of study (day 13). Values represent least squared means \pm SEM and statistical significance set at P< 0.05.

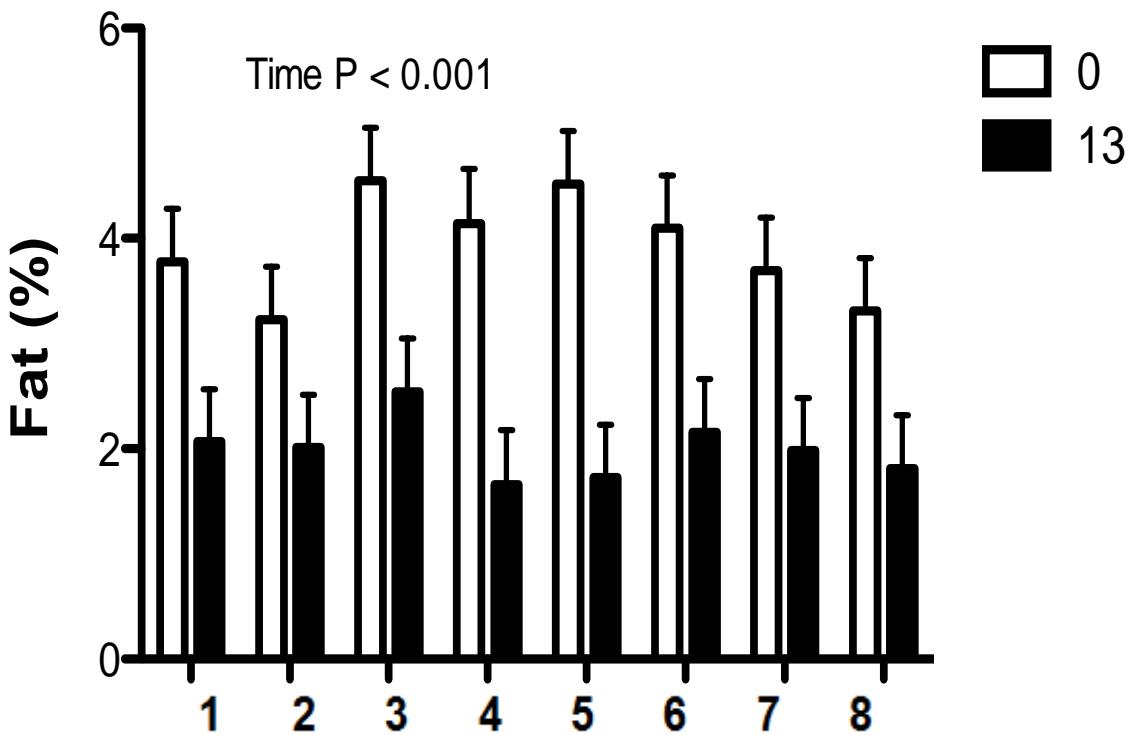


Figure 2.34. Effects of GOS and FOS dietary supplementation on percentage of fat (%) of pigs fed experimental milk replacer formula. Piglets ($1.628 +/_{.037}$ Kg BW) were randomly assigned to one of eight diets ($n = 6/\text{diet}$): 1) milk-based formula containing Bimuno GOS and Vivinal FOS (4 + 1 g/L); 2) soy-based formula containing Orafti (3g/L); 3) milk-based formula with no prebiotic added; 4) milk-based formula containing Bimuno GOS only (5g/L); 5) milk-based formula containing Vivinal GOS only (5g/L); 6) milk-based formula containing Bimuno GOS and Nutraflora FOS (4 + 1 g/L); 7) milk-based formula containing Vivinal GOS and Orafti FOS (4 + 1 g/L); 8) soy-based formula with no prebiotic added. Diets were isonitrogenous, isocaloric and fed at 250mL/kg body weight every 4 hours for a 2-week period. Dual energy X-ray absorptiometry (DEXA) scans were performed before study (day 0) and at the end of study (day 13). Values represent least squared means \pm SEM and statistical significance set at $P < 0.05$.

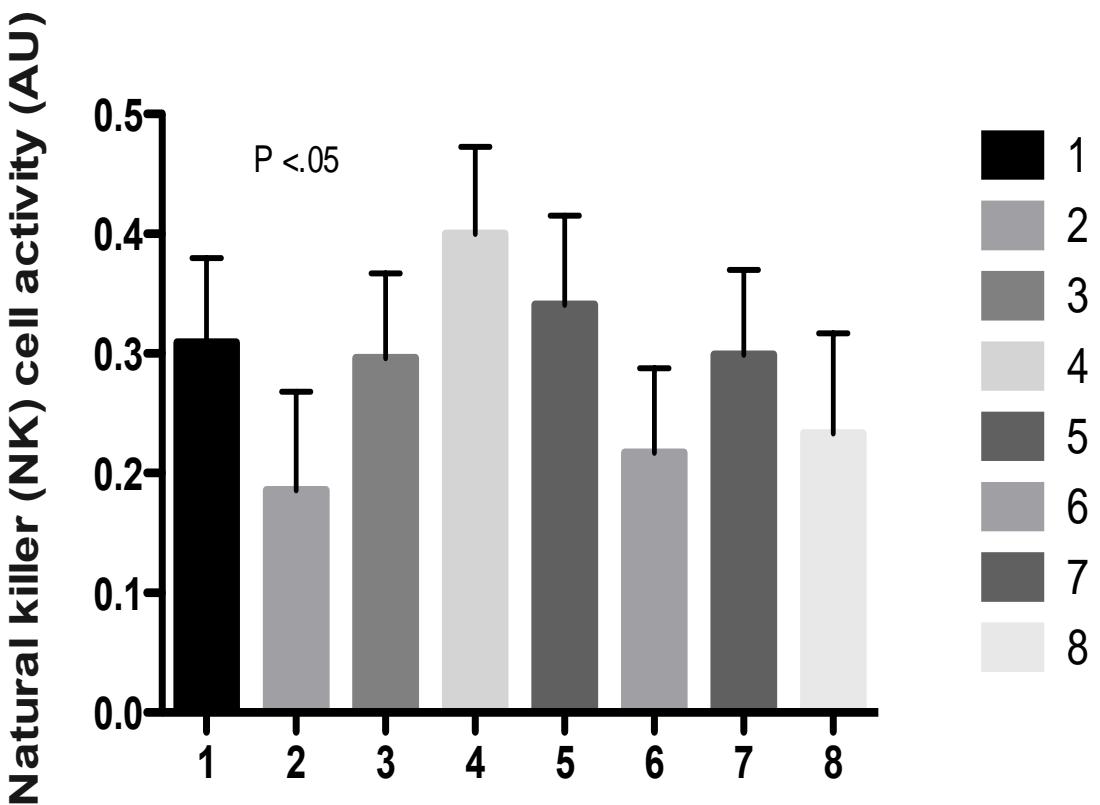


Figure 2.35. Effects of GOS and FOS dietary supplementation on natural killer (NK) cell activity measured in arbitrary units (AU) of pigs fed experimental milk replacer formula. Piglets ($1.628 +/_ .037$ Kg BW) were randomly assigned to one of eight diets ($n = 6/\text{diet}$): 1) milk-based formula containing Bimuno GOS and Vivinal FOS (4 + 1 g/L); 2) soy-based formula containing Orafti (3g/L); 3) milk-based formula with no prebiotic added; 4) milk-based formula containing Bimuno GOS only (5g/L); 5) milk-based formula containing Vivinal GOS only (5g/L); 6) milk-based formula containing Bimuno GOS and Nutraflora FOS (4 + 1 g/L); 7) milk-based formula containing Vivinal GOS and Orafti FOS (4 + 1 g/L); 8) soy-based formula with no prebiotic added. Diets were isonitrogenous, isocaloric and fed at 250mL/kg body weight every 4 hours for a 2-week period. Assay was performed on blood samples taken the day of sacrifice. Values represent least squared means \pm SEM and statistical significance set at $P < 0.05$.

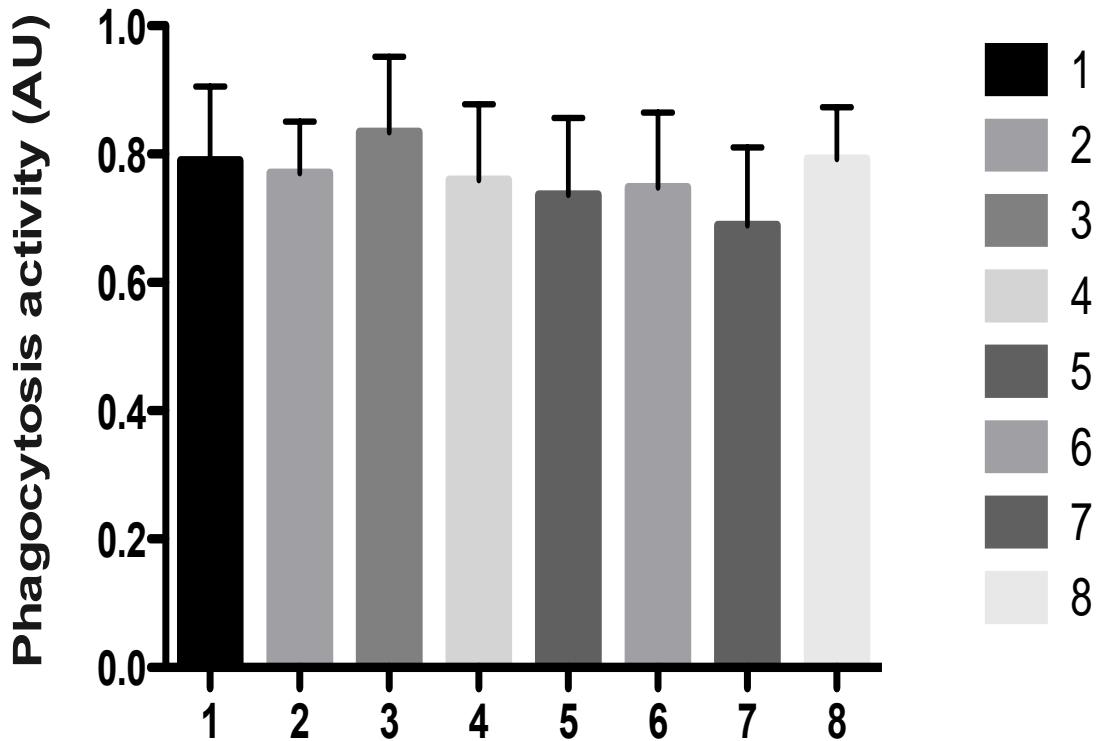


Figure 2.36. Effects of GOS and FOS dietary supplementation on phagocytosis activity measured in arbitrary units (AU) of pigs fed experimental milk replacer formula. Piglets ($1.628 +/_ .037$ Kg BW) were randomly assigned to one of eight diets (n = 6/diet): 1) milk-based formula containing Bimuno GOS and Vivinal FOS (4 + 1 g/L); 2) soy-based formula containing Orafti (3g/L); 3) milk-based formula with no prebiotic added; 4) milk-based formula containing Bimuno GOS only (5g/L); 5) milk-based formula containing Vivinal GOS only (5g/L); 6) milk-based formula containing Bimuno GOS and Nutraflora FOS (4 + 1 g/L); 7) milk-based formula containing Vivinal GOS and Orafti FOS (4 + 1 g/L); 8) soy-based formula with no prebiotic added. Diets were isonitrogenous, isocaloric and fed at 250mL/kg body weight every 4 hours for a 2-week period. Assay was performed on blood samples taken the day of sacrifice. Values represent least squared means \pm SEM and statistical significance set at P< 0.05.

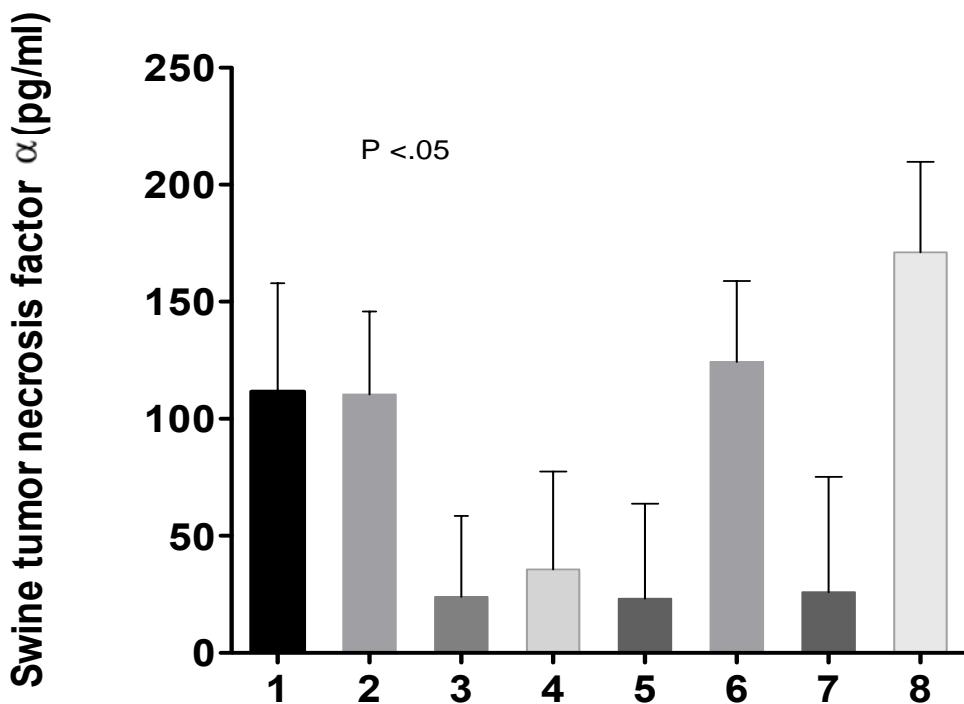


Figure 2.37. Effects of GOS and FOS dietary supplementation on Swine tumor necrosis factor α (TNF α) of pigs fed experimental milk replacer formula. Piglets ($1.628 +/_{.037}$ Kg BW) were randomly assigned to one of eight diets ($n = 6/\text{diet}$): 1) milk-based formula containing Bimuno GOS and Vivinal FOS (4 + 1 g/L); 2) soy-based formula containing Orafti (3g/L); 3) milk-based formula with no prebiotic added; 4) milk-based formula containing Bimuno GOS only (5g/L); 5) milk-based formula containing Vivinal GOS only (5g/L); 6) milk-based formula containing Bimuno GOS and Nutraflora FOS (4 + 1 g/L); 7) milk-based formula containing Vivinal GOS and Orafti FOS (4 + 1 g/L); 8) soy-based formula with no prebiotic added. Diets were isonitrogenous, isocaloric and fed at 250mL/kg body weight every 4 hours for a 2 -week period. Assay was performed on blood samples taken the day of sacrifice. Values represent least squared means \pm SEM and statistical significance set at $P < 0.05$.

CHAPTER III

Effects of Galactooligosaccharides and Fructooligosaccharides on gut microbiota and intestinal histopathology

ABSTRACT

Breast-feeding remains the most favorable method for promoting normal growth by meeting the nutritional requirements of the neonate. The objective of this study was to determine the effect of two oligosaccharides, GOS and FOS, supplementation in nursery pig formula on numbers of select gut bacterial groups and intestinal histopathology of the neonatal pig gastrointestinal tract. Forty-eight 4-day old crossbred pigs ($1.628 \pm .037$ kg BW) were randomly assigned to 1 of 8 diets that varied in the type of formula base (milk or soy), prebiotic (FOS, GOS or a combination). Diets were isonitrogenous, isocaloric and fed at 250 mL/kg body weight every 4 hrs for a 2-week period. Cross sections of ileal tissue were stained with hematoxylin and eosin (HE) the depth of the crypts and length of the villi were measured. Additional small sections from the ileum and cecum were taken at sacrifice for quantification of select bacterial groups. The numbers of total bacteria, *Lactobacillus*, *Bacteroides*, *E.coli* and *Bifidobacterium* in ileal and cecal tissues were quantified using qPCR. The average crypt depth and villi height were not significantly affected by treatment. However, the abundance of select bacterial groups present within the gut were significantly affected by the animal's diet. There was no statistical difference in log copies /g tissue of *Bacteroides*, *E.coli*, and *Bifidobacterium* in ileal and cecal tissues of pigs on different diets. However, differences in abundance of total bacteria (16S), and

Lactobacillus were significantly affected by diet in both the ileum and cecum tissue.

Piglets consuming a milk formula diet with both FOS and GOS had the largest increases in *Lactobacillus* in the cecum. In conclusion, it does appear that the addition of GOS and FOS does influence the gut microbiota present within the neonatal porcine gastrointestinal tract.

INTRODUCTION

It is well known that breast milk is the most advantageous vehicle to deliver sufficient quantities of high quality nutrients in order to sustain normal growth and development of the gastrointestinal tract. However, a variety of factors can make breast-feeding insufficient or impractical. The difference in the health of breast-fed infants compared to formula-fed infants, especially the gut microbiota, remains a concern of high priority for infant formula manufacturers today. As a result, manufacturers constantly strive to produce formulas supplemented with new ingredients that safely mimic the natural components of human breast milk.

Generally, *Bifidobacterium* and *Lactobacillus* predominate the microbiota of breast-fed infants (Haarman & Knol, 2006), therefore, the majority of studies in this area are focused specifically on these two types of bacteria. Supplementation with certain prebiotics has been strongly linked with modulation of the intestinal microbiota and shifting the balance of bacteria in favor of beneficial microorganisms, mainly bifidobacteria and lactobacilli species (Boehm et al., 2003).

It is speculated that in the infant gut an elevated bifidobacteria may be associated with the health advantages that breast-fed infants may have over formula-fed infants (Collins & Gibson, 1999).

It has been demonstrated that the addition of galactooligosaccharides and fructooligosaccharides to infant formula can modify fecal bacteria to more closely resemble the pattern of breast-fed infants with respect to *Bifidobacterium* and *Bacteroides* (Veereman-Wauters et al., 2011). Other clinical investigations of infant formulas supplemented with galactooligosaccharides and fructooligosaccharides at a range of concentrations and a range of blends have revealed increases in the numbers of fecal bifidobacteria in pre-term infants and in the numbers of both bifidobacteria and lactobacilli in-term infants compared with infants receiving non-supplemented formulas (Nakamura et al., 2008).

Therefore, this study aimed to investigate the effect of galactooligosaccharides and fructooligosaccharides on abundance of *Lactobacillus*, *Bacteroides*, *E.coli*, and *Bifidobacterium* present in the neonatal porcine intestine. Intestinal histopathology of the gastrointestinal tract was also examined, specifically crypt depth and villi length to ensure no adverse effects of the development of the GIT.

MATERIALS AND METHODS

Animals and experimental design:

Forty-eight 2-day old female crossbred pigs were acquired from Murphy Brown facilities. Piglets were acclimated to their environment for 24h and housed individually and kept on a standard light cycle (12h light/dark) at 80-85 degrees Fahrenheit. At 5 days old pigs (1.628 +/_ .037 kg BW) were randomly assigned to one of eight diets: 1) milk-based formula containing no prebiotic 2) milk-based

formula containing Type 1 GOS and Type 1 FOS 3) milk-based formula containing Type 1 GOS and Type 2 FOS 4) milk-based formula containing Type 2 GOS and Type 1 FOS 5) milk-based formula containing Type 1 GOS only 6) milk-based formula containing Type 2 GOS only 7) soy-based formula containing no prebiotic 8) soy-based formula containing Type 1 FOS. Diets were isonitrogenous, isocaloric and fed at 250mL/kg body weight every 4 hours for a 2-week period, refer to Table 2.2 for full diet compositions. Body weight was measured every two days and amount of formula was adjusted accordingly.

Sample Collection

On the last day of the study tissue samples were taken from the ileum and cecum for gut microbiota analysis. The samples were flushed gently with saline and then immediately frozen in liquid nitrogen.

Gut Microbiota

DNA extractions

Extractions were performed using the Qiagen DNeasy tissue kit (Qiagen, Valencia, CA) with an optimized chemical and mechanical lysis step designed to target gut microorganisms. Each sample consisted of 10-25 mg ileum or cecum tissue cut into small pieces using an ethanol flamed scalpel and placed in a 1.5 ml freestanding Screw-Cap Microcentrifuge Tubes (VWR®, Radnor, PA). A 100 mg mixture of 2.3MM, 0.5MM and 0.1MM silicone disruption beads (Research Products International, Mount Prospect, IL) and 180 ul of ATL tissue lysis buffer (from Qiagen kit) were added. The tubes were then placed in the TissueLyser II (Qiagen) for 10 minutes at

maximum speed. After which 2 mg/ml lysozyme (manufacturer) and 5 µl of mutanolysin (1 u/µl) (Sigma-Aldrich, St. Louis, MO) were added. Samples were incubated overnight shaking (125 rpm) at 37°C in an Innova® 42 (Eppendorf, Enfield, CT) incubator with vortexing every 5 min throughout incubation for the first 30 min. After mutanolysin lysis, 20 µl of Qiagen Proteinase K (20 mg/ml) (Qiagen, Valencia, CA) was added and incubated for 30 min shaking (125 rpm) at 37°C with vortexing every 5 min throughout incubation. After incubation, 200µl of Buffer AL and 200ul 99% ethanol was added. Samples were then placed in the bead-beater for 5 minutes at maximum speed. Manufacturers instructions were followed for the remainder of the extraction protocol.

Culturing conditions for bacterial standards

Lactobacillus rhamnosus (American Type Culture Collection (ATCC) 53103) was thawed from a freezer stock and cultures in chopped meat broth (Anaerobe Systems, CA), incubated overnight at 37°C, transferred to MRS (Difco Sparks, MD) and then grown overnight at 37°C. A few drops of thawed freezer stock of *Bacteroides fragilis* (VPI 13785) were placed into 7 ml chopped meat broth (Anaerobe Systems, CA), incubated overnight at 37°C, and then transferred into two 7 ml tubes of PYG broth and two 7 ml tubes of MTGE broth. *Escherichia coli* (ATCC 43890) was used to inoculate tryptic soy broth (TSB; Difco Sparks, MD) and grown overnight shaking at 37°C. *Bifidobacterium animalis* subsp. *lactis* (ATCC 700541) was put into chopped meat broth (Anaerobe Systems, CA), incubated overnight at 37°C, separated and then resuspended into two 7 ml tubes of PYG broth and two 7 ml tubes of MTGE broth and then grown overnight at 37°C.

Quantitative Real-Time PCR

DNA from pure culture bacteria were extracted using the Puregene Yeast/Bact. Kit B (Qiagen, Valencia, CA) per manufacturer's instructions. To create a standard curve DNA was serially diluted 10-fold from 100 ng/ml to 0.001 ng/ml and each concentration was run in triplicate. Each 25 µl reaction consisted of 12.5 µl of HotStart-IT™SYBR®Green qPCR Master Mix 2X (USB® 75762 Cleveland, OH, USA), 0.4 µM of both forward and reverse primers, and 10 ng of template DNA (except for the Total Bacteria primer set which consisted of 0.6 µM of both and reverse primers and 1ng of template DNA). Primers and their respective annealing temperatures are listed in Table 3.2. The PCR conditions consisted of a HotStart Binding Protein inactivation for 2 min at 95°C, followed by 40 cycles of denaturation at 95°C for 15 sec, a variable annealing temperature for 30 sec (listed in Table 3.2) and 72°C extension for 60 sec (acquire real-time data at this step). Standard curves were done in duplicate and three replicates of samples were run. Melting curve analysis of the PCR products was conducted to distinguish specific PCR products from non-specific. Amplification was carried out with a CFX96 Real-Time System (Bio-Rad, Hercules, CA).

Intestinal histopathology

At sacrifice, one-inch cross-sections were taken from the ileum of each pig. Each section was then placed into a conical tube with formalin. Prior to placement in the conical tubes, tiny slits were made on each end of the tissue to ensure complete penetration of the formalin. The ileum tissue sections were then shipped to Histo-

scientific research laboratories (HSRL) for staining with hematoxylin and eosin (H&E). The H&E slides were then viewed on a Nikon digital sight and crypt depth and villi length were measured with NIS-Elements AR 3.10 software (Nikon Instruments INC).

Data analysis

Statistical analyses were performed using SAS (version 9.3, SAS Institute, Cary, NC) statistical software. The *proc Glimmix* procedure of SAS was used to determine the main effect of diet. All data were analyzed using the *proc glimmix* procedure of SAS 9.3. Pig, treatment, type, and block were all included in the class statement. Comparisons were made within milk treatments, soy treatments, and all treatments. The statistical model $Y_i = \mu + D_i + e_i$ in which μ = overall mean, D_i = effect of the i th diet, and e_i = error was used. Statistical significance was set at a P-value of less than 0.05.

Results

Gut microbiota results

The amount of total bacteria in both the ileum and cecum tissue was affected by treatment ($P < 0.0001$; Figures 3.1 and 3.6). Milk diets containing Vivinal GOS and either Orafti or Nutrafbra FOS, treatments 1 and 6, were associated with a reduced abundance of total bacteria in ileal tissue compared to the other diets (Figure 3.1). Treatment also affected the amount of *Lactobacillus* present in the ileum ($P < 0.05$), with

increased abundance in the soy-based diets (Figure 3.2) and a small reduction in prebiotic supplemented milk formulas relative to the milk control formula.

In the cecum, the numbers of total bacteria increased in treatments containing both GOS and FOS (treatments 1 and 6), however, only the treatment containing Vivinal GOS and Nutraflora FOS (treatment 1) was statistically different than the milk control diet (Figure 3.6). The bg copies/g of total bacteria in cecal contents were reduced in the soy-based diets compared to the milk-based diets (Figure 3.6). *Lactobacillus* abundance in the cecum was significantly increased (1.5 bg copies/g) in pigs consuming treatment 1 (milk-based formula containing Vivinal (Type 2) GOS and Nutraflora (Type 1) FOS (4 + 1 g/L)) compared to the other seven treatments (Figure 3.7). The abundance of bg copies/g of *Bacteroides*, *Escherichia coli*, and *Bifidobacterium* were not statistically different for any diet in both tissues.

Intestinal histopathology results

Average crypt depth and villi height were 34.67 μm and 99.75 μm , respectively. Crypt depth (Figure 3.11) and villi height (Figure 3.12) were not significantly affected by treatment, which reflects normal development of the gastrointestinal mucosa.

DISCUSSION

It has been well established that GOS and FOS are able to modify the microbiota of the gastrointestinal tract. With respect to *Lactobacillus*, our results showed that in the cecum, a treatment with both GOS and FOS had significantly increased abundance (bg copies/g) compared to the controls treatments. This finding parallels a number of studies investigating the effect of a GOS/FOS mixture on the gut microbiota. A

study using fecal samples from both breast-fed and formula-fed infants fed a supplemented formula with a mixture of GOS and FOS found that the supplemented formula-fed infants showed a significant increase in abundance of fecal *lactobacilli* after supplementation with the mixture as compared to prior to supplementation (Haarman & Knol, 2006). A range of concentrations and blends (Nakamura et al, 2008) as well as different molecular weights (Moro et al, 2002) of GOS and FOS also showed an increase in fecal *lactobacilli* in infant fed supplemented formula (Nakamura et al, 2008). In this study, small decreases in ileal *Lactobacillus* were seen in milk -based supplemented formulations, which may reflect the use of tissue samples as opposed to fecal samples. Variation has been shown between communities that adhere to the mucosa and communities in the stool, which could be due to the fact that fecal samples may contain nonadherent populations in addition to species shed from the mucosa (Eckburg et al, 2005). In conclusion, the results from this study demonstrate that a mixture of Vivinal GOS and Nutraflora FOS are the most effective at increasing amounts of *Lactobacillus* in the cecum.

The results of this study show a significant difference in the amount of total bacteria. The milk-based diets containing Vivinal GOS and either Orafti or Nutraflora FOS, (treatments 1 and 6), were associated with a reduced abundance of total bacteria in ileal tissue compared to the other diets. This could be due to the origins of the types of FOS. The Orafti FOS is derived from chicory inulin (Liong and Shah, 2005; Semjonovs et al, 2007) while Nutraflora FOS is naturally synthesized from sucrose (Kaplan and Hutkins, 2000). A study investigating the utilization of carbohydrates by bacteria found that *lactobacilli* and *bifidobacteria* grew poorly on inulin (Watson et al, 2013), which is a source of the Orafati FOS used in this study. This could explain why the abundance of

Lactobacillus was different in the cecum tissue of the pigs fed diets supplemented with the Nutraflora FOS (treatments 2 and 7) given that this type of FOS comes from sucrose and not inulin.

Bifidobacterium is one of the most predominant bacteria in the gut of neonatal humans and pigs that flourish on glycans found in milk (Seh and Mills, 2014). Results in this study showed no significant differences in abundance of *Bifidobacterium* in either tissue. Although, the soy-based treatments did have a slightly higher abundance of *Bifidobacterium* compared to the milk-based treatments in the ileum. The results from this study showing no effect of GOS and FOS on *Bifidobacteria* are consistent with another infant formula study that found no significant differences between the GOS/FOS supplemented group and the control group (Bakker-Zierikzee et al, 2005). However, our results do not correlate with the majority of studies, which have shown a stimulatory effect of GOS and FOS on the growth of bifidobacterium, although, these effects were seen in fecal samples as opposed to tissue (Moro et al, 2002; Knol et al, 2005; Haarman and Knol, 2005; Xiao-Ming, et al, 2008).

Given that *bifidobacteria* is known for utilizing lactose, a main component of milk, it is surprising that in the ileum tissue this study showed more abundance of *bifidobacterium*. Although, this finding is similar to a study that tested the effect of fermented soy milk on intestinal bacteria and found that the populations of *Bifidobacterium* increased during the period of soymilk consumption (Cheng et al, 2005). This could be due to the fact that oligosaccharides in soybeans have been shown to have a prebiotic effect (Piacentini et al, 2010). In conclusion, the results of this study make it hard to elucidate the effects of solely GOS and FOS on *bifidobacterium* due to the influence of the soy-based diets compared to the milk-based diets.

Our results showed no significant difference in *Bacteroides* in the ileum or cecum of the supplemented formulas compared to the controls. These results are consistent with a study comparing breast-fed infants to infants fed formula supplemented with galactooligosaccharides, oligofructose, and long-chain inulin fructooligosaccharides that showed comparable numbers of *Bacteroides* but no significant differences between the supplemented treatments and the controls (Veereman-Wauters et al, 2011).

A study conducted *in vitro* using an intestinal tissue model for the investigation of bacterial association in the pig small intestine under different dietary regimes showed a significant reduction ($P<0.05$) in the numbers of *E.coli* in jejunal organ cultures of pigs fed a diet supplemented with FOS (Naughton et al, 2001). This study also examined tissue from pigs fed a diet supplemented with commercial GOS and found no significant effects on the numbers of *E.coli* (Naughton et al, 2001). For GOS these results are similar with the results found in the present study that showed no significant differences in *E.coli* numbers between the supplemented treatment groups and the controls.

Although our results for the different types of bacteria in the gut were for the most part consistent with other findings, the analysis in the current study was performed on tissue samples from the ileum and cecum in contrast to most infant formula studies that use fecal samples. Another factor that could have impacted the results of this study is only analyzing one sample from one time point throughout the experiment. Samples were not taken at the beginning of the study and therefore we were unable to compare the bacterial counts at the end of study with counts prior to treatment with GOS and FOS. Given that the piglets were not all from the same litter and their gut would have been colonized first by gut bacteria from the sow, there could have been slight variation between the gut microbiota of the piglets at the beginning of the study.

which could have had an impact on the abundance of the different types of bacteria at the end of the study.

The findings from this study are important because they show abundance of bacterial types that are able to adhere to tissue, while most studies use fecal samples. This study shows that Nutrafibra FOS is more beneficial for increasing numbers of *Lactobacillus*, which is valuable knowledge when supplementing infant formula. However, there are some further questions that need research. The increased numbers of *Bifidobacterium* in the soy-based diets could have been due to the fact that soy products are known to have a prebiotic effect, although, further research is needed to elicit the effects of the GOS and FOS in the presence of other potential prebiotics such as soy. Another area needing more research is the utilization of the types of GOS and FOS used in this study by bacteria. While some studies have been done, more are still needed. Another future direction would be to test the effects of GOS and FOS on the gut microbiota in a challenge study.

Table 3.1. Culturing conditions for PCR bacterial standards.

Bacterial Stain	Media	Growth Conditions
<i>Escherichia coli</i> ATCC 43890	Tryptic Soy broth (TSB; Difco, Sparks, MD)	Inoculated 5ml TSB from freezer stock using a sterile loop. Grew overnight, shaking at 37°C.
<i>Lactobacillus rhamnosus</i> ATCC 53103	Chopped meat broth (Anaerobe Systems, CA), MRS Broth (Difco, Sparks, MD)	Thawed freezer stock. Used filtered eye dropper to transfer approximately 5 drops of stock to 7ml chopped meat broth. Incubated overnight at 37°C. Using filtered eye dropper, transferred approximately 5 drops of culture to ~50ml MRS broth. Grew overnight at 37°C.
<i>Bacteroides fragilis</i> VPI 13785	Chopped meat broth (Anaerobe Systems, CA), PYG broth, MTGE broth	Thawed freezer stock. Used filtered eye dropper to transfer approximately 5 drops of stock to 7ml chopped meat broth. Incubated overnight at 37°C. Using filtered eye dropper, transferred approximately 5 drops of culture into two 7ml tubes of PYG broth and two 7ml tubes of MTGE broth. Grew overnight at 37°C.
<i>Bifidobacterium animalis</i> <i>subsp. lactis</i> ATCC 700541	Chopped meat broth (Anaerobe Systems, CA), PYG broth, MTGE broth	Thawed freezer stock. Used filtered eye dropper to transfer approximately 5 drops of stock to 7ml chopped meat broth. Incubated overnight at 37°C. Separated resuspended culture into two 7ml tubes of PYG broth and two 7ml tubes of MTGE broth. Grew overnight at 37°C.

Table 3.2. Primer sets used to quantify abundance of key members of swine microbial community using real-time PCR

Target Bacterial Group	Primer Sequence	Annealing Temperature °C	Reference
Total Bacteria	ACTCCTACGGGAGGCAGCAG ATTACCGCGCTGCTGG	60	Lane, 1991;
<i>Bacteroides</i>	GAGAGGAAGGTCCCCCAC CGCTACTTGGCTGGTTCA	60	Layton et al., 2006
<i>Bifidobacterium</i>	GGGTGGTAATGCCGGATG CCACCGTTACACCGGGAA	60	Satokari et al., 2001
<i>E.coli</i>	GACCTCGGTTAGTTCACAGA CACACGCTGACGCTGACCA	60	Rekha et al. 2006
Lactobacilli	GCAGCAGTAGGAAATCTTCCA GCATTYCACCGCTACACATG	60	Walter et al., 2001

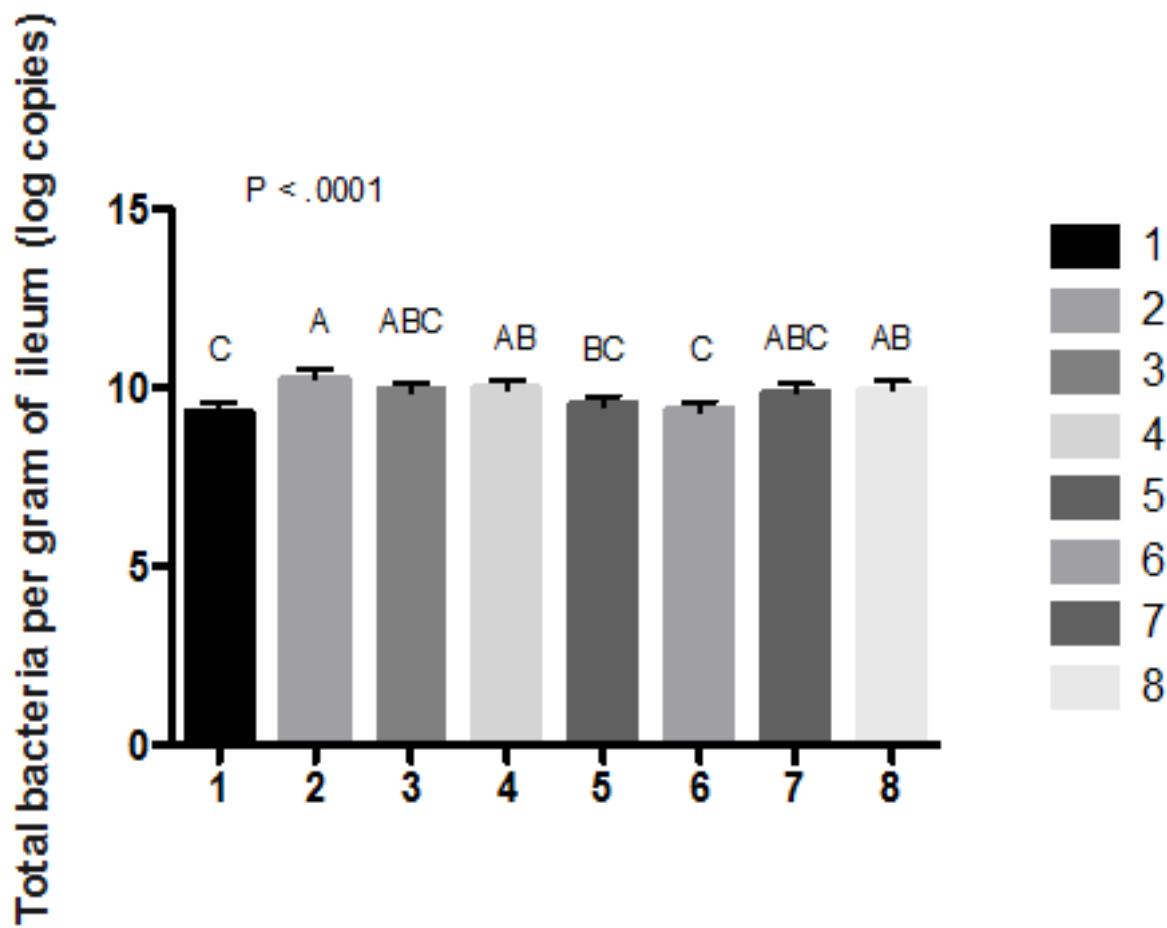


Figure 3.1. Effects of GOS and FOS dietary supplementation on total bacteria (log copies) per gram of ileum tissue quantified by rtPCR. Piglets ($1.628 +/-.037$ Kg BW) were randomly assigned to one of eight diets ($n = 6/\text{diet}$): 1) milk-based formula containing Bimuno GOS and Vivinal FOS (4 + 1 g/L); 2) soy-based formula containing Orafti (3g/L); 3) milk-based formula with no prebiotic added; 4) milk-based formula containing Bimuno GOS only (5g/L); 5) milk-based formula containing Vivinal GOS only (5g/L); 6) milk-based formula containing Bimuno GOS and Nutraflora FOS (4 + 1 g/L); 7) milk-based formula containing Vivinal GOS and Orafti FOS (4 + 1 g/L); 8) soy-based formula with no prebiotic added. Diets were isonitrogenous, isocaloric and fed at 250mL/kg body weight every 4 hours for a 2-week period. Values represent least squared means \pm SEM and statistical significance set at $P < 0.05$.

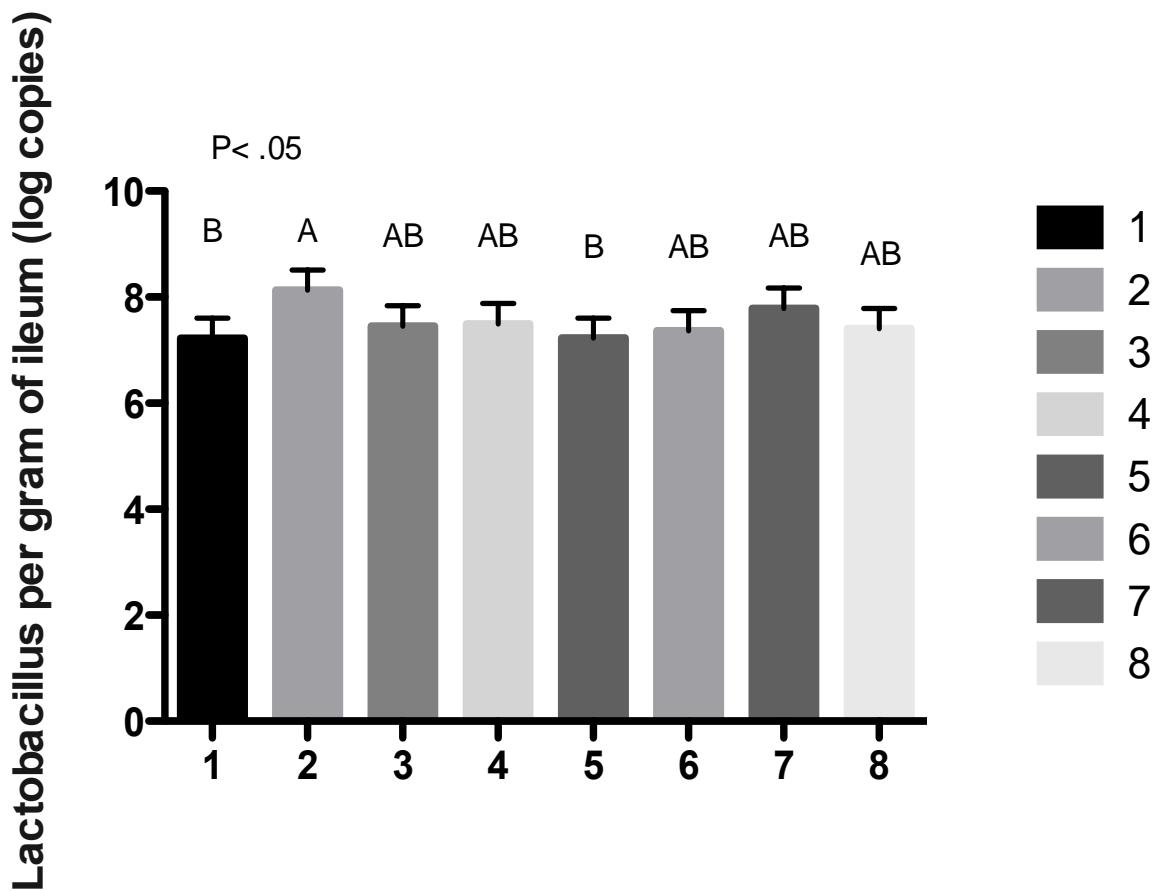


Figure 3.2. Effects of GOS and FOS dietary supplementation on *Lactobacillus* (log copies) per gram of ileum tissue quantified by rtPCR. Piglets ($1.628 +/_ .037$ Kg BW) were randomly assigned to one of eight diets ($n = 6/\text{diet}$): 1) milk-based formula containing Bimuno GOS and Vivinal FOS (4 + 1 g/L); 2) soy-based formula containing Orafti (3g/L); 3) milk-based formula with no prebiotic added; 4) milk-based formula containing Bimuno GOS only (5g/L); 5) milk-based formula containing Vivinal GOS only (5g/L); 6) milk-based formula containing Bimuno GOS and Nutraflora FOS (4 + 1 g/L); 7) milk-based formula containing Vivinal GOS and Orafti FOS (4 + 1 g/L); 8) soy-based formula with no prebiotic added. Diets were isonitrogenous, isocaloric and fed at 250mL/kg body weight every 4 hours for a 2-week period. Values represent least squared means \pm SEM and statistical significance set at $P < 0.05$.

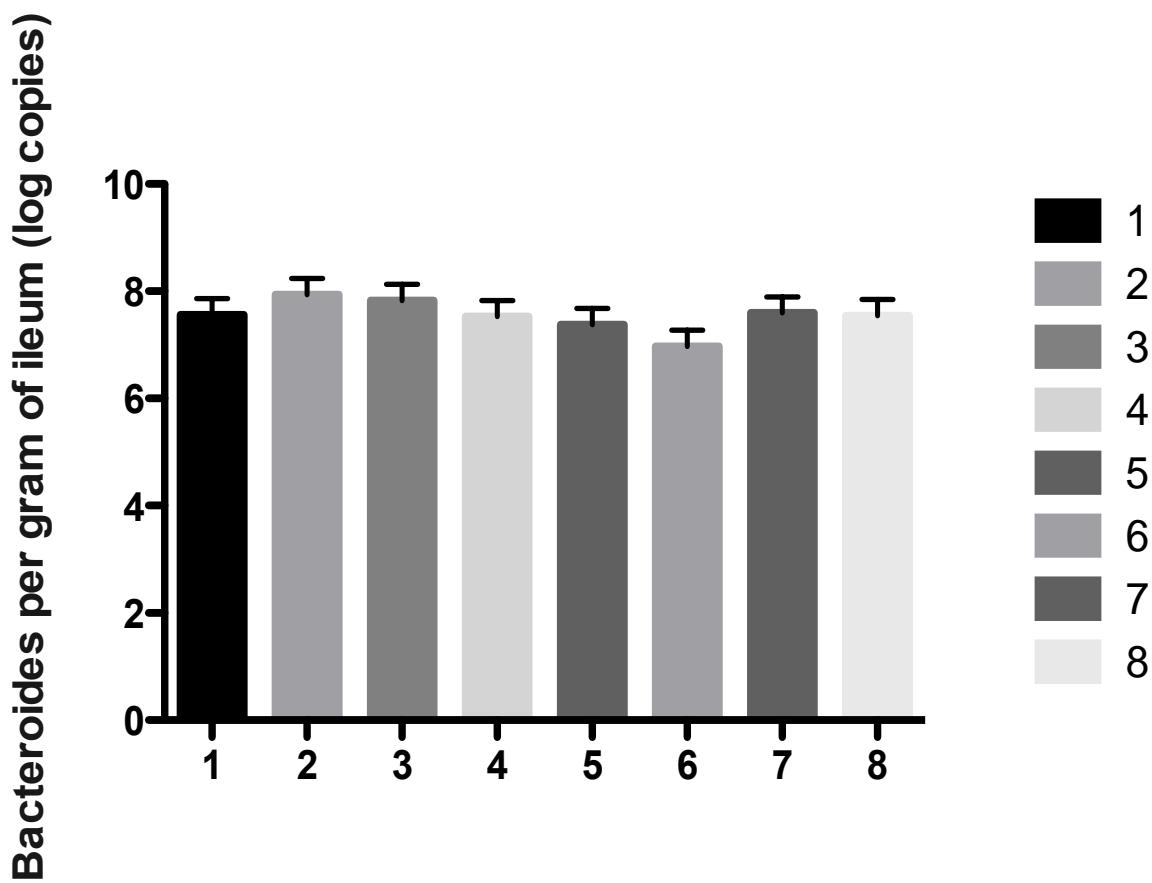


Figure 3.3. Effects of GOS and FOS dietary supplementation on *Bacteroides* (log copies) per gram of ileum tissue quantified by rtPCR. Piglets ($1.628 +/_{.037}$ Kg BW) were randomly assigned to one of eight diets ($n = 6/\text{diet}$): 1) milk-based formula containing Bimuno GOS and Vivinal FOS (4 + 1 g/L); 2) soy-based formula containing Orafti (3g/L); 3) milk-based formula with no prebiotic added; 4) milk-based formula containing Bimuno GOS only (5g/L); 5) milk-based formula containing Vivinal GOS only (5g/L); 6) milk-based formula containing Bimuno GOS and Nutraflora FOS (4 + 1 g/L); 7) milk-based formula containing Vivinal GOS and Orafti FOS (4 + 1 g/L); 8) soy-based formula with no prebiotic added. Diets were isonitrogenous, isocaloric and fed at 250mL/kg body weight every 4 hours for a 2-week period. Values represent least squared means \pm SEM and statistical significance set at $P < 0.05$.

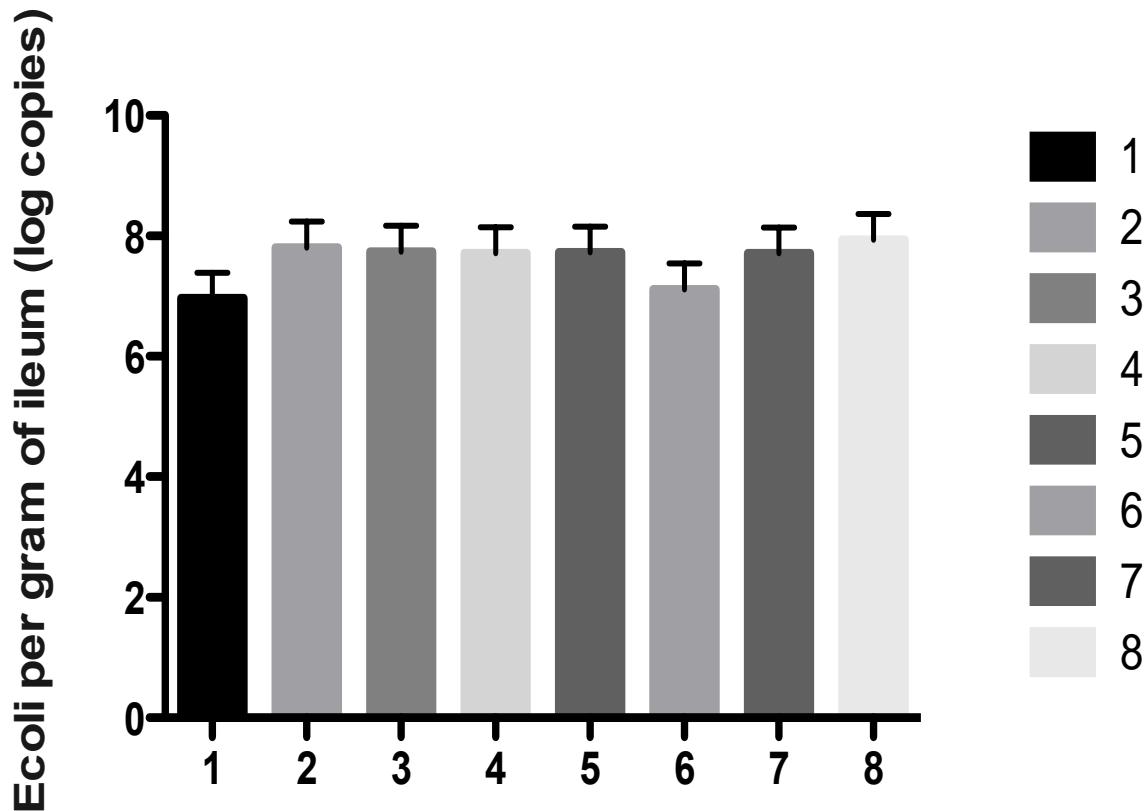


Figure 3.4. Effects of GOS and FOS dietary supplementation on *Escherichia coli* (log copies) per gram of ileum tissue quantified by rtPCR. Piglets ($1.628 +/_ .037$ Kg BW) were randomly assigned to one of eight diets ($n = 6/\text{diet}$): 1) milk-based formula containing Bimuno GOS and Vivinal FOS (4 + 1 g/L); 2) soy-based formula containing Orafti (3g/L); 3) milk-based formula with no prebiotic added; 4) milk-based formula containing Bimuno GOS only (5g/L); 5) milk-based formula containing Vivinal GOS only (5g/L); 6) milk-based formula containing Bimuno GOS and Nutraflora FOS (4 + 1 g/L); 7) milk-based formula containing Vivinal GOS and Orafti FOS (4 + 1 g/L); 8) soy-based formula with no prebiotic added. Diets were isonitrogenous, isocaloric and fed at 250mL/kg body weight every 4 hours for a 2-week period. Values represent least squared means \pm SEM and statistical significance set at $P < 0.05$.

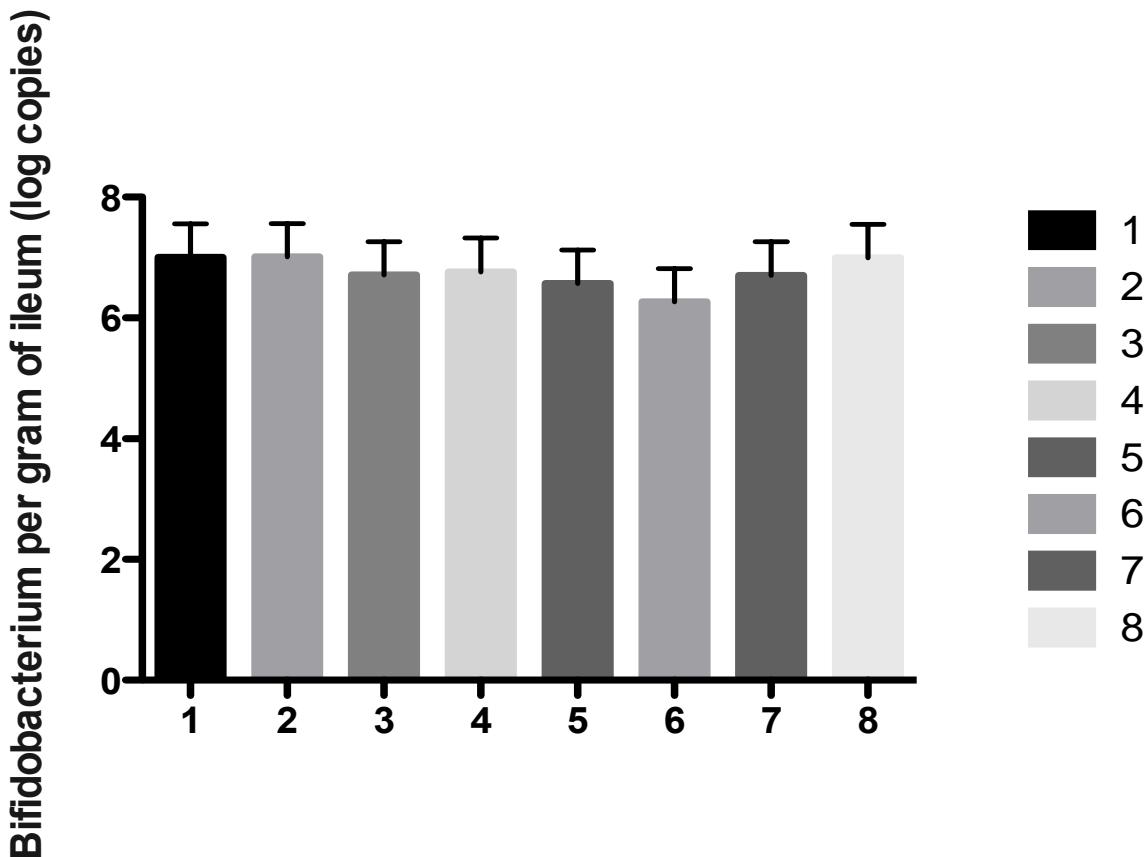


Figure 3.5. Effects of GOS and FOS dietary supplementation on Bifidobacterium (log copies) per gram of ileum tissue quantified by rtPCR. Piglets ($1.628 +/_{.037}$ Kg BW) were randomly assigned to one of eight diets ($n = 6/\text{diet}$): 1) milk-based formula containing Bimuno GOS and Vivinal FOS (4 + 1 g/L); 2) soy-based formula containing Orafti (3g/L); 3) milk-based formula with no prebiotic added; 4) milk-based formula containing Bimuno GOS only (5g/L); 5) milk-based formula containing Vivinal GOS only (5g/L); 6) milk-based formula containing Bimuno GOS and Nutraflora FOS (4 + 1 g/L); 7) milk-based formula containing Vivinal GOS and Orafti FOS (4 + 1 g/L); 8) soy-based formula with no prebiotic added. Diets were isonitrogenous, isocaloric and fed at 250mL/kg body weight every 4 hours for a 2-week period. Values represent least squared means \pm SEM and statistical significance set at $P < 0.05$.

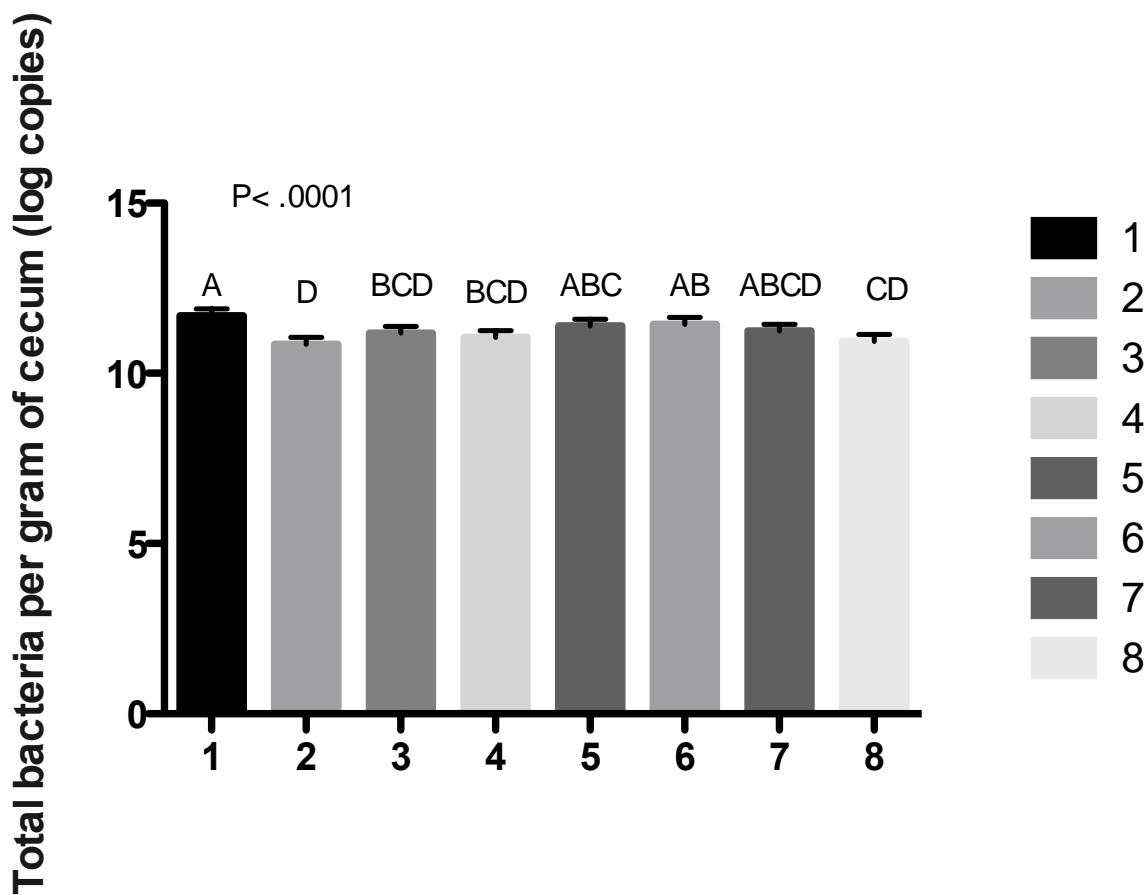


Figure 3.6. Effects of GOS and FOS dietary supplementation on total bacteria (log copies) per gram of cecum tissue quantified by rtPCR. Piglets ($1.628 \pm .037$ Kg BW) were randomly assigned to one of eight diets (n = 6/diet): 1) milk-based formula containing Bimuno GOS and Vivinal FOS (4 + 1 g/L); 2) soy-based formula containing Orafti (3g/L); 3) milk-based formula with no prebiotic added; 4) milk-based formula containing Bimuno GOS only (5g/L); 5) milk-based formula containing Vivinal GOS only (5g/L); 6) milk-based formula containing Bimuno GOS and Nutraflora FOS (4 + 1 g/L); 7) milk-based formula containing Vivinal GOS and Orafti FOS (4 + 1 g/L); 8) soy-based formula with no prebiotic added. Diets were isonitrogenous, isocaloric and fed at 250mL/kg body weight every 4 hours for a 2-week period. Values represent least squared means \pm SEM and statistical significance set at P < 0.05.

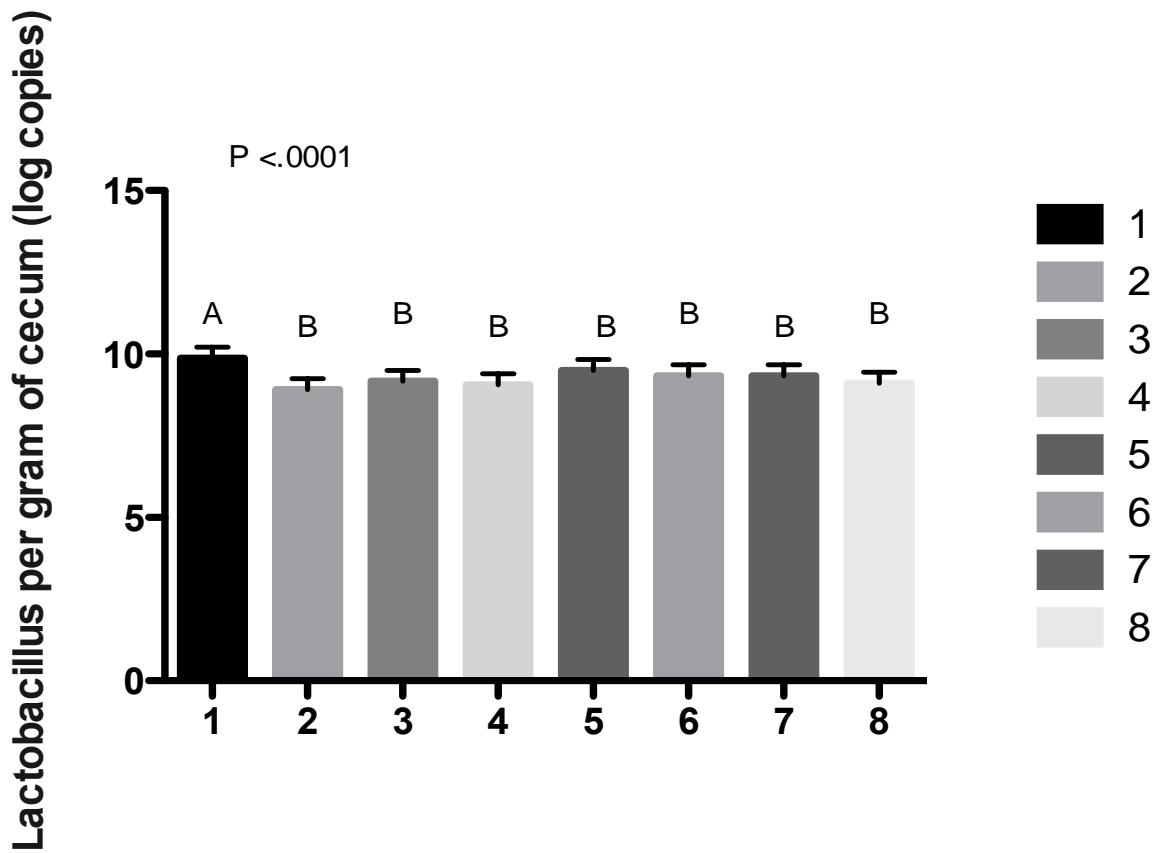


Figure 3.7. Effects of GOS and FOS dietary supplementation on *Lactobacillus* (log copies) per gram of cecum tissue quantified by rtPCR. Piglets ($1.628 + / - .037$ Kg BW) were randomly assigned to one of eight diets ($n = 6/\text{diet}$): 1) milk-based formula containing Bimuno GOS and Vivinal FOS (4 + 1 g/L); 2) soy-based formula containing Orafti (3g/L); 3) milk-based formula with no prebiotic added; 4) milk-based formula containing Bimuno GOS only (5g/L); 5) milk-based formula containing Vivinal GOS only (5g/L); 6) milk-based formula containing Bimuno GOS and Nutraflora FOS (4 + 1 g/L); 7) milk-based formula containing Vivinal GOS and Orafti FOS (4 + 1 g/L); 8) soy-based formula with no prebiotic added. Diets were isonitrogenous, isocaloric and fed at 250mL/kg body weight every 4 hours for a 2-week period. Values represent least squared means \pm SEM and statistical significance set at $P < 0.05$.

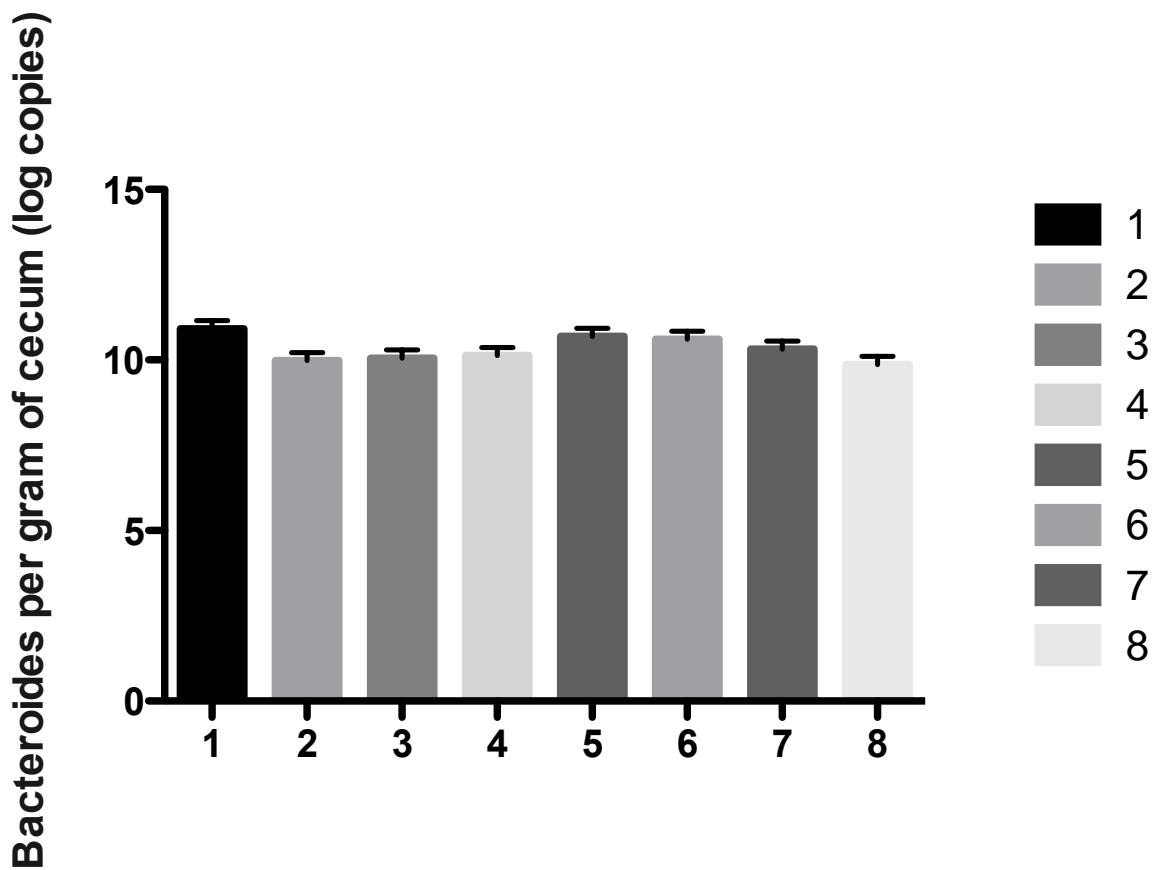


Figure 3.8. Effects of GOS and FOS dietary supplementation on *Bacteroides* (log copies) per gram of cecum tissue quantified by rtPCR. Piglets ($1.628 + / - .037$ Kg BW) were randomly assigned to one of eight diets ($n = 6/\text{diet}$): 1) milk-based formula containing Bimuno GOS and Vivinal FOS (4 + 1 g/L); 2) soy-based formula containing Orafti (3g/L); 3) milk-based formula with no prebiotic added; 4) milk-based formula containing Bimuno GOS only (5g/L); 5) milk-based formula containing Vivinal GOS only (5g/L); 6) milk-based formula containing Bimuno GOS and Nutraflora FOS (4 + 1 g/L); 7) milk-based formula containing Vivinal GOS and Orafti FOS (4 + 1 g/L); 8) soy-based formula with no prebiotic added. Diets were isonitrogenous, isocaloric and fed at 250mL/kg body weight every 4 hours for a 2-week period. Values represent least squared means \pm SEM and statistical significance set at $P < 0.05$.

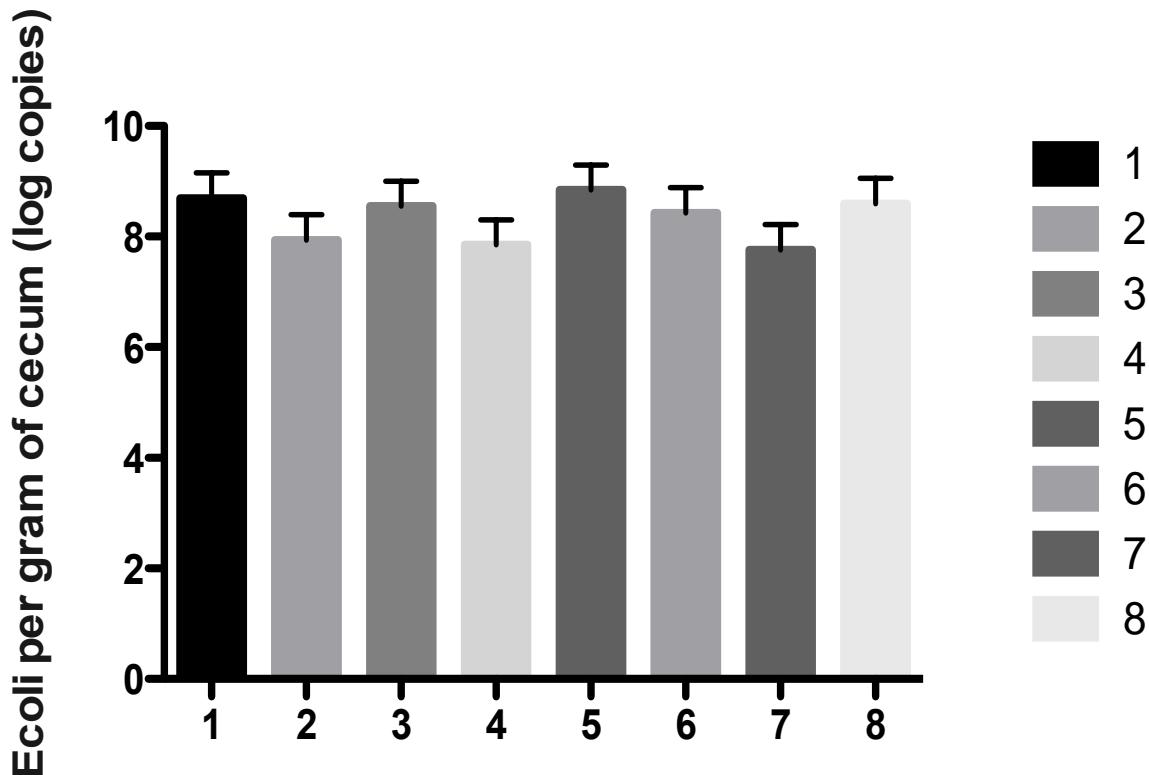


Figure 3.9. Effects of GOS and FOS dietary supplementation on *Escherichia coli* (log copies) per gram of cecum tissue quantified by rtPCR. Piglets ($1.628 +/_{.037}$ Kg BW) were randomly assigned to one of eight diets ($n = 6/\text{diet}$): 1) milk-based formula containing Bimuno GOS and Vivinal FOS (4 + 1 g/L); 2) soy-based formula containing Orafti (3g/L); 3) milk-based formula with no prebiotic added; 4) milk-based formula containing Bimuno GOS only (5g/L); 5) milk-based formula containing Vivinal GOS only (5g/L); 6) milk-based formula containing Bimuno GOS and Nutraflora FOS (4 + 1 g/L); 7) milk-based formula containing Vivinal GOS and Orafti FOS (4 + 1 g/L); 8) soy-based formula with no prebiotic added. Diets were isonitrogenous, isocaloric and fed at 250mL/kg body weight every 4 hours for a 2-week period. Values represent least squared means \pm SEM and statistical significance set at $P < 0.05$.

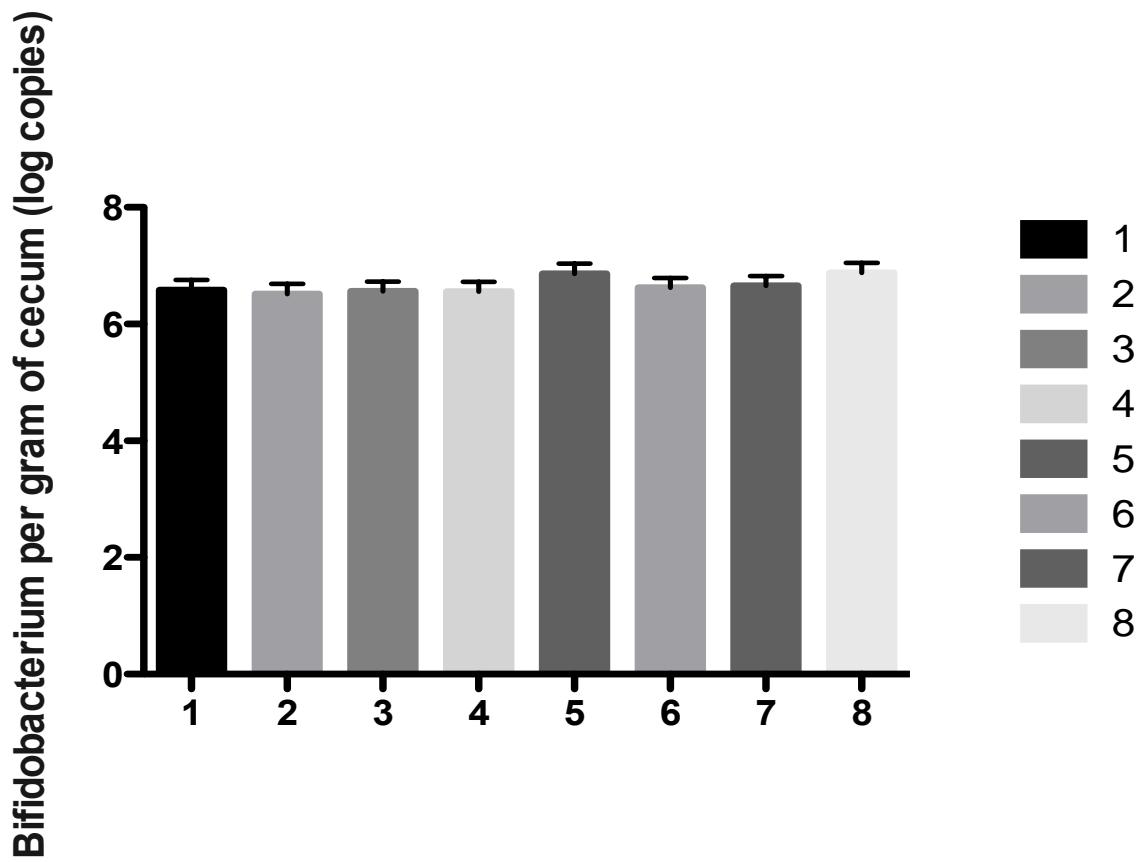


Figure 3.10. Effects of GOS and FOS dietary supplementation on *Bifidobacterium* (log copies) per gram of cecum tissue quantified by rtPCR. Piglets ($1.628 +/_{.037}$ Kg BW) were randomly assigned to one of eight diets ($n = 6/\text{diet}$): 1) milk-based formula containing Bimuno GOS and Vivinal FOS (4 + 1 g/L); 2) soy-based formula containing Orafti (3g/L); 3) milk-based formula with no prebiotic added; 4) milk-based formula containing Bimuno GOS only(5g/L); 5) milk-based formula containing Vivinal GOS only (5g/L); 6) milk-based formula containing Bimuno GOS and Nutraflora FOS (4 + 1 g/L); 7) milk-based formula containing Vivinal GOS and Orafti FOS (4 + 1 g/L); 8) soy-based formula with no prebiotic added. Diets were isonitrogenous, isocaloric and fed at 250mL/kg body weight every 4 hours for a 2 -week period. Values represent least squared means \pm SEM and statistical significance set at $P < 0.05$.

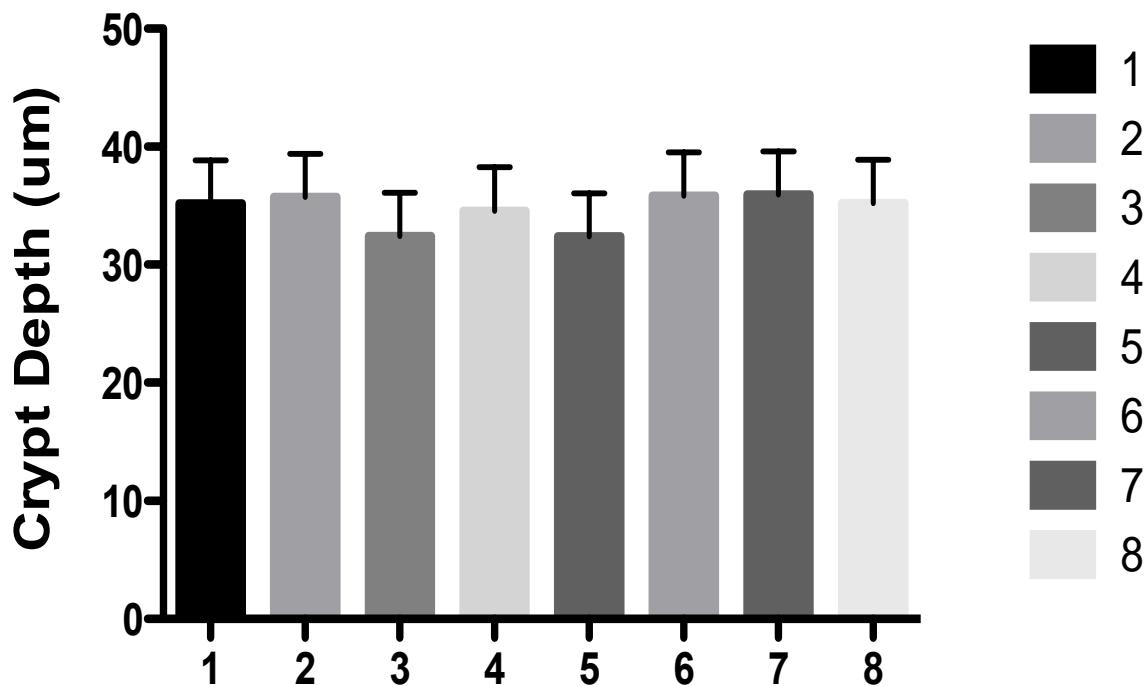


Figure 3.11. Effects of GOS and FOS dietary supplementation on average crypt depth (um) in ileum of pigs fed milk replacer formulas. Piglets ($1.628 +/_{.037}$ Kg BW) were randomly assigned to one of eight diets ($n = 6/\text{diet}$): 1) milk-based formula containing Bimuno GOS and Vivinal FOS (4 + 1 g/L); 2) soy-based formula containing Orafti (3g/L); 3) milk-based formula with no prebiotic added; 4) milk-based formula containing Bimuno GOS only (5g/L); 5) milk-based formula containing Vivinal GOS only (5g/L); 6) milk-based formula containing Bimuno GOS and Nutraflora FOS (4 + 1 g/L); 7) milk-based formula containing Vivinal GOS and Orafti FOS (4 + 1 g/L); 8) soy-based formula with no prebiotic added. Diets were isonitrogenous, isocaloric and fed at 250mL/kg body weight every 4 hours for a 2-week period. Values represent least squared means \pm SEM and statistical significance set at $P < 0.05$.

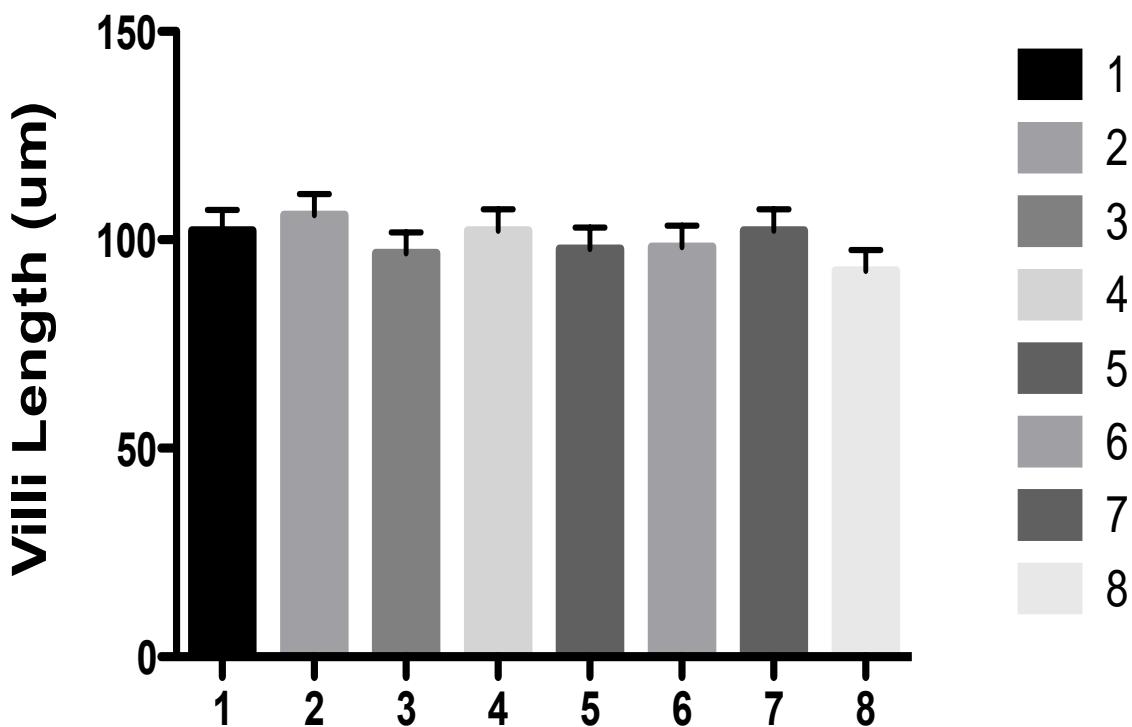


Figure 3.12. Effects of GOS and FOS dietary supplementation on average villi length (um) in ileum of pigs fed milk replacer formulas. Piglets ($1.628 +/_{.037}$ Kg BW) were randomly assigned to one of eight diets ($n = 6/\text{diet}$): 1) milk-based formula containing Bimuno GOS and Vivinal FOS (4 + 1 g/L); 2) soy-based formula containing Orafti (3g/L); 3) milk-based formula with no prebiotic added; 4) milk-based formula containing Bimuno GOS only (5g/L); 5) milk-based formula containing Vivinal GOS only (5g/L); 6) milk-based formula containing Bimuno GOS and Nutraflora FOS (4 + 1 g/L); 7) milk-based formula containing Vivinal GOS and Orafti FOS (4 + 1 g/L); 8) soy-based formula with no prebiotic added. Diets were isonitrogenous, isocaloric and fed at 250mL/kg body weight every 4 hours for a 2-week period. Values represent least squared means \pm SEM and statistical significance set at $P < 0.05$.

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