NUTRITIONAL AND IMMUNOLOGICAL OUTCOMES AS AFFECTED BY A NOVEL CARBOHYDRATE COMPLEX COMPOSED OF GALACTOGLUCOMANNAN OLIGOSACCHARIDES AND ARABINOXYLAN

BY

TREVOR AUSTIN FABER

DISSERTATION

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Animal Sciences in the Graduate College of the University of Illinois at Urbana-Champaign, 2012

Urbana, Illinois

Doctoral Committee:

Professor Emeritus George C. Fahey, Jr., Chair
Professor Carl M. Parsons
Professor Kelly A. Tappenden
Assistant Professor Ryan N. Dilger
Dr. Neil P. Price, National Center for Agricultural Utilization Research, ARS-USDA
ABSTRACT

The objective of this research was to evaluate a novel galactoglucomannan oligosaccharide-arabinoxylan (GGMO-AX) complex for properties that could positively impact nutritional and immunological outcomes. Five studies were designed to address three major research objectives: 1) determine the structural and chemical composition of the GGMO-AX substrate and select fractions, 2) determine the hydrolytic digestibility and fermentative capacity of the GGMO-AX substrate in vitro and in vivo, and 3) determine the immunological effects of GGMO-AX in a pathogen-challenged avian model. Study 1 evaluated the structural composition of the GGMO-AX components as determined by a combination of limited hydrolysis, monosaccharide composition and linkage analysis, size exclusion fractionation and MALDI-TOF/MS analysis of component GGMO, and 1D and 2D NMR techniques. Study 2 evaluated the hydrolytic digestibility, fermentative capacity, and microbiota modulating properties of GGMO-AX and four fractions of GGMO-AX. Study 3 evaluated nutritional effects and prebiotic potential of spray-dried GGMO-AX when added to canine diets and tested in a dose-response experiment. Studies 4 and 5 determined the effects of supplemental GGMO-AX in diets with emphasis on growth performance, fermentative effects, and immune indices in an avian model challenged with an acute coccidial (Eimeria acervulina; EA) or Salmonella typhimurium (ST) infection. Results indicated that GGMOs have a degree of polymerization (DP) from 4 to 13, with the major component being DP 5-8. The structure of these oligosaccharides is a β-1,4-linked backbone of Man and Glc residues, with occasional α-1,6 branching by single galactosyl units. The GGMO-AX substrate is resistant to hydrolytic digestion, well-fermented, and positively modulates microbial populations as measured in vitro and in vivo. When chicks were challenged with EA, a strain of avian coccidiosis, and
supplemented with select concentrations of GGMO-AX, chick performance was decreased, but GGMO-AX supplementation improved select fermentation indices and the innate intestinal immune response. During a ST infection, GGMO-AX elicited a prebiotic effect and appeared to decrease the virulence of the ST within the digestive tract, but did not limit ST intestinal colonization or shedding. Overall, GGMO-AX appears to be well fermented in vitro and in vivo and able to elicit a prebiotic effect in select animal models. Dietary GGMO-AX supplementation is able to improve the innate immune response to an EA infection and potentially decrease ST virulence.
To my family and my wife, Danielle
ACKNOWLEDGEMENTS

I extend many thanks to Dr. George Fahey, Jr. for his support, guidance, and encouragement over the years, enabling me to grow and learn as a scientist. I am very appreciative of the opportunity to further my education in animal nutrition under his guidance.

I also thank my Committee members, Drs. Ryan Dilger, Carl Parsons, Neil Price, and Kelly Tappenden, for their time and assistance in reviewing this dissertation. It was an honor to have you on my Committee. I am grateful to Laura Bauer for her guidance and instruction throughout my college career. I thank Temple-Inland, especially Anne Hopkins and Pat Aldred, for partially funding this dissertation, and allowing me the opportunity to conduct this research. I greatly appreciate the assistance of Drs. Mark Jenkins, Robin Anderson, and Isaac Caan, and Michael Iakiviak, for assistance with the chick-challenge trials. The studies would not have been possible without their assistance.

I express my appreciation to my lab mates throughout the years who have provided me their time, knowledge, and assistance. This research would have not been possible without them. Thank you for the friendship and memories over the years. Recognition needs to be given the to the Poultry Farm staff, in particular Pam and Chet Utterback and Doug Hilgendorf, for their knowledge and assistance with the chick trials.

Last, but not least, I express my gratitude to my parents, Jerry and Glenda, my siblings, and my wife, Danielle, for their encouragement, understanding, and advice over the years. I appreciate my parents instilling the virtues and values that have allowed me to achieve all that I have. I thank my siblings for being positive role models and for defining a path for me to follow. I thank Danielle for her love, patience, and support throughout my graduate career.
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CHAPTER 1: INTRODUCTION

Dogs and cats have evolved into being considered important members of the family. This has resulted in pet owners spending more money to enhance quality of life of their pet. Owners desire diets that are high quality and provide a health and wellness benefit to the pet. In response, the pet food industry is providing choices in the form of ingredient sources (e.g., natural, organic), high quality ingredients (e.g., food-grade ingredients), and novel dietary ingredients (e.g., venison, oatmeal). In addition, functional ingredient (defined as an ingredient that affects physiological function(s) of the body in a targeted way so as to have a positive effect(s) that may, in due course, justify health claims; Roberfroid, 2000) inclusion has greatly increased due to their perceived ability to improve animal health. A popular functional ingredient is dietary fiber, particularly prebiotic fibers. This stems from research indicating that dietary fiber inclusion may improve laxation, attenuate glycemic response and, perhaps, improve intestinal health (Dietary Reference Intakes, 2006; Anderson et al., 2009).

Dietary fibers are polymers of monosaccharides that vary in monosaccharide composition and structure. These characteristics impact fermentability, fermentative end-product production, and the microbial changes that often occur (Slavin, 2010). Many novel dietary fiber sources exist, including galactoglucomannan oligosaccharide-arabinoxylan (GGMO-AX) complex, a coproduct of fiberboard manufacturing. Based on structure and composition, GGMO-AX has the potential to be a promising dietary fiber ingredient and prebiotic; however, in vitro and in vivo research on this substance is limited. Therefore, a series of experiments was conducted to determine the structure and biological effects of dietary inclusion of GGMO-AX.

First, a detailed structural composition of GGMO-AX and select fractions was needed to accurately understand their properties, so that the mechanism of action on biological systems could be determined. Chapter 3 details the structural composition of the GGMO-AX complex as
determined by a combination of limited hydrolysis, monosaccharide composition and linkage analyses, size-exclusion chromatography (SEC) fractionation, and Matrix-Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS) analysis and 1D and 2D NMR techniques.

*In vitro* digestion and fermentation is a valuable methodology to evaluate the hydrolytic digestibility and fermentability of novel dietary fibers prior to conducting *in vivo* animal experiments. The GGMO-AX complex is composed of numerous types of oligosaccharides, including mannanoligosaccharides (MOS), arabinoxylans, and glucooligosaccharides (Moreira and Filho, 2008). Several *in vitro* studies have reported that these oligosaccharides are moderately to well fermented as indicated by increased production of short-chain fatty acids, lowered pH, and altered microbial populations (Sunvold et al., 1995; Flickinger et al., 2000; Vickers et al., 2001; Smiricky-Tjardes et al., 2003; Stewart and Slavin, 2006; Hughes et al., 2007). In addition, studies have indicated that fermentative end-products may vary depending on degree of polymerization of the oligosaccharide (Van Laere et al., 2000; Van de Wiele et al., 2007). Chapter 4 evaluates the hydrolytic digestibility, fermentability, and prebiotic potential of the GGMO-AX complex and purified GGMO fractions isolated from the complex.

It is important to evaluate dietary fibers *in vivo* to determine both positive and negative effects of dietary inclusion and the dietary concentration required to elicit a biological response. Dietary fiber influences digestion by altering microbial enzyme activities and gut anatomical characteristics (Boisen et al., 1985; Jørgensen et al., 1996). Dietary fiber sometimes will result in unwanted outcomes such as diarrhea and other signs of gastrointestinal upset. It is important to evaluate the GGMO-AX complex in dogs to evaluate its nutritional effects and prebiotic
potential in a dose-response experiment, as described in Chapter 5. High concentrations were fed to evaluate tolerance.

Finally, dietary fiber fermentation in the large bowel may benefit the gut-associated lymphoid tissues (GALT) through several potential mechanisms including modulation of the GALT and enhanced intestinal defenses such as increased mucin thickness, modulation of epithelial inflammation by short-chain fatty acids, quantity and type of microbiota, and limited bacterial translocation (Schley and Field, 2002; Cavaglieri et al., 2003; Tedelind et al., 2007; Stecher and Hardt, 2008; Hedemann et al., 2009; Ito et al. 2009). In addition, some oligosaccharides, particularly MOS, may prevent pathogenic bacteria from binding to epithelial cells in the small intestine and prevent colonization and proliferation in the intestinal tract (Ofek et al., 1977; Oyofo et al., 1989). The GGMO-AX complex may beneficially improve the immune status of the intestinal tract of the animal and improve overall gut health when pathogenically challenged. Chapters 6 and 7 determine the effects of the supplemental GGMO-AX complex in diets with emphasis on microbial effects, immune indices, and fermentative effects in an avian model challenged with select intestinal pathogens (Eimeria acervulina and Salmonella typhimurium).

LITERATURE CITED


CHAPTER 2: LITERATURE REVIEW

Introduction

Fermentable carbohydrates are polymers of monosaccharides that resist hydrolytic digestion in the small intestine and enter the large bowel where they are utilized by the resident microbiota (Wong et al., 2006). As a result of fermentation, short-chain fatty acids (SCFA) are produced, resulting in a drop in luminal pH, and potential modulation of the microbiome (Wong et al., 2006). These changes may result in positive health outcomes for the human or animal, such as improved laxation, attenuated glycemic response and, perhaps, a reduction in the incidence of disease (Young et al., 2005; Dietary Reference Intakes, 2006). As a result, many new and novel fermentable carbohydrates are being discovered that could potentially elicit beneficial health outcomes for both humans and animals.

In parallel with the human food industry, the petfood industry has turned its attention to improving quality of life and health of dogs and cats. In particular, ingredients such as fermentable fibers and immunomodulating ingredients are growing in demand. Evidence suggests that fermentable carbohydrates not only improve large bowel immune status, but also the immune status of the host (Anderson et al., 2009). In addition, fermentable carbohydrates may enhance intestinal health by either preventing or lessening the effects of pathogen challenge. This may be through prevention of colonization or enhancing intestinal immune status to help overcome infection (Liévin-Le Moal and Servin, 2006). This improves the ability of an animal to fight a systemic infection and maintain an overall positive health status.
**Fermentable carbohydrate fermentation**

**Digestion and Fermentation**

Fermentable carbohydrates, both soluble and insoluble, consist of oligosaccharides resistant to digestion by mammalian enzymes in the small intestine. Fermentable carbohydrates enter the large bowel or cecum of the animal and are fermented by the host’s microbiome (Anderson et al., 2009). The fermentation process involves microbes converting organic matter into metabolizable energy for the microbe and releasing fermentative end-products utilized by the host. Fermentative end-products include SCFA, gases (CO₂, CH₄, and H₂), branched-chain fatty acids (BCFA), and putrefactive compounds (ammonia, phenols, indoles, and biogenic amines; Roberfroid et al., 2010).

**Microbiota**

The microbiome of the large bowel represents a diverse population of microbial species that all play a role in the fermentation of organic matter. The microbiome is mainly anaerobic and is very complex in terms of numbers of organisms and their diversity. The large intestine of monogastrics and chickens contains an estimated 10¹¹–10¹² bacteria per gram of colonic or cecal contents (Suau et al., 1999; Apajalahti et al., 2004; O’Hara and Shanahan, 2006). Sequencing of the highly conserved 16S region of ribosomal RNA has revealed there is an estimated 800 – 1,000 different bacterial species with over 7,000 strains (O’Keefe, 2008; Qin et al., 2010).

The microbiome may be modified in number and diversity by factors such as pH, peristalsis, substrate availability, transit time, and type of fermentable substrate entering the site (Roberfroid et al., 2010). These changes may be either beneficial or detrimental to gut health depending on the species of bacterium and their main fermentative end-products. Microbes such as *Bifidobacterium* spp. and *Lactobacillus* spp. are viewed as beneficial due to their ability to
produce greater concentrations of SCFA relative to other species. This increased production of
SCFA, particularly acetate and lactate, lowers the pH of the lumen, thus limiting growth and
proliferation of pathogenic microbes such as *Escherichia coli* and *Salmonella enteriditis* (SE;
Bohnoff et al., 1964; Wang and Gibson, 1993). In addition, bifidobacteria and lactobacilli
genera do not include any known pathogenic strains and are primarily carbohydrate-fermenting
bacteria, unlike groups such as bacteroides and clostridia that ferment proteins and amino acids
(Guarner, 2007). Other species, such as *Butyrivibrio* spp., are considered beneficial since they
produce primarily butyrate as their main fermentative end-product (Rumney et al., 1995).

*Short-chain Fatty Acids*

The main SCFA produced are acetate, propionate, and butyrate. These fatty acids serve
as an energy source for the epithelial cells in the large bowel, with butyrate being the primary
source (Topping and Clifton, 2001). Acetate is the main SCFA produced in the large bowel and
is readily absorbed. Acetate serves as an energy source for some tissues in the body (e.g., heart,
muscle, kidney) and is transported to the liver (in some species), adipose tissue, or mammary
gland as a substrate for lipogenesis (Wong et al., 2006). Propionate is utilized during
gluconeogenesis by being an anaplerotic substrate for succinyl CoA in the Krebs cycle.
However, propionate also may inhibit gluconeogenesis via succinyl CoA acting as an inhibitor of
pyruvate carboxylase, an important enzyme in the gluconeogenesis pathway (Wong et al., 2006).
Butyrate is the main energy source for epithelial cells in the large bowel and has been shown to
be a regulator of cell division and proliferation (Topping and Clifton, 2001; Wong et al., 2006).

*Intestinal pH*

Colonic pH decreases as a result of increased SCFA production during fiber
fermentation. A reduction in colonic pH is associated with a reduced risk of developing colonic
cancer (Malhotra, 1982; Samelson et al., 1985). This could be attributed to a lowered pH inhibiting the transformation of primary to secondary bile acids that are cytotoxic to colonic cells and may promote the formation of tumors (Young and Le Leu, 2004). In addition, a lowered pH limits absorption of potentially cytotoxic agents such as ammonia (Topping et al., 2008). Colonic pH is able to affect microbial populations and, thus, the profile of SCFA being produced. *In vitro*, when the pH of the fermentation vessel was maintained at 5.5 for 150 h, butyrate was the dominant SCFA measured (54% of the medium) due to increased growth of *Roseburia*-E. rectale and *Faecalibacterium* groups, which are butyrate-producing bacteria. When the pH was changed to 6.5, acetate became the dominate SCFA, while butyrate decreased to 9% of the medium, likely due to a decrease in butyrate-producing bacteria and increase in *Bacteroides*-related populations (Walker et al., 2005). Pathogenic bacterial (e.g., *E. coli*, *C. perfringens*, *Salmonella*, spp.) growth was suppressed by lowering the pH of the *in vitro* medium, which may reduce the risk of infectious diarrhea *in vivo* (McHan and Shotts, 1992; Wang and Gibson, 1993; Topping and Clifton, 2001). Others studies have shown that short-chain fructooligosaccharids (scFOS) promoted the greatest change in bifidobacteria populations at pH 6.8 in 24 h, whereas inulin, galactooligosaccharides (GOS), and lactulose resulted in the greatest change in bifidobacterial growth at pH 6.0 (Palframan et al., 2002).

**Properties of Fiber That Affect Fermentation**

The rate and extent of fiber fermentation is dependent upon numerous properties including degree of polymerization (DP), solubility, and particle size (Slavin, 2010). These properties relate to region of the large bowel where fiber is fermented, with rapidly fermented fibers being fermented in the proximal region and less fermentable fibers being fermented in the distal bowel or potentially excreted in the feces. Fermentability is an important consideration
when evaluating fibers for health implications, as it relates to the region of the bowel that is most affected. Commonly, as digesta moves through the large bowel, carbohydrates are fermented in the proximal colon, followed by nitrogenous compounds in the distal colon. Fermentation of nitrogenous compounds results in ammonia, phenols, indoles, and biogenic amines being produced. These may be carcinogenic and detrimental to intestinal health (Roberfroid et al., 2010). Thus, by ingesting dietary fibers differing in fermentability, carbohydrate fermentation may be promoted throughout the large bowel (Perrin et al., 2002).

Degree of Polymerization

The DP value indicates the number of monosaccharide units found in an oligosaccharide chain. As DP increases, transit time and rate of fermentability decrease, causing the fiber to be fermented distally in the large bowel (Rumessen and Gudmand-Høyer, 1998; Stewart et al., 2008). Using an in vitro system that mimicked the three segments of the large intestine, Van de Wiele (2007) noted a greater bifidogenic response with inulin (DP 3-60) as compared to oligofructose (2-20) in the simulated distal colon. Degree of polymerization also impacts microbial activity. Bifidobacterium angulatum was able to ferment long-chain inulin, while B. infantis and B. longum only fermented longer chain fructans in the presence of shorter ones, possibly due to induction of select bacterial enzymes (Perrin et al., 2002). Fructans with a DP of <10 increase bifidobacteria populations and are fermented at a faster rate than fructans with a DP of >10 (Van Laere et al., 2000; Roberfroid et al., 2007; Hernot et al., 2009). These results suggest that a mixed DP fiber substrate would be most beneficial due to certain bacteria preferring a select DP and promoting carbohydrate fermentation throughout the digestive tract.
Solubility

Fiber solubility is one method to characterize different types of non-starch polysaccharides. Soluble fiber tends to have a greater impact on the regulation of digestion and absorption in the upper intestinal tract, while insoluble fiber primarily acts in the large intestine where it increases fecal bulk, dilutes colonic contents, and decreases transit time (Bach Knudsen, 2001). Soluble fiber often is rapidly fermented, while insoluble fiber is more slowly fermented, and fermentation does not occur until the distal colon (Bach Knudsen, 2001). In addition, solubility impacts fermentative end-products and microbial populations. In 24 h in vitro fermentation experiments testing soluble and insoluble fibers, soluble fibers such as citrus pectin, FOS, and lactulose resulted in greater SCFA production compared to insoluble fibers such as pea and oat fibers and oat and rice brans (Titgemeyer et al., 1991; Sunvold et al., 1995). A mixture of soluble and insoluble fibers, such as beet pulp, resulted in intermediate production of SCFA. In an in vitro comparison of insoluble wheat fiber to a soluble, enzyme-treated wheat fiber, the soluble wheat fiber resulted in greater bifidobacteria and lactobacilli populations by an average of 0.69 and 0.32 log10 colony forming units (CFU), respectively (Napolitano et al., 2009).

Novel Fermentable Carbohydrates and Their Properties

Prebiotics

Fermentable carbohydrates that “allow specific changes, both in composition and (or) activity in the gastrointestinal microflora that confers benefits upon host well-being and health” are termed prebiotics (Roberfroid, 2007). Prebiotics are beneficial by producing SCFA, which lower luminal pH of the large bowel. The reduced pH creates an environment conducive to growth of beneficial bacteria and detrimental to growth of potential pathogens (Topping and Clifton, 2001). Fructooligosaccharides, GOS, and lactulose have repeatedly demonstrated the
ability to meet the prebiotic definition. Several other types of fermentable carbohydrates, such as xylooligosaccharides (XOS), mannanoligosaccharides (MOS), and glucooligosaccharides, have been shown to demonstrate prebiotic-like effects (Roberfroid, 2007). New and novel fermentable carbohydrates are continually being evaluated for prebiotic potential for use in both human and animal diets.

*Wood-derived Novel Carbohydrates*

A unique fermentable carbohydrate complex is a co-product of fiberboard manufacturing. Fiberboard is a wood product made of tightly packed wood fibers bonded together with a resin or gelling agent to hold the fibers together. One approach to obtain wood fiber is through mild autohydrolysis (Garrote et al., 1999). This process involves wood chips, water, and pressure. Wood chips are placed in a pressurized vat and exposed to steam and temperatures ranging from 200-220 °C for a few minutes (Li et al., 2005). Decompression of the vat results in partial destruction of cellulose, hemicelluloses, and lignin that release soluble sugars into the surrounding water, along with polyphenolic compounds from lignin. Hemicelluloses are depolymerized through hydronium ions from water, and compounds such as uronic, acetic, and phenolic acids are generated (Garrote et al., 1999). The resulting water solution contains high concentrations of sugars (3-4%), a concentration unsafe for disposal into wastewater streams. The wood chips are removed from the sugar solution and further processed into fiberboard (Michalka, 2007).

The remaining sugar solution can be condensed into a syrup, known to AAFCO (2010) as “hemicellulose extract”, with a final sugar concentration of 30-54% (Michalka, 2007). Sugars are mostly in the form of oligosaccharides as compared to free sugars. This particular
hemicellulose extract is composed mainly of oligosaccharides found in hemicelluloses including
arabinoxylans (AX) and galactoglucomannan oligosaccharides (GGMO; Wyman et al., 2005).

*Galactoglucomannan Oligosaccharides*

The main oligosaccharide derived from softwood hydrolysis is GGMO (Willför et al., 2005). The GGMO structure comprises a $\beta$ 1,4-linked backbone consisting of mannose, or mannose and glucose. Galactose residues are attached to the mannan-glucose backbone by $\alpha$-1,6-linkages. The mannose, glucose, and galactose sugars are found in a ratio of approximately 4:1:0.1 with a DP ranging from 2-150 units (Moreira and Filho, 2008).

Studies evaluating the fermentative properties of GGMO are limited. Chalupa and Montgomery (1979) evaluated Masonex®, a GGMO substrate derived from hardwoods, in an *in vitro* system using ruminal inoculum. The GGMO substrate was predicted to have a dry matter disappearance of 90-94% using regression analysis. Glucomannan oligosaccharides (GMO), a common gelling agent in foodstuffs extracted from the *Amorphophallus konjac* plant, is gaining attention for its ability to improve laxation and attenuate blood cholesterol concentrations (Arvill and Bodin, 1995; Chen et al., 2006). An *in vitro* comparison of commercial fiber supplements indicated that GMO resulted in higher fermentability and concentrations of total SCFA compared to other fiber supplements ranging in concentrations of soluble and insoluble fiber (Goñi et al., 1998). Similarly, *in vivo* trials using humans and mice indicated that supplemental GMO (4.5 g/d and 5% of diet, respectively) decreased large bowel pH, increased SCFA production, and increased lactobacilli and bifidobacteria populations compared to control diets (Chen et al., 2005; Chen et al., 2006; Chen et al., 2010).
Arabinoxylans

Arabinoxylan is the second most abundant oligosaccharide in hemicelluloses of softwoods. Arabinoxylan consists of a \( \beta \)-1,4-linked backbone of xylose with arabinose side chains. The xylose to arabinose ratio is approximately 8:1 with a DP of approximately 100 units (Wyman et al., 2005).

Arabinoxylan is readily fermented in the large bowel. *In vitro* studies have shown increases in SCFA production, particularly butyrate, when AX was fermented using human fecal inoculum or isolated probiotic strains of bacteria (Crittenden et al., 2002; Hughes et al., 2007). Both studies indicated that AX promoted bifidobacterial growth, and Crittenden et al. (2002) indicated that AX was not fermented by *E. coli*, *Clostridium perfringens*, or *Clostridium difficile*. Glitsø et al. (1998) evaluated rye dietary fibers in pig diets at an inclusion level of approximately 6%. Fibers with higher concentrations of AX were shown to result in a lower proximal colonic pH and higher SCFA concentrations, particularly butyrate. In chickens, bifidobacterial concentrations were increased \((P < 0.05)\) in birds fed 0.25% supplemental AX compared to the control (Courtin et al., 2008).

**Fermentable Carbohydrates and Intestinal Health**

The gastrointestinal tract (GIT) is exposed to a milieu of ingested ingredients, endogenous compounds, microorganisms, and substances from the external environment. To protect the intestinal tract and the host, the GIT contains numerous gut-associated lymphoid tissues (GALT) to properly distinguish nutrients and innocuous organisms from antigens and pathogenic organisms to protect the body from disease and illness. However, it is important that the GALT not be hypersensitive to organisms and compounds within the intestinal lumen in which an unwarranted response is initiated.
It may be possible to enhance the activity of the GALT through dietary methods, particularly fermentable carbohydrate feeding. Research has shown a positive correlation between the effect of fermentable carbohydrates and improved gut health (Anderson et al., 2009; Roberfroid et al., 2010). Fiber fermentation in the large bowel may benefit the GALT through several potential mechanisms including modulation of the GALT and enhanced intestinal defenses such as increased mucin thickness, modulation of epithelial inflammation by SCFA, quantity and type of microbiota, and limited bacterial translocation (Schley and Field, 2002; Cavaglieri et al., 2003; Tedelind et al., 2007; Stecher and Hardt, 2008; Hedemann et al., 2009; Ito et al. 2009). In addition, some oligosaccharides, particularly MOS, may prevent pathogenic bacteria from binding to epithelial cells in the small intestine and prevent colonization and proliferation in the intestinal tract (Ofek et al., 1977; Oyofo et al., 1989a; Grieshop et al., 2004; Gouveia et al., 2006; Baurhoo et al., 2009).

**Intestinal Immune System**

The GALT consists of aggregated tissue in the form of Peyer’s patches, mesenteric lymph nodes, lymphoid follicles, and also non-aggregated cells within the lamina propria and intraepithelial regions of the GIT (Forchielli and Walker, 2005). Avian species have an additional cluster of aggregated lymphoid tissue identified as cecal tonsils located at the proximal end of the ceca close to the ileocolonic junction (Castelelyn et al., 2010).

Peyer’s patches are located in the mucosa and submucosa layers of the small intestinal tract and serve as “sampling sites” for the intestinal tract. Peyer’s patches consist of lymphatic tissue containing CD4+ and CD8+ T-cells, naïve B-cells, macrophages, and dendritic cells. Overlying the Peyer’s patches are microfold (M) cells, specialized epithelial cells responsible for sampling digesta from the intestinal lumen that transport antigens to the Peyer’s patches (Cebra...
et al., 1998). Upon antigen detection, activated immune cells exit the Peyer’s patches through the mesenteric lymph nodes and enter systemic circulation. The cells then return back to the lamina propria and intraepithelial regions of the intestine (Schley and Field, 2002). The lamina propria contains T- and B- cells, plasma cells, mast cells, and macrophages covered by a single layer of epithelial cells. Intestinal epithelial cells (IEC) or enterocytes not only digest, absorb, and transport nutrients, but also regulate the immune cells within the lamina propria by producing chemokines and cytokines in response to select microbial molecules (Hurley and McCormick, 2004). Interspersed throughout the enterocytes of villi and crypts are intraepithelial lymphocytes (IEL). These lymphocytes are found in both the small and large intestine and consist mainly of T cells (Kunisawa et al., 2007). The IELs assist to survey and regulate the immune response to ensure intestinal homeostasis. This is achieved by adjusting their immunological function in response to various substances found within the small and large intestinal tract. However, little is known about these lymphocytes, but they are thought to be the first segment of the immune system to respond to antigens within the intestine (McKay and Perdue, 1993). Paneth cells are located at the bottom of small intestinal crypts of select mammals. These cells regulate intestinal barrier penetration by both commensal and pathogenic bacteria through the production of antimicrobial peptides (Porter et al., 2002; Vaishnava et al., 2008). This important process lessens the burden of the mesenteric lymphoid nodes to process the numerous penetrating bacteria (Vaishnava et al., 2008).

When activated, the GALT initiates a cell-mediated immune response to a pathogen or antigen. Cell-mediated immunity involves activation of specific cell-types, including macrophages, natural killer cells, and antigen-specific cytotoxic T-lymphocytes. Macrophages, in particular, produce cytokines that serve as cell-signaling molecules to elicit appropriate pro- or
anti-inflammatory responses. Cytokines stimulate B and T cell production that aid in immune cell activation and in developing adaptive immunity. In response to infection, the innate immune system is first activated and involves recruitment of cytotoxic T lymphocytes and natural killer cells to non-specifically kill invading pathogens. Subsequently, the adaptive immune system responds by producing T helper and B lymphocytes that bind to the invading pathogen with high specificity and ultimately signal their destruction to restore homeostasis (Iwasaki and Medzhitov, 2010). Pro-inflammatory cytokines associated with the innate immune system either activate other cytokines or help recruit immune cells to the site of infection to neutralize pathogens or stimulate the adaptive immune system (Lillehoj et al., 2004; Swaggerty et al., 2004; Ferro et al., 2005). In addition, pro-inflammatory cytokines (e.g., IL-1) stimulate the release of mucin to cleanse the mucosal layer of pathogens and prevent further invasion (Jarry et al., 1996).

**Gut Morphology**

Dietary fiber modulates the structure of the intestinal tract such that alterations in length and weight occur in addition to modifications in the morphology of the mucosa such as higher villi, deeper crypts, increased numbers of goblet cells, and a thicker mucus layer on the colonic epithelium (Sigelo et al., 1984; Kim, 2002). These alterations may improve barrier function and elements of immune function of the intestinal tract (Tungland and Meyer, 2002).

The mucin layer that forms within the intestinal tract can be beneficial and also detrimental. Mucins protect IEC against enzymes, bacteria, dietary consituents, and potentially harmful chemicals (Deplancke and Gaskins, 2001). In addition, the mucin layer acts as a physical barrier or retention zone to prevent bacterial penetration through the IEC; however, the glycoprotein matrix that forms the mucin may provide an attachment site for bacteria to bind and proliferate. This attachment can prevent clearance from the digestive tract. In addition, the
mucin layer provides nutrients to the attached bacteria, promoting proliferation and colonization (Aristoteli and Willcox, 2003). Mucins are classified as either neutral or acidic, and acidic types are further divided into sulfated (sulfomucins) and non-sulfated (sialomucins) types (Sheahan and Jervis, 1976). The large intestine contains mostly acidic mucins, which are thought to protect against bacterial translocation due to less degradation from bacterial glycosidases and host proteases (Sheahan and Jervis, 1976; Fontaine et al., 1996; Roberton and Wright, 1997). On the contrary, select bacterial strains, such as *Salmonella typhimurium* (ST), bind to the sialic acid glycoproteins and produce a sialidase to destroy and penetrate the mucin layer (Hoyer et al., 1992; Vimal et al., 2000).

Dietary fiber has been shown to modulate mucin thickness in rats. Both cellulose (insoluble fiber) and pectin (soluble fiber) were shown to increase colonic mucin thickness by 118 µm and 105 µm, respectively (Hedemann et al., 2009). Overall, mucosal layer thickness is affected by fermentable carbohydrate inclusion. Ito et al. (2009) demonstrated that ileal mucin secretion increased as fiber viscosity of a GMO substrate increased. In addition, as fiber viscosity increased, goblet cell numbers increased accordingly.

*Short-chain Fatty Acids*

Short-chain fatty acids produced from fiber fermentation may modulate the intestinal immune system by controlling cytokine release. Newly weaned piglets fed a diet supplemented with beet pulp (5%), inulin (0.75%), and lactulose (0.1%), which resulted in increased SCFA concentrations, had elevated IL-6 (a pro-inflammatory cytokine) expression in colon, but not ileum compared to piglets fed a control with no supplemental fermentable carbohydrate inclusion (Pié et al., 2007). On the contrary, acetate, butyrate and, particularly, propionate, were shown to decrease IL-6 expression using colon organ cultures (Tedelind et al., 2007). Cavaglieri et al. 
(2003) demonstrated that acetate, propionate, and butyrate did not impact IFN-γ expression, a pro-inflammatory cytokine, while an increase in IL-10 (an anti-inflammatory cytokine) expression was shown in rat mesenteric lymph nodes. Inan et al. (2000) demonstrated in vitro that butyrate suppressed expression of NF-κB, a regulator of the expression of numerous cytokines and cell adhesion molecules involved in immune and inflammatory responses. These results indicate that SCFA may modulate the GALT by modulating cytokine expression, thus affecting inflammation and immune responses.

Short-chain fatty acids also may directly inhibit cecal colonization. In SE-infected chickens fed microbeads covered in select SCFA, acetate resulted in an increase in the SE-colonization of ceca and internal organs, while butyrate-impregnated microbeads resulted in a decrease (P < 0.05) in the SE-colonization of the ceca (Van Immerseel et al., 2004).

Colonization Resistance

Colonization resistance is the prevention of colonization and enteric disease caused by pathogenic bacteria (enteropathogens) in the normal gut due to the density of the commensal microbiota (Stecher and Hardt, 2008). If the commensal microbial population is disturbed (i.e., antibiotics, gut inflammation), enteropathogens could colonize the intestine and pathogenesis could occur (Stecher et al., 2007). Commensal bacteria use a variety of strategies to prevent enteropathogens from colonizing the gut. These include producing antimicrobials or toxins (e.g., bacteriocins and SCFA), utilization of adhesion receptors to prevent attachment, stimulation of mucin release, maintaining the gut mucosal barrier, improving gut motility, and limiting nutrients for pathogenic bacteria (Stecher and Hardt, 2008).

Prebiotics enhance the population of perceived beneficial microbiota such as Lactobacillus and Bifidobacterium spp. These bacteria play an important role in the commensal
bacteria due to their ability to destroy pathogenic bacteria. *Lactobacillus* and *Bifidobacterium* spp. produce antimicrobial compounds able to destroy pathogens that cause diarrhea (Servin, 2004). In addition, *Lactobacillus* spp. are able to inhibit the growth of different strains of *E. coli* and *Salmonella* through mechanisms related to the destruction of the bacterial cell membrane and inhibition of the pathogens ability to internalize itself into host cells (Coconnier et al., 1993; Bernet-Camard et al., 1997; Cocconnier et al., 2000; Gopal et al., 2001; Cocconnier-Polter et al., 2005; Fayol-Messaoudi et al., 2005).

Fermentable carbohydrates, especially prebiotics, provide nutrients to the microbiota, particularly *Lactobacillus* spp. and *Bifidobacteria* spp., of the large bowel and may increase overall population density of bacteria (Roberfroid et al., 2010). This increase in population density of the commensal microbiota may prevent enteropathogenic colonization and prevent a systemic bacterial infection. In addition, commensal microbiota of the GIT contribute greatly to the development and activity of the GALT and the defense against pathogenic organisms (Cebra, 1999). Germ-free (GF) animals possess defects in GALT development, antibody production compared to conventional animals, and are susceptible to colonization by pathogenic bacteria (Bohnoff et al., 1964; Nardi et al., 1989; Falk et al., 1998; Macpherson and Harris, 2004; Rhee et al., 2004).

**Translocation**

During times of enteropathogenic infection, bacteria can penetrate the intestinal barrier and enter the lymph and blood circulation. Once in the circulation, the bacteria translocate to other lymphatic organs such as the mesenteric lymph nodes, spleen, and liver.

The feeding of both soluble and insoluble fermentable carbohydrates has been shown to limit or decrease bacterial translocation in both healthy and immune-challenged rat models
(Deitch et al., 1993; Frankel et al., 1995; Nettelbladt et al., 1998; Buddington et al., 2002; Kleesen and Blaut, 2005). On the contrary, research using ST- and SE-challenged rats and mice indicated that fermentable carbohydrates, FOS, pectin, and XOS, may actually increase bacterial translocation to the mesenteric lymph nodes, spleen, and liver (Bovee-Oudenhoven et al., 2003; Ten Bruggencate et al., 2003; Ten Bruggencate et al., 2004; Ten Bruggencate et al., 2005; Petersen et al., 2009). The conflicting results may be related to the rate of fermentation of the fiber substrates. Rapidly fermented substrates (scFOS, XOS, pectin) result in increased bacterial translocation, while fibers that were slowly to moderately fermented (inulin, cellulose, polydextrose) resulted in decreased bacterial translocation. Rapid fermentation can result in a lower pH in the proximal colon compared to moderate to slowly fermented fibers, which decreases pH throughout the large bowel. The low pH in the proximal colon can lead to intestinal irritation, resulting in decreased barrier function and allow enteropathogens to cross the epithelial layer (Argenzio and Meuten, 1991; Lin et al., 2002). In addition, in studies by Bovee-Oudenhoven et al. (2003) and Ten Bruggencate et al. (2003; 2004; 2005), rats were fed a low calcium diet (20-30 mmol/kg of diet) that may have impaired barrier function. When Ten Bruggencate et al. (2004) fed rats a calcium sufficient diet (100 mmol/kg of diet) containing inulin and FOS, the decrease in cecal pH was blunted and bacterial translocation was limited compared to rats fed a low calcium diet containing either FOS or inulin.

The exact mechanism(s) by which fermentable carbohydrates affect translocation has yet to be determined. As previously mentioned, one potential mechanism may be fermentable carbohydrates increasing mucin thickness, thus creating a physical barrier and decreasing translocation. Another potential mechanism is through increased butyrate production. Butyrate has been shown in vitro to improve the intestinal barrier by enhancing the tight junction integrity.
of epithelial cells (Peng et al., 2009). This improvement in tight junction integrity has been shown to decrease bacterial translocation across epithelial cells (Kanauchi et al., 1999; Van Deun et al., 2008).

**Bacteria-oligosaccharide Binding**

As stated earlier, the mucosal layer of the small intestine acts as a barrier to prevent bacterial penetration and translocation, but may provide a substrate for bacterial attachment. Binding to the mucosal layer prevents bacteria from being carried out of the intestinal tract, particularly the small intestine where the digesta flow rate is considerably greater (Sonnenburg et al., 2004). Enterobacteria species, such as certain strains of *E. coli* and *Salmonella*, utilize appendages, fimbriae, to attach to the mucosal surface of the intestine and colonize the digestive tract (Ofek et al., 1977). Fimbriae are long, filamentous appendages present on the surface of microbes and are attracted to specific receptors, either glycoproteins or glycolipids, located on the mucosal layer of the intestines (Beachey et al., 1988). In particular, type-1 fimbriae recognize and adhere to only mannose and mannan moieties that are either free in the intestinal tract or bound to proteins (glycoproteins) or lipids (glycolipids; Mirelman and Ofek, 1986; Neeser et al., 1986).

Feeding D-mannose and mannann oligosaccharides may prevent colonization of pathogenic bacteria in the digestive tract by competitive inhibition. Dietary mannose acts as a receptor analog by being structurally similar to glycoprotein receptors for type-1 fimbriae and, thus, able to prevent attachment to the mucosal layer (Mirelman et al., 1980; Firon et al., 1984; Ofek et al., 2003). Oyofo et al. (1989b) demonstrated *in vitro* that the addition of 2.5% D-mannose to media blocked 95% of type-1 fimbriae from adhering to small intestinal epithelial cells. When D-mannose was added to the drinking water of broiler chickens, colonization by
type-1 fimbriated ST was reduced by 50-61% compared to chickens consuming water without D-mannose (Oyofo et al., 1989a).

Studies evaluating MOS also have been shown to decrease intestinal populations of type-1 fimbriae bacteria. Chicks challenged with either *E. coli* or *Salmonella* spp. and fed MOS ranging between 0.2-2.5% resulted in decreased colonization by the pathogenic bacteria in either the ceca or jejunum (Spring et al., 2000; Fernandez et al., 2002; Yang et al., 2008; Baurhoo et al., 2009). Grieshop et al. (2004) noted decreased (P < 0.04) fecal *E. coli* colony forming units in dogs fed a diet containing 1% MOS. Gouveia et al. (2006) noted an antibiotic treatment plus 2 g supplemental MOS eliminated pathogenic *E. coli* in 86% of dogs with enterocolitis compared to 25% of dogs given the antibiotic treatment alone. Burkey et al. (2004) noted no decrease in fecal shedding of ST in pigs fed 0.15% MOS and challenged with ST. It is possible that dietary concentration of MOS was not great enough to elicit an effect. Overall, dietary mannose and MOS appear to benefit the host animal when pathogenically challenged.

**Disease States Potentially Affected by Fermentable Carbohydrates**

**Coccidiosis**

**Pathogenesis**

Coccidiosis, or *Eimeria*, is an infectious protozoa that penetrates the intestinal tract and damages the epithelial cells of numerous species of animals. Currently, seven species of *Eimeria* (*E. acervulina, E. maxima, E. tenella, E. necatrix, E. mitis, E. brunetti, and E. praecox*) have been recognized to infect chickens (Carvalho et al., 2011). In particular, *E. acervulina* invades the duodenal intestinal epithelium resulting in intestinal inflammation and, potentially, intestinal hemorrhaging (Lillehoj and Trout, 1996). The intestinal damage results in decreased animal growth and feed efficiency, as well as diarrhea and mortality, all of which have significant
implications for the commercial poultry industry, where coccidiosis-associated production losses amount to an estimated $3 billion U.S. annually (Williams, 1999; Shirley et al., 2004; Dalloul and Lillehoj, 2006). The decrease in growth and feed efficiency is partially attributed to chicks experiencing a decrease in digestibility of energy, glucose, fat, and amino acids, as indicated in both in vitro and in vivo experiments (Ruff and Wilkins, 1980; Adams et al., 1996; Persia et al., 2006; Jacobs, 2011). Preston-Mafham and Sykes (1970) showed that the decrease in feed intake due to infection accounts for approximately 30-70% of the decrease in weight gain as determined in uninfected chicks that were pair-fed with infected chicks. Moreover, decreases in nutrient digestibility are known to occur concomitantly with changes in intestinal enzyme activity, which are associated with Eimeria-induced damage of duodenal tissue (Adams et al., 1996).

**Immune Response**

In response to an *E. acervulina* infection, the GALT utilizes various mechanisms to overcome the infection, including natural killer (NK) cells, mast cells, macrophages, cytokines, and parasite-specific antibodies (IgM, IgG, and IgA; Lillehoj and Trout, 1996). Interferon-γ expression has been well characterized during an avian *Eimeria* infection. It activates the phagocytic potential of macrophages and cytotoxic potential of NK cells to destroy the *Eimeria* protozoa (Lillehoj and Trout, 1996). In addition, IFN-γ is able to decrease intestinal invasion and development of the parasite and improve body weight gain during *E. acervulina* and *E. tenella* infections (Kogut and Lange, 1989a,b; Lowenthal et al., 1997; Lillehoj and Choi, 1998). Little is known about the involvement of most cytokines in response to an *Eimeria* infection, except that the mRNA expression is elevated during infection.
Effects of Dietary Fiber

Few studies have evaluated the effects of dietary fiber on *Eimeria* infection. Mannanoligosaccharides are the most common fiber sources evaluated (Elmusharaf et al., 2006, 2007; Gao et al., 2009). Chicks infected with either *E. tenella* or *E. acervulina* and fed diets containing 0.1 or 1% YCW, respectively, had reduced numbers of schizonts, an intermediate life stage during *Eimeria* oocyst reproduction, in the lamina propria of the cecum and oocyte shedding in excreta (Elmusharaf et al., 2006, 2007). Gao et al. (2009) suggested that YCW supplementation (0.25 and 0.5%) promoted overall immune function in *E. tenella*-infected chicks as indicated by improved responses of both the innate immune response and antibody production. Persia et al. (2006) reported improved weight gain for infected chicks fed 5.0% Grobiotic® (a mixture of yeast products and various solubles), while Jacobs (2011) failed to observe a similar response. Jacobs (2011) reported that 1.2% Dairylac-80 (a lactose substrate), or a 1.0% lactose-containing diet, was unable to improve weight gain in *E. acervulina*-infected chicks.

*Salmonella*

Pathogenesis

*Salmonella* spp. are common intestinal pathogens for most animals, and one of the major causes of food-borne illness in the United States with an estimated one million reported cases per year (Scallan et al., 2011). *Salmonella typhimurium* and SE infections result in gastroenteritis, diarrhea, and fever in most animals (Ohl and Miller, 2001). Following ingestion, ST travels through the digestive tract and attempts to colonize the ileum and large intestine (or cecum). Using fimbria, ST adheres and colonizes the intestinal mucin layer. *Salmonella typhimurium* penetrates the epithelial layer in two ways. Most often, ST penetrates the M-cells of Peyer’s
patches and replicates in the submucosa and Peyer’s patches (Carter and Collins, 1974). Salmonella also may be translocated to the lamina propria by macrophages and phagocytes. Intestinal epithelial cells utilize pattern-recognition receptors expressed on cellular membranes to detect pathogen-associated molecular patterns (PAMP) exhibited by pathogenic bacteria (Eckmann, 2006). Salmonella possess several PAMP, including lipopolysaccharide (LPS), peptidoglycan, mannose, and flagellin. Once a PAMP is detected, macrophages and phagocytes are recruited to the epithelial cells to endocytose the bacterium, resulting in epithelial cells initiating an inflammatory response (Zheng et al., 2003). Once engulfed by a macrophage or phagocytes, ST is able to grow and replicate within the cell (Alpuche-Aranda et al., 1994). These cells migrate to other organs of the lymphatic system, allowing ST to disseminate and initiate a systemic infection (Ohl and Miller, 2001). Colonization results in intestinal inflammation, which suppresses and alters growth of the commensal bacteria due to the release of antibacterial peptides and lectins (Cash et al., 2006; Dann and Eckmann, 2007) and phagocyte activation (Stecher et al., 2008). However, ST often is able to overcome these responses and, with the decrease in commensal bacteria, is able to colonize the digestive tract (Stecher et al., 2008).

**Immune Response**

The detection of salmonella in epithelial cells by macrophages and phagocytes initiates a cell-mediated response resulting in release of cytokines (e.g., IFN-γ, IL-6, and IL-1β) from macrophages (Trebičavský, 1999). Additional cytokines, such as tumor necrosis factor (TNF)-α, IFN-γ, and IL-12, initiate intestinal inflammation and recruitment of macrophages to destroy the pathogen and suppress ST growth (Mastroeni et al., 2000). Clearance of a ST infection involves IFN-γ-mediated Th1 responses that increase activity of lymphocytes and macrophages,
which then destroy and clear ST from the body (Mastroeni and Menager, 2003; Withanage et al., 2005).

*Effect of Dietary Fiber*

Dietary fiber can potentially prevent ST colonization in several ways as mentioned previously. The most common dietary fiber evaluated is MOS due to type-1 fimbria bacteria binding to mannans as discussed previously. In addition, AXs ($\geq 0.4\%$ of the diet) have been shown to decrease ST and SE colonization and shedding in chicks (McHan et al., 1991; Eeckhaut et al., 2008). Numerous studies have evaluated the effect of prebiotics on pathogenic organisms, mainly ST and *E. coli*. Studies using puppies, piglets, and chicks as *in vivo* models have indicated mainly positive results as indicated by decreased ileal enterocyte sloughing, ST shedding and colonization, and incidence of diarrhea (Chambers et al., 1997; Letellier et al., 2001; Correa-Matos et al., 2003; Apanavicius et al., 2007), while few have indicated no effect (Letellier et al., 2000). Studies using rodents have yielded mixed results, possibly related to a greater number of studies leading to more variances in diet, fiber concentration, and inoculum dose, thus affecting results (Lomax and Calder, 2009).

**Summary**

The intestinal and systemic health benefits of fermentable carbohydrates in human and animal nutrition suggest fiber to be an important dietary ingredient. The discovery of novel fermentable carbohydrates that are able to exhibit prebiotic-like effects will benefit both humans and animals due to their ability to positively modulate the microbiome and fermentative end-products. This, in turn, could enhance the GALT, increase mucin thickness, modulate epithelial inflammation through SCFA, increase quantity and type of microbiota, and limit bacterial translocation to improve the ability of the GIT to protect and destroy intestinal pathogens. In this
thesis, a novel GGMO-AX complex carbohydrate will be evaluated for these characteristics. Information gained from this research will enhance the knowledge base about the effects of fermentable carbohydrates on intestinal inflammation and their ability to improve intestinal and systemic health.

**LITERATURE CITED**


CHAPTER 3: GALACTOGLUCOMANNAN OLIGOSACCHARIDES (GGMOs) FROM A MOLASSES BYPRODUCT OF PINE (PINUS TAEDA) FIBERBOARD PRODUCTION

ABSTRACT: “Temulose” is the trade name for a water-soluble molasses produced on a large scale (300-400 tonnes per year) as a byproduct of the fiberboard industry. The feedstock for Temulose is predominantly a single species of pine (Pinus taeda) grown and harvested in stands in southeastern Texas. Because of the method of production, the molasses was predicted to consist of water soluble hemicelluloses, mainly arabinoxylan-type and galactoglucomannan-type oligosaccharides, plus minor components of lignin, but no detailed structural study had been reported. The structure and composition of the molasses has now been deduced by a combination of MALDI-TOF mass spectrometry, size exclusion chromatography, proton and $^{13}$C NMR techniques, and classic carbohydrate analysis. Limited acid hydrolysis released a series of galactoglucomannan oligosaccharides (GGMOs) that were selectively recovered from the acid-labile arabinogalactan by precipitation with ethanol. The precipitate was named “Temulose brown sugar” because of its appearance, and is shown to consist of GGMOs with a degree of polymerization (DP) from 4 to 13, with the major component being DP 5-8. The structure of these oligosaccharides is a $\beta$-1,4-linked backbone of Man and Glc residues, with occasional $\alpha$-1,6 branching by single galactosyl units.

INTRODUCTION

Temulose molasses is a commercial byproduct in the production of medium-density fiberboard. The water-soluble molasses is produced on a large scale (typically 0.9 tonne per day).

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1 Dr. Neil Price and Trina Hartman are acknowledged for conducting the research and writing this chapter.
and has a brown-colored, viscous appearance. The feedstock for Temulose is loblolly pine
\((Pinus taeda)\), predominantly from natural stands in eastern and central Texas. Loblolly pine is
often referred to as southern yellow pine, which is not a single tree species but rather a variety of
species, such as longleaf pine \((Pinus palustris\) Mill.\), shortleaf pine \((Pinus echinata\) Mill.\),
loblolly pine \((P. taeda L.)\), and slash pine \((Pinus elliottii\) Engelm.\), that grow well in the acidic
red clay soil found in most of the southern United States. Previous studies have shown that pine
wood is typically composed of lignocellulosics, arabinoxylan hemicelluloses, and
galactoglucomannan (GGM).

Pine wood hemicelluloses are typically two-thirds GGM and about one-third arabino-4-
O-methylglucuronoxylan. The GGM backbone generally consists of \(\beta-(1\rightarrow4)\)-D-
mannopyranosyl and \(\beta-(1\rightarrow4)\)-D-glucopyranosyl residues, with occasional \(\alpha-(1\rightarrow6)\)-D-
galactopyranosyl branching (Timell, 1965; Spiridon and Popa, 2004) with a degree of
polymerization (DP) between 100 and 150 residues (16-24 kDa). Mannose, glucose, and
galactose ratios are typically in a molar ratio of 3:1:1 (Aspinall and McKay, 1962; Aspinall and
Wood, 1963; Brasch, 1983; Puls et al., 1992; Willf"or et al., 2003; Willf"or et al., 2005). The
majority of the mannosyl units (for Norway spruce, about 65\%) are partially substituted by O-
acetyl groups at either the C-2 or C-3 position in a ratio of 2.2:1 (Willf"or et al., 2005). Another
study of the spruce \((Picea abies)\) GGM structure showed a DP from 11 to 20, with the
Man/Glc/Gal molar ratio of 4:1:0.1 (Lundqvist et al., 2002; Willf"or et al., 2003). About one-
third of D-mannosyl units were O-acetylated, almost equally distributed between C-2 and C-3.
The acetyl content varied between 5.9 and 8.8\% (Puls et al., 1992). The ratio between mannose
and glucose residues reported for other softwood glucomannans also varies, such as Norway
spruce between 3.5 and 4:1 (Capek et al., 2000; Capek et al., 2002; Lundqvist et al., 2002;
Lundqvist et al., 2003; Willför and Holmbom, 2004; Xu et al., 2010), red cedar 2.5:1 (Hamilton and Partlow, 1958), western hemlock 3:1 (Hamilton and Thompson, 1957; Hamilton and Kircher, 1958), loblolly pine 2.7:1 (Jones et al., 1957), Scots pine 3.1-3.7:1 (Willför and Holmbom, 2004), and Sitka spruce 2.5:1 (Dutton and Hunt, 1958). Other reports of softwood GGM have included those from red pine (Hoffman and Timell, 1970), southern pine (Hamilton et al., 1960), white spruce (Tyminsk and Timell, 1960), and poplar (Kubačková et al., 1992).

To be consistent with these reported data, we expected that the loblolly pine-derived Temulose molasses may consist of predominantly water-soluble arabinoxylan and GGM type hemicelluloses, plus minor components of lignin-derived phenolic esters. The material has a noticeable but pleasant odor that GC-MS analysis showed to be predominantly due to complex vanillin esters (data not shown). Here we report the structural composition of the Temulose carbohydrate components as determined by a combination of limited hydrolysis, monosaccharide composition and linkage analysis, size exclusion fractionation and MALDI-TOF/MS analysis of component GGM oligosaccharides, and 1D and 2D NMR techniques.

**MATERIALS AND METHODS**

Materials and supplies were obtained commercially from Sigma-Aldrich Chemicals, St. Louis, MO. Temulose molasses was kindly provided by Temple Inland Inc., Diboll, TX.

*Compositional and Linkage Analysis by Gas Chromatography-Mass Spectrometry (GC-MS)*

Duplicate samples were hydrolyzed on a reaction block using aqueous trifluoroacetic acid (TFA, 2.0 M, 110 °C, 1 h). After cooling, the solvent was removed by evaporation, and aldononitrile acetate or alditol acetate derivatives were prepared as described previously (Jones and Albersheim, 1972; Price, 2004). Permethylation linkage analysis was performed as
described (Ciucano and Kerek, 1984). GC-MS analysis was performed on an Agilent (Santa Clara, CA) 6890N gas chromatograph interfaced with an Agilent 5973N mass-selective detector configured in EI mode and with a Hewlett-Packard (Santa Clara, CA) 7683 series autoinjector. A Hewlett-Packard DB-5 ms column (30 m; 0.2 mm i.d.) was used in split injector mode with helium as the carrier and a linear gradient from 150 to 300 at 10 °C/min. Mass spectra were recorded in positive-ion mode over the range m/z of 50-550. Injector and detector/interface temperatures were 275 and 300 °C, respectively. Data analysis was done off-line using HP Chemstation software.

**Size Exclusion Chromatography (SEC)**

The Temulose “brown sugar” was recovered by ethanol precipitation from acid-hydrolyzed (0.2 M TFA, reflux, 1 h) Temulose and fractionated by SEC on a 1.27 m Bio-Gel P2 column. A typical hydrolysis used 20 mL of 1.0 M aqueous TFA plus 100 mL of Temulose molasses and was precipitated with an equal volume of ethanol. The brown sugar precipitate was recovered by filtration. The SEC column was eluted with deionized water, and fractions (5 mL) were collected using an automated fraction collector. The fractions were analyzed by MALDI-TOF/MS, and those containing oligosaccharides of equivalent molecular mass were pooled and concentrated by rotary evaporation.

**MALDI-TOF/MS Analysis**

MALDI-TOF mass spectra were recorded on a Bruker Daltonic Omniflex instrument (Bruker Daltonics, Billerica, MA) operating in reflectron mode. Samples were typically dissolved in acetonitrile, and the matrix used was 2,5-dihydrobenzoic acid. Ion source 1 was set to 19.0 kV and source 2 to 14.0 kV, with lens and reflector voltages of 9.20 and 20.00 kV, respectively. A 200 ns pulsed ion extraction was used with matrix suppression up to 200 Da.
The instrument was calibrated externally on a DP series of malto-oligosaccharides. Excitation was at 337.1 nm, typically at 60% of 150 μJ maximum output, and 80 shots were accumulated. The linear mass resolution (fwhm) for m/z 2465 (ACTH 18-39) was >3500.

**NMR Spectroscopy**

All NMR experiments were performed on a Bruker Avance spectrometer (Bruker BioSpin Corp., Billerica, MA) operating at 500.11 MHz using a standard 5 mmz-gradient BBI probe at 27 °C. Chemical shifts are reported as parts per million from tetramethylsilane calculated from the lock solvent. The deuterated solvents used were obtained from Cambridge Isotope Laboratories (Andover, MA). The pulse sequences used were those supplied by Bruker, and processing was done with the Bruker TOPSPIN software package (version 1.3).

**RESULTS AND DISCUSSION**

**Monosaccharide Compositional Analysis and MALDI-TOF MS**

The total monosaccharide composition of the Temulose was determined by GC-MS analysis of peracetylated aldononitrile acetate derivatives. The nonhydrolyzed (free) monosaccharides in the Temulose were predominantly arabinose (52.2%) and xylose (17.6%), with smaller quantities of free mannose, glucose, and galactose. The free mannose content of the nonhydrolyzed Temulose was 6.6%. Following mild acid hydrolysis with aqueous trifluoroacetic acid, the predominant monosaccharides present were mannose (56.5%), glucose (13.5%), and xylose (10.7%). Comparable results were obtained on filtered Temulose (Table 3.1). To investigate the mass distribution of these sugars, the Temulose was diluted and dialyzed against a 3500 Da cutoff membrane. GC-MS compositional analysis of the eluent retentate
showed that the Man-Glc-Gal component was evenly distributed, whereas the arabinoxylan was predominantly retained (Figure 3.1).

To investigate the molecular mass distribution (degree of polymerization, DP) further, the Temulose molasses was analyzed by matrix-assisted laser dissociation/ionization time-of-flight mass spectrometry (MALDI-TOF/MS). The molasses consisted of a complex series of oligosaccharides predominantly in the mass range of 400-1500 Da (Figure 3.2). These were assigned by the mass of the observed [M + Na]^+ sodium adduct ions and were composed of hexosyl oligosaccharides (Man, DP 3-8), both mono- and diacetylated hexosyl oligosaccharides (ManAc, DP 4-8, and ManAc2, DP 3-8), plus pentosyl oligosaccharides (Xyl, DP 3-10), and two methyl-glucuronate-containing pentosyl oligosaccharides (Xyl8MeGA and Xyl9MeGA).

Biphasic fractionation of the diluted molasses with butanol, ethyl acetate, or diethyl ether was undertaken, and the phases were analyzed by MALDI-TOF/MS. Ether was observed to preferentially extract the partially acetylated hexosyl oligosaccharides and, to a great extent, the series of pentosyl oligosaccharides (Figure 3.2). A more detailed oligosaccharide compositional analysis by MALDI-TOF/MS for the mass range of 600-1000 Da is shown in Figure 3.3. Cellulo-oligosaccharides (Cel, DP 3-5) were used as controls to assign hexosyl oligosaccharide masses and xyloooligosaccharides (Xyl, DP 5-7) as pentosyl oligosaccharide controls. The isobaric oligosaccharidic ions from Temulose were assigned at m/z 689, 851, and 1013 (Man, DP 4, 5, and 6, respectively) and m/z 701, 833, and 965 (Xyl, DP 5, 6, and 7, respectively).

Sodium adduct ions at m/z 731 and 893 correspond to 689 + 42 and 851 + 42 Da, respectively, where 42 Da is the mass of a single acetyl group (ManAc). Similarly, ions at m/z 773 and 935 arise from diacetylated oligosaccharides (ManNAc2), with masses 689 + 42 + 42 and 851 + 42 + 42 Da (Figure 3.3). These data are consistent with the untreated Temulose molasses containing
eight xylan or arabinobioxylen oligosaccharides plus two methylglucuronyl-containing
arabinosepeyins/xylans, most probably derived from the wood hemicelluloses (Table 3.2). The
pentose-containing components were not acetylated. In addition, five free GGMO were detected
in this mass range, together with five monoacetylated and five diacetylated GGMO. Hence, a
total of 25 free oligosaccharides is reported for the untreated Temulose material.

**Susceptibility to Mild Acid Hydrolysis**

The Temulose molasses was subjected to a regimen of acid hydrolysis using a variety of
acidic conditions (Supplementary Figure S.3.1 and S.3.2). Strong acid hydrolysis, with either 0.5
M hydrochloric acid or 2.0 M trifluoroacetic acid, resulted in predominantly monosaccharides
and small DP oligosaccharides as determined by MALDI-TOF/MS. Milder acid hydrolysis (0.2
M TFA, reflux, 1 h) resulted in a mass series typical of hexose-containing oligosaccharides of
DP 2-12. The observed \([M + Na]^+\) pseudomolecular ions differed by 162 Da, indicative of the
mass difference of one hexose unit. The lowest mass ion m/z 365 corresponds to \([M + Na]^+\) for a
DP 2 hexosyl disaccharide and the highest recorded mass ion m/z 2149 for a DP 13-mer. This
series was assigned as mild acid-resistant GGMO. No partially acetylated GGMO were
observed, indicating that all of the acetyl groups were removed by the mild acid treatment.
Moreover, no hemicellulosic arabinobioxylen or xylan pentosyl oligosaccharides were seen by
MALDI-TOF/MS after the mild acid hydrolysis, indicating that these are susceptible to
degradation by the weaker acidic conditions.

To confirm this apparent selective susceptibility to acid hydrolysis (Xu et al., 2008), the
Temulose was treated with mild acid (aqueous trifluoroacetic acid, 0.2 M) and reduced with
sodium borohydride. Hence, monosaccharides released by the mild acid treatment were
converted to alditols, whereas the acid-resistant GGMOs remained intact except for their
reducing residues. Continued treatment with strong acid (aqueous trifluoroacetic acid, 2.0 M) resulted in hydrolysis of the remaining GGMOs to component monosaccharides, which were subsequently converted to aldononitriles. Following peracetylation, the weakly acid-labile monosaccharide alditols formed alditol acetates, and the strongly acid labile components formed aldononitrile acetates (PAANs). This mixture of derivatives was analyzed by GC-MS and compared to the PAAN profile from fully hydrolyzed Temulose (Figure 3.4). Noticeably, the major alditol acetates observed were arabinitol acetate and xylitol acetate, indicating that the arabinoxylan hemicellulosics were hydrolyzed by the mild acid treatment. Less mannitol acetate and glucitol acetate were produced and almost no galactitol acetate. Conversely, the major aldononitrile acetates seen were mannose-PAAN, glucose-PAAN, and galactose-PAAN, resulting from the strong acid hydrolysis of the GGMOs. Peaks for the arabinose-PAAN and xylose-PAAN were small, because these two weakly acid labile monosaccharides were converted to alditols by the borohydride treatment. Hence, this experiment confirmed that the arabinoxylan was degraded by mild acid and that the GGMOs were more resistant to this treatment.

The resistance of the GGMOs to mild acid hydrolysis provided a straightforward way to purify the GGMOs from the arabinoxylan. A large-scale mild acid treatment of Temulose with 0.2 M trifluoroacetic acid followed by precipitation with ethanol resulted in a light-colored brown sugar in good yield (Figure 3.5). Analysis of the brown sugar by MALDI-TOF/MS showed it to consist of hexose-containing oligosaccharides from DP 2 to 13, and mainly DP 4, 5, and 6 (Figure 3.6). These medium-sized GGMOs were entirely free of arabinoxylan or xylan oligosaccharides, and GC-MS monosaccharide analysis of the ethanol-soluble phase showed that it contained predominantly free xylose and arabinose (data not shown).
The selective acid hydrolysis of GGMOs has been studied previously, when it was noted that 50% of southern pine galactoglucomannan was converted to monosaccharides by refluxing in a 0.05 N oxalic acid solution for 6 h (Hamilton et al., 1960). Under these conditions (Hamilton et al., 1960), the galactose side chain was shown to be more labile than the glucomannan backbone. We tested the acid susceptibility of the Temulose molasses to several food-grade organic acids. Aqueous oxalic acid (1.0 M) results were comparable to those obtained with dilute trifluoroacetic acid. The MALDI MS spectrum of the ethanol-precipitated pellet was a simple hexose containing series, free from pentosyl oligosaccharides. This series was from DP 1 (minor amount) to DP 11 (minor amount) with DP 6 and 7 being most abundant when peak intensities were compared. The other acids tested (acetic, malonic, and citric) all contained a pentosyl oligosaccharide series and were at various states of hydrolysis with many intermediates after 1 h. To summarize, food grade oxalic acid can be substituted for the more toxic and expensive trifluoroacetic acid for the selective degradation of the arabinoxylan component of Temulose molasses. Following this treatment, the GGMO are readily recovered by a straightforward precipitation with ethanol. The typical yield of the GGMO was 25% w/w, equivalent to about 100 tonnes per year of oligosaccharide material. This also represents a significant potential source of mannose, perhaps as much as 50-70 tonnes per year.

Size Exclusion Chromatography, Permethylation Linkage Analysis, and NMR Analysis

The Temulose molasses was partially hydrolyzed with mild acid (0.2 M TFA, 100 °C, 30 min). This hydrolyzed the arabinoxylan present to arabinose and xylose monosaccharides, but retained the hexosyl GGMO. The latter were precipitated from the hydrolysate by adding ethanol. Free Ara and Xyl remained in the ethanol solution, whereas the GGMO are selectively precipitated. These were redissolved in water and reprecipitated with ethanol before lyophilizing,
resulting in a clean white product. MALDI-TOF/MS and GC-MS compositional analysis indicated a series of GGMO from DP 2 to 16, with the major products around DP 6-7. This material was permethylated with NaOH/DMSO/methyl iodide as described (Ciucano and Kerek, 1984). The completeness of methylation was monitored by MALDI-TOF/MS. The permethylated GGMO then was hydrolyzed with 2.0 M TFA and dried. Permethylated aldononitrile acetate (Me-PAAN) derivatives were prepared and analyzed by GC-MS linkage analysis.

Three GC peaks were apparent from the permethylation analysis and were assigned by reference to the electron impact ionization MS spectrum. The major peak (retention time of 7.17 min, 79.12%) was assigned as 2,3,6-MeMan PAAN and is indicative of 1,4-linked mannose residues. A smaller peak at 7.39 min (2,3,6-MeGlc, 16.90%) was assigned as due to a 1,4-linked glucosyl residues, suggesting that the 1,4-mannosyl backbone is interspersed with 1,4-glucosyl groups at an average frequency of five mannose to one glucose units. This conforms reasonably well to the compositional analysis. A third peak at 6.21 min was assigned as a 2,3,4,6-MeHexose PAAN and is most probably due to the branched-chain galactosyl residues. This is in a ratio of ~1:20 with the larger peak at 7.17 min, that is, about 5% Gal branching of the β-1,4-linked glucomannan oligosaccharide backbones. However, the permethylation analysis did not provide information on the linkage or frequency of occurrence of the galactosyl residues observed in the compositional analysis. GGMO fractions from the size exclusion column were also analyzed by permethylation, with essentially the same result. This suggested that structural heterogeneity in the linkage composition remained after SEC, and as confirmed by NMR analysis.
Temulose NMR Analysis

Large quantities (10-15 g) of individual size-fractionated GGMOs were required to enable biological testing and to enable the linkage and NMR analysis. All of the fractions were analyzed by MALDI-TOF/MS, and those containing equivalent molecular masses (DP 5-13) were pooled for further analysis (Figure 3.6). The ethanol-precipitated mild acid (0.2 M TFA, 100 °C 20 min) hydrolysate of Temulose was deuterium-exchanged by lyophilization from D₂O (99.9 atom % D), and was redissolved in D₂O (99.96 atom % D) for analysis by NMR. Similarly, NMR data were obtained on the fractions purified by gel filtration. Spectra were obtained on a Bruker 500 at 27 and 40 °C and displayed signals typical of nonacetylated oligosaccharides (Table 3.3). The major anomeric H-1 proton signal was observed at 4.69 ppm with a J-coupling constant of 2 Hz, as determined from a J-resolved spectrum. The coupled H-2 proton was apparent in the COSY spectra at 4.07 ppm. Heteronuclear coupling, observed from a HSQC spectrum, identified the coupled ¹³C nuclei C-1 and C-2 at 100.0 and 70.0 ppm, respectively. A second β-1,4-linked mannose residue was characterized by a smaller anomeric NMR signal at 4.67 ppm, coupled to an H-2 proton at 4.01 ppm. The coupled anomeric ¹³C nucleus was also observed at 100.0 ppm, where it overlapped the other β-1,4-Man carbon-1. The presence of two distinct β-1,4-linked mannosyl residues is due to one being linked to Glc and the other to a second Man in the oligosaccharide backbone, or to the difference between branched and unbranched Man residues. The presence of β-1,4-Glc in the backbone was confirmed by anomeric NMR signals at 4.45 and 102.5 ppm, which COSY showed to be coupled to β-1,4-Glc H-2 at 3.30 ppm. The anomeric values are in good agreement with those reported for β-1,4-linked mannose residues (Ramesh et al., 2001; Hannuksela and Hervé du Penhoat, 2004) and with previous assignments for galactoglucomannan (Kubačková et al., 1992).
Proton NMR signals for the α-H-1 and β-H-1 of the reducing mannose residues were observed at 5.12 and 4.85 ppm, respectively. These were coupled to overlapping $^{13}$C NMR signals at 94 ppm, as reported previously (Sata et al., 2005). Two small α-anomeric (J~4 Hz) peaks were observed at 4.95 and 4.97 ppm, coupled to overlapping H-2 protons at 3.79 ppm. Coupled carbon-13 signals for these protons were observed by HSQC to overlap at 98.5 ppm. These signals were assigned as due to α-1,6-galactosyl side chains, in agreement with previous assignments (Vieira et al., 2007). Several smaller anomic signals were observed, even for the gel filtration-purified samples. These are likely due to compositional heterogeneity arising from the nonselective nature of the mild acid hydrolysis. The HSQC experiment was used to assign the C-6 (60.5 ppm) and H-6a/H-6b (3.65 and 3.80 ppm) signals due to the 6-methylene group as the only carbon atom coupled to two protons. The C-6 $^{13}$C resonance is in good agreement with β-D-mannopyranosyl residues unbranched at the 6-OH, as branching at this position tends to shift the signal upfield to 67.4 ppm (Hannuksela and Hervé du Penhoat, 2004). Other HSQC signals were assigned for H-3/C-3 (3.74, 71.5 ppm), H-4/C-4 (3.75, 77.0 ppm), and H-5/C-5 (3.50, 75.0 ppm) on the basis of published assignments (Kubačková et al., 1992; Ramesh et al., 2001; Hannuksela and Hervé du Penhoat, 2004).

In summary, most prebiotic oligosaccharides are currently produced by transglycosylation or enzymatic cleavage of polysaccharides and include fructo-oligosaccharides, gentio-oligosaccharides, xylo-oligosaccharides, and galacto-oligosaccharides. Moreover, there is a growing need in the food and feed industries for novel carbohydrates and new prebiotic oligosaccharides. Galactoglucomannan oligosaccharides (GGMO) are attractive alternative carbohydrates having biological activities worthy of study. The structural analysis reported indicates that GGMO can be recovered from Temulose molasses by a straightforward
ethanol precipitation after mild acid hydrolysis of xyloarabinan components. The DP of the GGMO (mean DP 5-6) is similar to that of proven prebiotic oligosaccharides, and GGMO is able to maintain the selective growth of bifidobacterium (Sela et al., 2008). Because Temulose molasses is produced in large amounts (about 0.9 tonne per day), it is a raw material that provides an abundant source of potential bioactive novel oligosaccharides.

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<th>Free sugar</th>
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Table 3.1. Temulose compositional analysis by GC-MS of sugar aldonitrile acetate derivatives.
Table 3.2. MALDI-TOF MS analysis of component oligosaccharides of Temulose molasses following solvent partitioning between ether and water (total of 25 oligosaccharides in mass range 400 – 1400 Da).

Aqueous Layer: Contained 21 oligosaccharides:
- Hemicelluloses (xylans and/or arabinoxylans): Xyl₃ – Xyl₈ (6 components)
- Galactoglucomannan oligosaccharides (GGMO): Man₃ – Man₈ (5 components)
- Acetyl galactoglucomannans (GGMOAc): Man₄Ac, Man₅Ac, Man₆Ac, Man₇Ac, Man₈Ac (5 components)
- Diacetyl galactoglucomannans (GGMOAc₂): Man₄Ac₂, Man₅Ac₂, Man₆Ac₂, Man₇Ac₂, Man₈Ac₂ (5 components)

Ether Layer (emulsion): Contained 17 oligosaccharides:
- Hemicelluloses (xylans and/or arabinoxylans): Xyl₃ – Xyl₁₀ (8 components)
- 4-O-methylglucuronic-xylans (Xyl-MeGAs): Xyl₈MeGA, Xyl₉MeGA (2 components)
- Galactoglucomannan oligosaccharides (GGMO): Man₃, Man₄, Man₅ (3 components)
- Acetyl galactoglucomannans (GGMOAc): Man₄Ac, Man₅Ac, Man₆Ac (3 components)
- Diacetyl galactoglucomannans (GGMOAc₂): Man₄Ac₂ (1 component)
Table 3.3. $^1$H and $^{13}$C NMR assignments.

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$^a$The $J$ coupling constants are in parentheses.

NA = Not assigned
Figure 3.1. GC-MS monosaccharide compositional analysis of dialyzed material (3500 Da cut-off). Total carbohydrates were acid hydrolyzed and analyzed as aldononitrile acetates.
Figure 3.2. MALDI-TOF mass spectrometric analysis of Temulose molasses following ether partitioning. The nomenclature refers to hexosyl oligosaccharides (Man) and pentosyl oligosaccharides (Xyl), with the superscript referring to the degree of polymerization (DP). Monoacetylated (Ac) and diacetylated (Ac₂) hexosyl oligosaccharides, and methyl-glucuronylated (MeGA) pentosyl oligosaccharides, are also assigned.
Figure 3.3. MALDI-TOF mass spectra of oligosaccharides in Temulose molasses, shown in the mass range 600 – 1000 Da. A. Cellulo-oligosaccharide mass controls; B. Temulose galactoglucomannans, monoacetyl-galactoglucomannans, diacetyl-galactoglucomannans, and arabinoxylan hemicellulosic oligosaccharides; C. Xylo-oligosaccharide mass controls. The nomenclature refers to Table 2.
Figure 3.4. Susceptibility of Temulose sugars to acid hydrolysis. GC-MS profiles of strong (2 M TFA; top) and weak (0.2 M TFA, lower) acid hydrolysates. Weak acid-labile sugars are predominantly arabinose (Ara-ol) and xylose (Xyl-ol) derived from arabinoxylan (lower). Stronger acid also releases the GGMO monosaccharides to give predominantly Man, Glc, and Gal (top).
Figure 3.5. Ethanol-precipitated GGMO “brown sugar” (tube on the left) from mild acid hydrolyzed Temulose molasses.
Figure 3.6. Large-scale size-exclusion fractionation of galactoglucomannan oligosaccharides, (GGMOs). MALDI-TOF mass spectra of GGMOs from ethanol-precipitated Temulose “brown sugar”. Sufficient quantities (several grams) of GGMOs of various lengths (DP 5 – 13) were produced by size-exclusion separation on a 1.27 m Bio-Gel P2 column. The MS spectra (range m/z 700 – 2300) are shown in pileup.
Supplementary Figure. S.3.1. Susceptibility of Temulose oligosaccharides to acid hydrolysis. Temulose molasses samples were hydrolyzed with mild or strong acid, ethanol-precipitated, and mass-analyzed by MALDI-TOF mass spectrometry.
Supplementary Figure. S.3.2. MALDI-TOF MS analysis of total acid-stable oligosaccharides in the Temulose “Brown Sugar” precipitate. Left; total [M + Na]⁺ adduct ions GGMO in the mass range DP 2–12. Right; expansion of the isotopomer ion profile for to the DP 5 GGMO.
CHAPTER 4: IN VITRO DIGESTION AND FERMENTATION CHARACTERISTICS OF GALACTOGLUCOMANNAN OLIGOSACCHARIDE-ARABINOXYLAN (GGMO-AX) MOLASSES, A CO-PRODUCT OF FIBERBOARD PRODUCTION, AND SELECT GGMO-AX FRACTIONS USING CANINE FECAL INOCULUM

ABSTRACT: It is of interest to discover new fermentable carbohydrate sources that function as prebiotics. This study evaluated the hydrolytic digestibility, fermentative capacity, and microbiota modulating properties of galactoglucomannan oligosaccharide-arabinxylan (GGMO-AX) molasses, four hydrolyzed fractions of GGMO-AX molasses, short-chain fructooligosaccharides (scFOS), and a yeast cell wall preparation (Safmannan). These substrates resisted in vitro hydrolytic digestion. Each substrate was fermented in vitro using dog fecal inoculum, and fermentation characteristics were quantified at 0 and 12 h. All GGMO-AX molasses substrates decreased pH by at least 0.64 units and resulted in greater (P < 0.05) butyrate and total short-chain fatty acid (SCFA) production compared to scFOS and Safmannan. The GGMO-AX molasses substrates resulted in higher (P < 0.01) or equal Bifidobacterium spp. concentrations compared to scFOS. The GGMO-AX molasses substrate and its fractions demonstrated prebiotic characteristics as indicated by low hydrolytic digestibility, high fermentability, and enhanced growth of microbiota considered to be beneficial to health.

INTRODUCTION

Fermentable carbohydrates have garnered increased attention as a result of their ability to improve bowel health of both humans and animals. These carbohydrates are able to resist hydrolytic digestion and are fermented in the large bowel yielding short-chain fatty acids (SCFA), particularly butyrate, an energy source for colonocytes (Topping and Clifton, 2001). In
addition, fermentable carbohydrates may potentially increase populations of beneficial bacteria, such as bifidobacteria and lactobacilli and decrease concentrations of pathogenic bacteria, such as *Escherichia coli* and *Clostridium perfringens*.

The galactoglucomannan oligosaccharide-arbinoxylan (GGMO-AX) molasses, derived from the fiberboard manufacturing process, may be a beneficial fermentable carbohydrate source. Production of this novel carbohydrate involves steaming wood chips obtained from Southern Yellow Pine, which includes *Pinus taeda*, *Pinus echinata*, *Pinus palustris*, and *Pinus elliotii*, using high temperature and pressure. When the pressure is released quickly, soluble wood sugars and oligosaccharides are released and washed into the surrounding water. The resulting sugar solution is condensed through evaporation, resulting in a viscous ingredient referred to as GGMO-AX molasses.

The GGMO-AX molasses is composed of numerous types of oligosaccharides, including mannanoligosaccharides (MOS), xylooligosaccharides (XOS), and glucooligosaccharides (GOS) (Price et al., 2011). Several *in vitro* studies have reported that these oligosaccharides are moderately to well fermented as indicated by increased production of SCFA’s, lowered pH, and altered microbial populations (Sunvold et al., 1995; Flickinger et al., 2000; Vickers et al., 2001; Smiricky-Tjardes et al., 2003; Stewart and Slavin, 2006; Hughes et al., 2007). In addition, studies have indicated that fermentative end-products may vary depending on the degree of polymerization (DP) of the oligosaccharide (Van Laeare et al., 2000; Van de Wiele et al., 2007).

The GGMO-AX molasses and select fractions of GGMO-AX molasses, varying in DP, were evaluated for fermentative and microbiota modulating properties *in vitro* using canine fecal inoculum.
MATERIALS AND METHODS

Galactoglucomannan Oligosaccharide-Arabinobioylan Molasses Substrate

Production of GGMO-AX molasses involves wood chips, water, and pressure, but does not use strong acids or bases unlike other wood pulping production processes (Garrote et al., 1999). This results in an ingredient safe for consumption by animals (Garrote et al., 1999). The effects of time and steam pressure on the “wood chip digester” result in depolymerization of cellulose, hemicelluloses, and lignin that releases soluble sugars and polyphenolic compounds that are recovered by water washing. The resulting solution contains high concentrations of sugars (3-4%), which is further condensed into a molasses with a 60-65% solids content. Sugars are mostly in the form of oligosaccharides as compared to free sugars. The GGMO-AX molasses has a DP ranging from 2-50 units.

Another substrate “ethanol-precipitated GGMO-AX brown sugar (GBS)” was produced according to the procedure described by Price et al. (2011). Briefly, 5 mL of GGMO-AX molasses was partially hydrolyzed with 7 mL of 0.2M aqueous trifluoroacetic acid (TFA) and precipitated in ethanol and freeze-dried. The hydrolysis procedure removed arabinose and xylose from GGMO-AX molasses, producing a galactoglucomannan oligosaccharide (GGMO) product. This fraction had a DP of 4 – 13, with the major component having a DP of 5 – 8. An aliquot of GBS then was fractionated using size-exclusion chromatography (SEC) into select DP ranges according to the procedure of Price et al. (2011). One substrate, “SEC-purified small GGMO oligosaccharides (GS)” had a degree of polymerization of 2-5, while “SEC-purified medium GGMO oligosaccharides (GM)” had a DP of 6-8, and “SEC-purified large GGMO oligosaccharides (GL)” had a DP of 9-13.
The yeast cell wall preparation, Safmannan® (Lesaffre Yeast Corporation, Milwaukee, WI), and short-chain fructooligosaccharide (scFOS; NutraFlora, GTC Nutrition Co., Johnstown, CO) were obtained commercially and evaluated for comparison to the GGMO-AX substrates.

The GGMO-AX molasses and fractions were analyzed for dry matter (DM), organic matter (OM), and ash using AOAC (2002) methods. Free monosaccharide concentrations were determined according to Smiricky et al. (2002). Hydrolyzed monosaccharides (i.e., sugars obtained after acid hydrolysis) were determined according to Hoebler et al. (1989) and Bourquin et al. (1990). Briefly, an internal standard of 1 mg of inositol/mL in 72% (wt/wt) sulfuric acid was prepared. One milliliter of the internal standard was added to a screw-cap tube, containing 50 mg of finely ground sample, and vortexed gently. After 30 min, the samples were diluted to 2 N sulfuric acid by adding 11 mL of distilled, deionized water. Samples were hydrolyzed for 2 h in a boiling water bath. The hydrolyzed samples were filtered through Whatman GF/D glass fiber filters (Whatman Inc., Florham Park, NJ) and then neutralized by passing through a preparation column containing 15 g of AG 4-X4 anion exchange resin (BioRad, Hercules, CA). Effluents were collected in 200-mL volumetric flasks and brought up to volume with distilled, deionized water. Hydrolyzed monosaccharides were quantified using a Dionex DX500 high-performance liquid chromatography (HPLC) system (Dionex Corp., Sunnyvale, CA). Standards for quantification included arabinose, fucose, galactose, glucose, inositol, mannose, rhamnose, and xylose.

In vitro Digestion

The method described by Boisen (1991) was used to simulate gastric and small intestinal (hydrolytic) digestion. Briefly, at each fermentation sampling time (0 and 12 h), 0.2 g of substrate were weighed in triplicate and incubated with pepsin/hydrochloric acid for 6 h and
pancreatin for 18 h. Tubes containing reagents, but no substrate, were run as blanks. The tubes were analyzed for free released monosaccharides using HPLC (Smiricky et al., 2002) following simulated hydrolytic digestion. The remaining residues were lyophilized and used for the *in vitro* fermentation stage.

The released monosaccharide values correspond to the amount of monosaccharides resulting from hydrolytic digestion and would be expected to be absorbed *in vivo*. These sugars cannot be extracted from the *in vitro* tubes after the second stage of simulated digestion. Therefore, the released monosaccharide values were used to prepare a set of pure monosaccharide blank tubes for each substrate. These free sugar blank tubes went through the *in vitro* fermentation experiment along with the residues from the hydrolytic digestion stage. Short-chain fatty acids measured after 12 h of *in vitro* fermentation then were corrected with the appropriate blank (control) tube values.

**Donors**

Purpose-bred, healthy, female, adult dogs (*n* = 3; Butler Farms USA, Clyde, NY) with hound bloodlines, an average initial body weight of 23.1 kg (18.2–26.6 kg), and an average age of 4.4 yr (1–6 yr) served as sources of feces from which inoculum was prepared. Dogs consumed the same commercial diet (Iams Weight Control®; The Iams Co., Lewisburg, OH) composed of corn meal, chicken, whole grain sorghum, chicken by-product meal, ground whole grain barley, and fish meal. The dogs had not been exposed to antibiotics for 6 mo prior to the experiment. Dogs were housed individually in kennels in a temperature-controlled room (21 °C) at the animal care facility in the Edward R. Madigan Laboratory, University of Illinois at Urbana-Champaign. Animal care procedures were approved by the University of Illinois Animal Care and Use Committee prior to initiation of the experiment. On the designated collection day,
fresh feces from three dogs were collected in plastic bags, which were sealed after expressing excess air, and maintained at 37 °C until inoculum was prepared. Anaerobic inoculum was prepared from fresh fecal samples within 15 min of defecation.

**In vitro Fermentation Model**

The lyophilized residue remaining after simulated stomach and small intestinal digestion (*in vitro* hydrolytic digestion) was used in a model that simulated large bowel fermentation (Bourquin et al., 1993). The composition and the preparation of the *in vitro* medium have been described in detail elsewhere (Spears et al., 2007). An aliquot (26 mL) of the medium was aseptically transferred to the tubes containing the lyophilized residue remaining after simulated hydrolytic digestion and to the control tubes containing the appropriate amount of free monosaccharides. All tubes were stored at 4 °C for approximately 12 h to enable hydration of the substrates before initiating fermentations. Tubes were placed in a 37 °C water bath approximately 30 min before inoculation.

Fresh fecal samples were maintained at 37 °C until inoculum was prepared (within 10 min). Equal amounts of each fecal sample were mixed together and diluted 1:10 (wt/vol) in anaerobic dilution solution (Bryant and Burkey, 1993) by blending for 15 sec in a Waring blender under a stream of CO$_2$. Blended, diluted feces were filtered through four layers of cheesecloth and sealed in 125-mL serum bottles under CO$_2$.

Appropriate samples and control tubes were aseptically inoculated with 4 mL of diluted feces. Tubes were incubated at 37 °C with periodic mixing for 12 h. After 12 h, tubes were removed from the 37 °C incubator and processed immediately for analyses. First, the pH of the tube contents was measured with a standard pH meter (Denver Instrument Co., Arvada, CO).
Then, a 2-mL aliquot was taken from each tube for SCFA analyses. A second 2-mL aliquot was taken and frozen at -80 °C for bacterial analyses.

**Chemical Analysis**

Short-chain fatty acids and branched-chain fatty acid (BCFA) concentrations were determined by gas chromatography according to Erwin et al. (1961) using a Hewlett-Packard 5890A series II gas chromatograph (Palo Alto, CA) and a glass column (180 cm x 4 mm i.d.) packed with 10% SP-1200/1% H₃PO₄ on 80/100+ mesh Chromosorb WAW (Supelco Inc., Bellefonte, PA). Nitrogen was the carrier with a flow rate of 75 mL/min. Oven, detector, and injector temperatures were 125, 175, and 180 °C, respectively.

**Microbial Analysis**

Microbial populations were measured by DNA extraction from fermented samples, followed by quantitative PCR (qPCR). DNA was extracted from frozen samples using the RBB+C method described by Yu and Morrison (2004). Briefly, cells were lysed by employing bead beating in the presence of high concentrations of sodium dodecyl sulfate (SDS), salt, and EDTA. The DNA was purified using QIAamp columns (QIAamp DNA stool mini kit, Qiagen, Valencia, CA) according to manufacturer’s instructions. Extracted DNA was quantified using a NanoDrop ND-1000 spectrophotometer (Nano-Drop Technologies, Wilmington, DE).

Quantitative PCR was performed for bifidobacteria, lactobacilli, and *E. coli* genera, as well as *C. perfringens*. Specific primers were used for bifidobacteria (Matsuki et al., 2002), lactobacilli (Collier et al., 2003), *E. coli* (Malinen et al., 2003), and *C. perfringens* (Wang et al., 1994). Amplification was performed according to DePlanke et al. (2002). Briefly, a 10 µL final volume contained 5 µL 2X SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), 15 pmol of the forward and reverse primers for the bacterium of interest, and 10 ng of
extracted DNA from the sample. Standard curves were obtained by harvesting pure cultures of
the bacterium of interest in the log growth phase in triplicate followed by serial dilution.
Bacterial DNA was extracted from each dilution using a QIAamp DNA stool mini-kit and
amplified with the bacterial DNA to create triplicate standard curves using an ABI PRISM
7900HT Sequence Detection System (Applied Biosystems, Foster City, CA). Colony forming
units in each dilution were determined by plating on specific agars; lactobacilli MRS (Difco,
Detroit, MI) for lactobacilli, reinforced clostridial medium (bifidobacteria, C. perfringens), and
Luria Bertani medium (E. coli). The calculated log cfu/ml of each serial dilution was plotted
against the cycle threshold (Ct) to create a linear equation to calculate cfu/ml.

Statistical Analysis

Data were analyzed as a completely randomized design using the Mixed Models
procedure of SAS (SAS Inst, Inc, Cary, NC). All treatment least squares means were compared
with each other and a Tukey adjustment was used to control for experiment-wise error. Least
squares means were reported along with the pooled SEM for all response criteria. A probability
of $P < 0.05$ was accepted as statistically significant for released monosaccharides, SCFA, and pH
and a probability of $P < 0.01$ was accepted as statistically significant for microbiota. Means were
separated using the least significant difference method of SAS.

RESULTS

Substrates

All substrates were similar in DM content except GGMO-AX molasses that had a
considerably lower DM content (59.2%) (Table 4.1). Organic matter was high for all substrates
except for GBS. Size-exclusion chromatography purified GGMO oligosaccharides (GS, GM,
and GL) contained no free sugars. Besides SEC-purified GGMO oligosaccharides, Safmannan
contained the lowest total concentration of free sugars (2.1 mg/g), while GGMO-AX molasses and GBS had the highest free sugar concentrations (100.8 and 102.7 mg/g, respectively). The GGMO-AX molasses contained a high concentration of free arabinose (41.6 mg/g), while GBS contained a high concentration of mannose (37.2 mg/g) and xylose (22.3 mg/g).

All GGMO-AX substrates contained high concentrations of total hydrolyzed monosaccharides ranging between 615.8-1051.4 mg/g. Fucose and rhamnose were detected only in the GGMO-AX molasses. Arabinose and galactose concentrations were low but similar among all GGMO-AX substrates. The GGMO-AX molasses contained more than twice the amount of xylose compared to GBS and SEC-purified GGMO oligosaccharides. Glucose concentration was much lower in GGMO-AX molasses compared to GBS and the SEC-purified GGMO oligosaccharides. Mannose concentrations were high for all GGMO-AX substrates, with GL and GM being greatest (622.3 and 580.4 mg/g DMB, respectively). Safmannan and scFOS were not analyzed for hydrolyzed monosaccharide concentrations.

After in vitro hydrolytic digestion, all substrates released similar concentrations of fucose, rhamnose, and galactose (Table 4.2). The GGMO-AX molasses released a greater (P < 0.05) concentration of arabinose (13.9 mg/g DMB) than the other test substrates. Glucose release was lowest (P < 0.05) for GGMO-AX molasses (4.9 mg/g DMB) and greatest (P < 0.05) for Safmannan (27.8 mg/g DMB). The GGMO-AX molasses released a greater (P < 0.05) concentration of xylose (1.1 mg/g) compared to scFOS, GBS, and GM. The GGMO-AX brown sugar resulted in the greatest (P < 0.05) release of mannose among substrates. Short-chain FOS was the only substrate to release fructose (203.5 mg/g). Only glucose was released upon hydrolytic digestion of Safmannan. Short-chain FOS resulted in the greatest (P < 0.05) release of total monosaccharides, while GS, GM, and GL resulted in the lowest (P < 0.05) release of
total monosaccharides. The hydrolytic digestion value for the GGMO-AX substrates was only approximately 2%.

**Fermentation Metabolites**

Safmannan fermentation resulted in the smallest (P < 0.05) pH change among test substrates (-0.29) (Table 4.3). Short-chain FOS and GBS fermentation resulted in the greatest (P < 0.05) decrease in pH (-1.22) among test substrates. Size-exclusion chromatography-purified GGMO oligosaccharides resulted in intermediate changes in pH.

Safmannan fermentation resulted in the lowest (P < 0.05) concentrations of acetate, propionate, and total SCFA among substrates. Short-chain FOS fermentation resulted in lower (P < 0.05) concentrations of total SCFA, acetate, propionate, and butyrate compared to the GGMO-AX substrates. The GGMO-AX molasses and SEC-purified GGMO oligosaccharides resulted in a similar concentration of acetate, with these values being greater (P < 0.05) than that for GBS. Propionate production was similar between GS and GBS, but values were lower (P < 0.05) than that for the GGMO-AX molasses. Large DP GGMO fermentation produced the greatest (P < 0.05) concentration of propionate (170.7 mg/g), while GM fermentation resulted in the next highest concentration (142.8 mg/g). Among SEC-purified GGMO oligosaccharides, as DP increased, propionate production increased (P < 0.05). Butyrate production was similar for Safmannan, GGMO-AX molasses, GBS, and GL. Small DP GGMO fermentation resulted in the greatest (P < 0.05) butyrate concentration (22.5 mg/g) among substrates, while GM fermentation resulted in the next greatest concentration (18.7 mg/g). Among SEC-purified GGMO oligosaccharides, as DP increased, butyrate production decreased (P < 0.05). Short-chain FOS fermentation resulted in no butyrate production after 12 h. Total SCFA production was greatest (P < 0.05) for the SEC-purified GGMO oligosaccharides compared with GBS. Total SCFA
production increased ($P < 0.05$) as the DP of the GGMO fraction increased. Short-chain FOS and GGMO-AX molasses fermentation did not result in any BCFA production. Also, very low concentrations of BCFA were produced by any of the test substrates.

**Microbiota**

After 12 h of fermentation, GGMO-AX molasses resulted in the greatest ($P < 0.01$) *Bifidobacterium* count among substrates, while Safmannan resulted in the lowest ($P < 0.01$) count. *Lactobacillus* counts for GGMO-AX molasses were greater ($P < 0.01$) than those for Safmannan and GL, while remaining substrates were intermediate. *Escherichia coli* counts for scFOS and Safmannan were greater ($P < 0.01$) than for GL and GM, while other counts were intermediate. The GGMO-AX brown sugar resulted in the greatest ($P < 0.01$) *Clostridium perfringens* count, while Safmannan resulted in the lowest ($P < 0.01$) count.

**DISCUSSION**

The objective of this *in vitro* study was to evaluate the hydrolytic digestibility, fermentability, and prebiotic potential of GGMO-AX molasses and purified GGMO fractions isolated from the molasses. After 12 h of fermentation, test substrates resulted in a significant drop in pH and produced greater concentrations of SCFA than did the control substrates, scFOS, or Safmannan. The GGMO-AX substrates also resulted in beneficial shifts in microbial populations compared to the control substrates. To be classified as a prebiotic, the substrate must “be a selectively fermented ingredient that allows specific changes, both in composition and/or activity, in the gastrointestinal microflora that confers benefits upon host well-being and health” (Roberfroid et al., 1998). Based on this definition, the GGMO-AX molasses and SEC-purified GGMO oligosaccharides demonstrated potential to be prebiotic ingredients.
Safmannan and scFOS were used as control substrates as considerable information is known about each. Safmannan is a mannanoligosaccharide (MOS) derived from the cell wall of yeast. Short-chain FOS is a fermentable oligosaccharide proven to be a prebiotic.

Substrates tested were similar in OM content, except for GBS, which contained a much lower OM content. This substrate may contain an unknown inorganic substance, perhaps a residual salt from the fractionation step. The GBS fraction contained a lower OM concentration than did the GGMO-AX molasses from which GBS was derived. Size-exclusion chromatography removed the inorganic impurity as indicated by fractions of select DP having an OM concentration near 100%.

All substrates, except scFOS, had low hydrolytic digestibility values, resulting in a large amount of substrate for fermentation. The scFOS substrate used in this study was of low purity, which allowed a portion of the fructose to be cleaved during in vitro hydrolytic digestion.

It is evident from pH change data and total SCFA production data that GGMO-AX substrates were well fermented compared to control substrates. The GGMO-AX substrates contained various bound monosaccharides with high concentrations of xylose, mannose, glucose, and galactose. Fermentative end-product concentrations were similar to those reported from other in vitro studies evaluating pure forms of these oligosaccharides, with all studies noting that these oligosaccharides were well fermented (Sunvold et al., 1995; Flickinger et al., 2000; Vickers et al., 2001; Smiricky-Tjardes et al., 2003; Stewart and Slavin, 2006; Hughes et al., 2007). The GL fraction contained the highest concentration of mannose and total oligosaccharides that resulted in greater fermentation as indicated by greater acetate, propionate, and total SCFA production compared to the other substrates evaluated.
The GBS substrate produced much less acetate (approximately 100 mg/g) compared to the other GGMO-AX substrates. Bound monosaccharide compositional differences may have been a factor. The GBS substrate contained much less mannose and glucose than did the SEC-purified GGMO oligosaccharides. However, the SEC-purified GGMO oligosaccharides and the GGMO-AX molasses produced similar concentrations of acetate, despite GGMO-AX molasses and GBS being similar in monosaccharide composition except for bound xylose concentration.

In this study, we analyzed the six commonly produced SCFA. It is possible that other SCFAs such as formate, succinate, or malate were produced from GBS instead of acetate, but these were not analyzed. The GBS fraction resulted in the greatest pH change among all substrates tested, potentially indicative of higher fermentability and higher SCFA production.

Even though the SEC-purified GGMO oligosaccharides were derived from GBS, they produced greater concentrations of isovalerate compared to GBS that produced no BCFA. Safmannan, a yeast cell wall-derived MOS, produced a high concentration of BCFA, likely due to the protein commonly found in this ingredient.

The DP of a carbohydrate impacts the rate and site of fermentation in the large bowel and also potentially alters the microbial species that utilize the substrate (Roberfroid et al., 1998; Van Laeare et al., 2000; Van de Wiele et al., 2007; Hernot et al., 2009). Both Hernot et al. (2009) and Roberfroid et al. (1998) noted that fructans with a DP <10 increased bifidobacteria populations and were fermented at a faster rate than fructans with a DP >10. Van Laere et al. (2000) demonstrated that bifidobacteria preferentially fermented low DP substrates first and bacteroides were able to ferment substrates with a high DP.

Substrates fermented in this study were either SEC-purified GGMO oligosaccharides (GL, GM, and GS) or a mixture of DP fractions (GGMO-AX molasses and GBS). The large DP
fraction resulted in greater propionate and total SCFA production. The small and medium DP fractions, which both had a DP < 10, resulted in greater butyrate production than did GL. The GS substrate, but not GM, resulted in a greater bifidobacteria population. This result is similar to findings by Van Laere et al. (2000) who noted that lower DP oligosaccharides promote bifidobacteria populations. The mixed DP fractions, GGMO-AX molasses and GBS, resulted in the lowest butyrate and total SCFA concentrations, but resulted in the highest bifidobacteria populations. Substrates with an array of DP’s promoted bifidobacteria growth to a greater extent than select fractions of similar DP.

Roberfroid et al. (2007) indicated “the observation of 1 log-fold increase in bifidobacteria is a clear indication of a modification of the intestinal flora”. In the current study, Safmannan had a final bifidobacteria population of 4.8 cfu, log_{10}/ml. All other substrates were noted to be at least 0.9 log units greater than Safmannan, indicating that GGMO-AX substrates were able to clearly modify the bifidobacteria population. The GGMO-AX substrates resulted in either an equal or significantly greater bifidobacteria population compared to the proven prebiotic, scFOS. The GGMO-AX molasses resulted in the greatest increase in the bifidobacteria population after 12 h of fermentation. Moreover, GGMO-AX substrates inhibited the growth of *E. coli* to a greater extent than did Safmannan or scFOS. Inhibition of growth of *E. coli* and *C. perfringens* is viewed as beneficial to large bowel health. Only slight differences in *C. perfringens* and *Lactobacillus* spp. populations were detected among substrates.

In conclusion, GGMO-AX substrates resisted hydrolytic digestion and were well fermented as indicated by a decrease in pH, increased SCFA production, and beneficial microbial changes in comparison to control substrates. Based on these results, all GGMO-AX substrates exhibited prebiotic-like effects. Among substrates, GBS appears to be least
fermentable based on total SCFA production. The GGMO-AX molasses has the greatest prebiotic potential based on SCFA production and promotion of bifidobacteria and lactobacilli growth and inhibition of *E. coli* growth. Overall, GGMO-AX substrates were highly fermentable and may positively impact large bowel health.

**LITERATURE CITED**


Table 4.1. Dry matter, organic matter, free sugar concentrations, and hydrolyzed monosaccharide concentrations of control substrates, galactoglucomannan oligosaccharide-arbinoxylan (GGMO-AX) molasses, GGMO-AX brown sugar (GBS), and size-exclusion chromatography-purified GGMO.

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<th>Safmannan</th>
<th>GGMO-AX molasses</th>
<th>GBS&lt;sup&gt;b&lt;/sup&gt;</th>
<th>GL&lt;sup&gt;c&lt;/sup&gt;</th>
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<tr>
<th>Item</th>
<th>scFOS</th>
<th>Safmannan</th>
<th>GGMO-AX molasses</th>
<th>GBS</th>
<th>GL</th>
<th>GM</th>
<th>GS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrolyzed monosaccharides(^a)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fucose</td>
<td>8.1</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Arabinose</td>
<td>12.7</td>
<td>12.4</td>
<td>10.0</td>
<td>12.1</td>
<td>14.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rhamnose</td>
<td>3.7</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Galactose</td>
<td>73.2</td>
<td>82.8</td>
<td>118.8</td>
<td>114.2</td>
<td>108.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>115.0</td>
<td>148.8</td>
<td>284.5</td>
<td>273.8</td>
<td>261.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Xylose</td>
<td>132.8</td>
<td>24.2</td>
<td>15.9</td>
<td>34.0</td>
<td>53.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mannose</td>
<td>364.3</td>
<td>347.6</td>
<td>622.3</td>
<td>580.4</td>
<td>522.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>697.9</td>
<td>615.8</td>
<td>1051.4</td>
<td>1014.5</td>
<td>960.1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) scFOS – Short-chain fructooligosaccharides.
\(^b\) GBS – GGMO-AX molasses hydrolyzed with 0.2M trifluoracetic acid and precipitated with ethanol.
\(^c\) GL – Size-exclusion chromatography-purified galactoglucomannan oligosaccharide with a high degree of polymerization.
\(^d\) GM – Size-exclusion chromatography-purified galactoglucomannan oligosaccharide with a medium degree of polymerization.
\(^e\) GS – Size-exclusion chromatography-purified galactoglucomannan oligosaccharide with a low degree of polymerization.
\(^f\) Hydrolyzed monosaccharide concentrations were corrected for free sugar concentrations.
Table 4.2. Released monosaccharide concentrations (mg/g, DMB) of control substrates, galactoglucomannan oligosaccharide-arbinobinoxylan (GGMO-AX) molasses, GGMO-AX brown sugar (GBS), and size-exclusion chromatography-purified GGMO after simulated hydrolytic digestion\(^{a,b}\).

<table>
<thead>
<tr>
<th>Item</th>
<th>scFOS(^c)</th>
<th>Safmannan</th>
<th>GGMO-AX molasses</th>
<th>GBS(^d)</th>
<th>GL(^e)</th>
<th>GM(^f)</th>
<th>GS(^g)</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Released monosaccharides</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fucose</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Arabinose</td>
<td>0.0(^a)</td>
<td>0.0(^a)</td>
<td>13.9(^b)</td>
<td>0.6(^a)</td>
<td>0.0(^a)</td>
<td>0.1(^a)</td>
<td>0.3(^a)</td>
<td>0.19</td>
</tr>
<tr>
<td>Rhamnose</td>
<td>0.0(^a)</td>
<td>0.0(^a)</td>
<td>0.1(^b)</td>
<td>0.0(^a)</td>
<td>0.0(^a)</td>
<td>0.0(^a)</td>
<td>0.0(^a)</td>
<td>0.02</td>
</tr>
<tr>
<td>Galactose</td>
<td>0.0(^a)</td>
<td>0.0(^a)</td>
<td>0.6(^ab)</td>
<td>1.2(^b)</td>
<td>0.2(^a)</td>
<td>0.1(^a)</td>
<td>0.4(^a)</td>
<td>0.15</td>
</tr>
<tr>
<td>Glucose</td>
<td>21.5(^e)</td>
<td>27.8(^f)</td>
<td>4.9(^a)</td>
<td>13.6(^b)</td>
<td>19.3(^de)</td>
<td>17.3(^cd)</td>
<td>16.8(^c)</td>
<td>0.51</td>
</tr>
<tr>
<td>Xylose</td>
<td>0.3(^a)</td>
<td>0.7(^ab)</td>
<td>1.1(^b)</td>
<td>0.0(^a)</td>
<td>0.6(^ab)</td>
<td>0.3(^a)</td>
<td>0.6(^ab)</td>
<td>0.13</td>
</tr>
<tr>
<td>Mannose</td>
<td>0.0(^a)</td>
<td>0.0(^a)</td>
<td>0.4(^b)</td>
<td>4.9(^c)</td>
<td>0.0(^a)</td>
<td>0.1(^ab)</td>
<td>0.0(^a)</td>
<td>0.07</td>
</tr>
<tr>
<td>Fructose</td>
<td>203.5(^b)</td>
<td>0.0(^a)</td>
<td>0.0(^a)</td>
<td>0.0(^a)</td>
<td>0.0(^a)</td>
<td>0.0(^a)</td>
<td>0.0(^a)</td>
<td>1.00</td>
</tr>
<tr>
<td>Total</td>
<td>225.3(^c)</td>
<td>28.5(^b)</td>
<td>21.0(^ab)</td>
<td>20.3(^ab)</td>
<td>20.1(^a)</td>
<td>17.9(^a)</td>
<td>18.1(^a)</td>
<td>1.57</td>
</tr>
</tbody>
</table>

\(^a\) Sugars released from substrate after 6 h of HCl-pepsin digestion and 18 h of digestion with pancreatin. Concentrations were corrected for free sugars.

\(^b\) Within a row, means without a common superscript letter differ (P < 0.05).

\(^c\) scFOS – Short-chain fructooligosaccharides.

\(^d\) GBS – GGMO-AX molasses hydrolyzed with 0.2M trifluoracetic acid and precipitated with ethanol.

\(^e\) GL – Size-exclusion chromatography-purified galactoglucomannan oligosaccharide with a high degree of polymerization.
f GM – Size-exclusion chromatography-purified galactoglucomannan oligosaccharide with a medium degree of polymerization.

g GS – Size-exclusion chromatography-purified galactoglucomannan oligosaccharide with a low degree of polymerization.
Table 4.3. pH change and short-chain fatty acid (SCFA) and branched chain fatty acid (BCFA) production following 12 h of *in vitro* fermentation of control substrates, galactoglucomannan oligosaccharide-arbinoxylan (GGMO-AX) molasses, GGMO-AX brown sugar (GBS), and size-exclusion chromatography-purified GGMO*. 

<table>
<thead>
<tr>
<th>Item</th>
<th>Fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>scFOS</td>
</tr>
<tr>
<td>pH change</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-1.22</td>
</tr>
<tr>
<td>Short-chain fatty</td>
<td></td>
</tr>
<tr>
<td>acids</td>
<td></td>
</tr>
<tr>
<td>Acetate</td>
<td>86.1</td>
</tr>
<tr>
<td></td>
<td>25.3</td>
</tr>
<tr>
<td></td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>0.0</td>
</tr>
<tr>
<td>Total SCFA</td>
<td>162.2</td>
</tr>
<tr>
<td>Branched-chain fatty</td>
<td></td>
</tr>
<tr>
<td>acids</td>
<td></td>
</tr>
<tr>
<td>Isobutyrate</td>
<td>0.0</td>
</tr>
<tr>
<td>Isovalerate</td>
<td>0.0</td>
</tr>
<tr>
<td>Valerate</td>
<td>0.0</td>
</tr>
<tr>
<td>Total BCFA</td>
<td>0.0</td>
</tr>
</tbody>
</table>

* Within a row, means without a common superscript letter differ (P < 0.05).

* scFOS – Short-chain fructooligosaccharides.
c GBS – GGMO-AX molasses hydrolyzed with 0.2M trifluoracetic acid and precipitated with ethanol.
d GL – Size-exclusion chromatography-purified galactoglucomannan oligosaccharide with a high degree of polymerization.
e GM – Size-exclusion chromatography-purified galactoglucomannan oligosaccharide with a medium degree of polymerization.
f GS – Size-exclusion chromatography-purified galactoglucomannan oligosaccharide with a low degree of polymerization.
Table 4.4. Microbiota concentration ($\log_{10}$ cfu/ml of \textit{in vitro} fluid) in batch culture fermentation with control substrates, galactoglucomannan oligosaccharide-arbinoxylan (GGMO-AX) molasses, GGMO-AX brown sugar (GBS), and size-exclusion chromatography-purified GGMO after 12 h of fermentation with dog fecal inoculum\textsuperscript{a}.

<table>
<thead>
<tr>
<th>Item</th>
<th>scFOS\textsuperscript{b}</th>
<th>Safmannan</th>
<th>GGMO-AX molasses</th>
<th>GBS\textsuperscript{c}</th>
<th>GL\textsuperscript{d}</th>
<th>GM\textsuperscript{e}</th>
<th>GS\textsuperscript{f}</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{Bifidobacterium} spp.</td>
<td>5.8\textsuperscript{bc}</td>
<td>4.8\textsuperscript{a}</td>
<td>6.4\textsuperscript{e}</td>
<td>6.1\textsuperscript{d}</td>
<td>5.7\textsuperscript{b}</td>
<td>5.7\textsuperscript{b}</td>
<td>5.9\textsuperscript{cd}</td>
<td>0.06</td>
</tr>
<tr>
<td>\textit{Lactobacillus} spp.</td>
<td>8.2\textsuperscript{ab}</td>
<td>8.1\textsuperscript{a}</td>
<td>8.3\textsuperscript{b}</td>
<td>8.2\textsuperscript{ab}</td>
<td>8.1\textsuperscript{a}</td>
<td>8.1\textsuperscript{ab}</td>
<td>8.2\textsuperscript{ab}</td>
<td>0.04</td>
</tr>
<tr>
<td>\textit{Escherichia coli}</td>
<td>10.0\textsuperscript{c}</td>
<td>10.0\textsuperscript{c}</td>
<td>9.8\textsuperscript{bc}</td>
<td>9.8\textsuperscript{bc}</td>
<td>9.6\textsuperscript{a}</td>
<td>9.6\textsuperscript{a}</td>
<td>9.7\textsuperscript{ab}</td>
<td>0.05</td>
</tr>
<tr>
<td>\textit{Clostridium perfringens}</td>
<td>7.3\textsuperscript{bc}</td>
<td>7.0\textsuperscript{a}</td>
<td>7.2\textsuperscript{ab}</td>
<td>7.4\textsuperscript{c}</td>
<td>7.1\textsuperscript{ab}</td>
<td>7.1\textsuperscript{ab}</td>
<td>7.3\textsuperscript{bc}</td>
<td>0.06</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Within a row, means without a common superscript letter differ (P < 0.01).

\textsuperscript{b} scFOS – Short-chain fructooligosaccharides.

\textsuperscript{c} GBS – GGMO-AX molasses hydrolyzed with 0.2M trifluoracetic acid and precipitated with ethanol.

\textsuperscript{d} GL – Size-exclusion chromatography-purified galactoglucomannan oligosaccharide with a high degree of polymerization.

\textsuperscript{e} GM – Size-exclusion chromatography-purified galactoglucomannan oligosaccharide with a medium degree of polymerization.

\textsuperscript{f} GS – Size-exclusion chromatography-purified galactoglucomannan oligosaccharide with a low degree of polymerization.
CHAPTER 5: GALACTOGLUCOMANNAN OLIGOSACCHARIDE-ARABINOXYLAN (GGMO-AX) COMPLEX SUPPLEMENTATION AFFECTS NUTRIENT DIGESTIBILITY, FERMENTATION END-PRODUCT PRODUCTION, AND LARGE BOWEL MICROBIOTA OF THE DOG

ABSTRACT: A galactoglucomannan oligosaccharide-arabinoxylan (GGMO-AX) complex obtained from fiberboard production was evaluated as a dietary supplement for dogs. The GGMO-AX substrate contained high concentrations of oligosaccharides containing mannose, xylose, and glucose, with the mannose component accounting for 35% of DM. Adult dogs assigned to a 6 x 6 Latin square design were fed 6 diets, each containing a different concentration of supplemental GGMO-AX (0, 0.5, 1, 2, 4, and 8%) that replaced dietary cellulose. Total tract DM and OM apparent digestibilities increased (P < 0.001) linearly, while total tract CP apparent digestibility decreased (P < 0.001) linearly, as dietary GGMO-AX substrate concentration increased. Fecal concentrations of acetate, propionate, and total short-chain fatty acids (SCFA) increased (P ≤ 0.001) linearly, whereas butyrate concentration decreased (P ≤ 0.001) linearly with increasing dietary concentrations of GGMO-AX. Fecal pH decreased (P ≤ 0.001) linearly as dietary GGMO-AX substrate concentration increased, whereas fecal score increased quadratically (P ≤ 0.001). Fecal phenol (P ≤ 0.05) and indole (P ≤ 0.01) concentrations decreased linearly with GGMO-AX supplementation. Fecal biogenic amine concentrations were not different among treatments except for phenylethylamine, which decreased (P < 0.001) linearly as dietary GGMO-AX substrate concentration increased. Fecal microbial concentrations of *E. coli*, *Lactobacillus* spp., and *Clostridium perfringens* were not different among treatments. A quadratic increase (P ≤ 0.01) was noted for *Bifidobacterium* spp. as dietary GGMO-AX
substrate concentration increased. Data suggest positive nutritional properties of supplemental GGMO-AX when incorporated in a high quality dog food.

**INTRODUCTION**

A novel galactoglucomannan oligosaccharide (GGMO-AX) substrate (Previda, Temple-Inland, Diboll, TX) is derived from the fiberboard manufacturing process. During production, wood chips are steamed using high temperature and pressure. When the pressure is released quickly, soluble wood sugars and oligosaccharides are separated from the insoluble wood pulp and dissolve in the surrounding water. The resulting sugar solution is condensed through evaporation, resulting in a thick, molasses-like substance.

The GGMO-AX substrate is composed of numerous types of oligosaccharides, including mannooligosaccharides (MOS), xylooligosaccharides (XOS), and glucooligosaccharides (GOS). In addition, GGMO-AX contain select polyphenolic compounds. The GGMO-AX substrate has been shown to be resistant to hydrolytic digestion, but highly fermentable *in vitro* using canine fecal inoculum (Faber et al., 2011); however, *in vivo* data are lacking.

Because the GGMO-AX substrate contains a high concentration of select oligosaccharides and is easily fermented, it has the potential to elicit a prebiotic effect; however, the prebiotic potential has yet to be evaluated in an animal model. To be classified as a prebiotic, the substrate must “allow specific changes, both in composition and/or activity in the gastrointestinal microflora that confers benefits upon host well-being and health” (Roberfroid, 2007). The objective of this study was to evaluate nutritional effects and prebiotic potential of a spray-dried GGMO-AX substrate when added to canine diets and tested in a dose-response experiment.
MATERIALS AND METHODS

All animal care procedures were approved by the University of Illinois Institutional Animal Care and Use Committee before initiation of the experiment.

Galactoglucomannan Oligosaccharide Substrate

Production of the GGMO-AX substrate involves wood chips, water, and pressure, but does not use strong acids or bases unlike other fiberboard production processes. This results in an ingredient potentially safe for consumption by animals. During hydrolysis, hemicelluloses are depolymerized through hydronium ions from water and other compounds such as uronic, acetic, and phenolic acids (Garrote et al., 1999). The release of pressure on the “wood chip digester” results in destruction primarily of cellulose, hemicelluloses, and lignin that releases soluble sugars into the surrounding water, along with polyphenolic compounds from lignin. The resulting water solution contains high concentrations of sugars (3 to 4%), a concentration unsafe for disposal into wastewater streams. Thus, the sugar solution is removed from the wood chips and further condensed into a syrup with a final sugar concentration of 30 to 54% (Michalka, 2007). Sugars are mostly in the form of oligosaccharides as compared to free sugars.

The GGMO-AX syrup substrate was spray-dried (PCS P-0.1; Pulse Combustion Systems, Payson, AZ) with a contact temperature of 360°C and exit temperature of 102°C. The starting substrate was diluted to 50.25% solids before drying. Spray-drying allowed the substrate to be mixed in a diet matrix that was extruded and a kibble formed.

Substrate Chemical Analyses

The GGMO-AX substrate was analyzed for DM, OM, and ash using AOAC (2006) methods. Crude protein was calculated from Leco total N values (AOAC, 2006). Total lipid content (acid-hydrolyzed fat) of the substrate was determined according to the methods of the
AACC (1983) and Budde (1952). Gross energy was measured using an oxygen bomb calorimeter (model 1261, Parr Instruments, Moline, IL). Free monosaccharide and oligosaccharide concentrations were determined according to Smiricky et al. (2002). Hydrolyzed monosaccharides (i.e., sugars covalently bound to each other) were determined according to Hoebler et al. (1989) and Bourquin et al. (1990). Polyphenolic compound concentrations were determined according to Jung et al. (1983) and Titgemeyer et al. (1991).

**Animals and Diets**

Six female dogs with hound bloodlines (3.4 ± 0.0 yr; 22 ± 2.1 kg) were utilized. Dogs were housed in individual kennels (2.4 x 1.2 m) in a temperature-controlled room with a 16 h light:8 h dark cycle. Six diets were formulated to contain approximately 30% CP and 20% fat (as-is basis). Each diet contained a specified concentration of the GGMO-AX substrate (0, 0.5, 1, 2, 4, or 8%), which replaced cellulose (Solka floc) in the diet. Low ash poultry by-product meal, poultry fat, brewer’s rice, ground corn, and vitamin and mineral premixes made up the remainder of the dry, extruded, kibble diet (Table 5.1). Diets were formulated to meet or exceed the NRC (2006) requirements for adult dogs at maintenance. Diets were extruded at the Kansas State University Bioprocessing and Industrial Value-Added Program facility (Manhattan, KS) under the supervision of a private consultant (Pet Food and Ingredient Technology, Inc., Topeka, KS). Dogs were offered 160 g of the diet twice daily (0800 and 1700 h) to meet the required energy needs based on estimated ME of the diet. Chromic oxide (0.2%) was added to the diet as a digestibility marker. Fresh water was offered to the dogs ad libitum.

**Sample Collection**

A 6 x 6 Latin square design experiment with 14 d periods was conducted. The first 10 d were an adaptation period, followed by 4 d of total fecal collection. Although total tract nutrient
digestibility values were based on the concentration of chromic oxide recovered in feces, total feces excreted during the collection phase of each period were taken from the pen floor, weighed, and frozen at -20°C until further analyses. All fecal samples during the collection period were subjected to a consistency score according to the following scale: 1 = hard, dry pellets, and small hard mass; 2 = hard, formed, dry stool, and remains firm and soft; 3 = soft, formed, and moist stool, and retains shape; 4 = soft, unformed stool, and assumes shape of container; and 5 = watery, liquid that can be poured.

**Sample Handling**

Fecal samples were dried at 55°C in a forced-air oven and ground in a Wiley mill (model 4, Thomas Scientific, Swedesboro, NJ) through a 2-mm screen. On d 11 of each period, fresh fecal samples were collected within 15 min of defecation. An aliquot of fresh feces was immediately transferred to sterile cryogenic vials (Nalgene, Rochester, NY) and snap-frozen in liquid nitrogen. Once frozen, vials were stored at -80°C until DNA extraction for microbial analysis. Aliquots for analysis of phenols, indoles, and biogenic amines were frozen at -20°C immediately after collection. One aliquot was collected and placed in 5 mL of 2 N hydrochloric acid for ammonia and short-chain fatty acid (SCFA) analysis. Additional aliquots were used for pH measurement and fresh fecal DM determination.

**Chemical Analyses**

Diet and fecal samples were analyzed for DM, OM, and ash using AOAC (2006) methods. Crude protein was calculated from Leco total N values (AOAC, 2006). Total lipid content (acid hydrolyzed fat) of the samples was determined according to AACC (1983) and Budde (1952). Total dietary fiber (TDF) was analyzed according to Prosky et al. (1984). Gross energy of the diets was measured using an oxygen bomb calorimeter (Parr Instruments).
Chromium concentrations of diet and fecal samples were analyzed according to Williams et al. (1962) using atomic absorption spectrophotometry (model 2380, Perkin-Elmer, Norwalk, CT). Fecal SCFA and branched-chain fatty acid (BCFA) concentrations were determined by gas chromatography according to Erwin et al. (1961) using a gas chromatograph (Hewlett-Packard 5890A series II, Palo Alto, CA) and a glass column (180 cm x 4 mm i.d.) packed with 10% SP-1200/1% H₃PO₄ on 80/100+ mesh Chromosorb WAW (Supelco Inc., Bellefonte, PA). Nitrogen was the carrier with a flow rate of 75 mL·min⁻¹. Oven, detector, and injector temperatures were 125, 175, and 180°C, respectively. Fecal ammonia concentrations were determined according to the method of Chaney and Marbach (1962). Fecal phenol and indole concentrations were determined using gas chromatography according to the methods described by Flickinger et al. (2003). Biogenic amines concentrations were quantified using HPLC according to methods described by Flickinger et al. (2003).

**Microbial Analyses**

Fecal microbial populations were analyzed using methods described by Middelbos et al. (2007a) with minor adaptations. Briefly, fecal DNA was extracted from freshly collected samples that had been stored at −80°C until analysis, using the repeated bead beater method described by Yu and Morrison (2004) with a DNA extraction kit (QIAamp DNA Stool Mini Kit, Qiagen, Valencia, CA) according to the manufacturer’s instructions. Extracted DNA was quantified using a spectrophotometer (NanoDrop ND-1000, Nano-Drop Technologies, Wilmington, DE). Quantitative PCR was performed using specific primers for *Bifidobacterium* spp. (Matsuki et al., 2002), *Lactobacillus* spp. (Collier et al., 2003), *E. coli* (Malinen et al., 2003), and *C. perfringens* (Wang et al., 1994). Amplification was performed according to DePlancke et al. (2002). Briefly, a 10-µL final volume contained 5 µL of 2× SYBR Green PCR
Master Mix (Applied Biosystems, Foster City, CA), 15 pmol of the forward and reverse primers for the bacterium of interest, and 10 ng of extracted fecal DNA. Standard curves were obtained by harvesting pure cultures of the bacterium of interest in the log growth phase in triplicate, followed by serial dilution. Bacterial DNA was extracted from each dilution using a DNA extraction kit (Qiagen) and amplified with the fecal DNA to create triplicate standard curves (ABI PRISM 7900HT Sequence Detection System, Applied Biosystems, Foster City, CA). Colony forming units in each dilution were determined by plating on specific agars; lactobacilli MRS (Difco, BD, Franklin Lakes, NJ) for lactobacilli, reinforced clostridial medium (bifidobacteria, *C. perfringens*), and Luria Bertani medium (*E. coli*). The calculated log cfu·mL⁻¹ of each serial dilution was plotted against the cycle threshold to create a linear equation to calculate cfu·g⁻¹ dry feces.

**Calculations**

Dry matter recovery was calculated by dividing Cr intake (mg·d⁻¹) by Cr concentrations in feces (mg Cr·g feces⁻¹). Fecal nutrient flows were calculated by multiplying DM flow by nutrient concentrations in the fecal DM. Total tract nutrient digestibilities were calculated as nutrient intake (g·d⁻¹) minus fecal nutrient flow (output, g·d⁻¹); this value then was divided by nutrient intake (g·d⁻¹).

**Statistical Analysis**

Data for continuous variables were analyzed by the MIXED procedure, and data for discontinuous variables were analyzed by the GLIMMIX procedure (SAS Inst. Inc., Cary, NC). The statistical model included the random effects of animal and period and the fixed effect of treatment. Least squares means were separated using least squares differences with a Tukey adjustment and linear and quadratic contrasts. Outlier data were removed from analysis after
analyzing data using the UNIVARIATE procedure to produce a normal probability plot based on residual data and visual inspection of the raw data. Outlier data were defined as data points 3 or more standard deviations from the mean. Differences among treatment level least squares means with \( P \leq 0.05 \) were accepted as statistically significant, whereas mean differences with \( P \leq 0.10 \) were accepted as trends.

**RESULTS**

**Substrate Composition**

Dry matter and OM concentrations of the GGMO-AX substrate were greater than 94%, while concentrations of CP and acid hydrolyzed fat were less than 1% (Table 5.2). Of the free monosaccharides, arabinose, xylose, and galactose were highest in concentration, while fructose and sucrose were lowest. After hydrolysis, free monosaccharide concentrations were highest for mannose, glucose, and xylose, while fucose and rhamnose were lowest. Oligosaccharide concentrations were highest for raffinose, cellotriose, and maltopentaose, while cellopentaose and maltotriose concentrations were lowest. No free phenolic compounds were detected in the GGMO-AX substrate. Of the bound phenolics, vanillin and sinapyl acid were highest in concentration.

**Chemical Composition of Diets**

Chemical composition of diets was similar. Crude protein concentrations were near the desired 30% value (as-is basis). Acid hydrolyzed fat concentrations were near the desired 20% value (as-is basis; Table 5.1). An uncorrected TDF concentration value and a corrected TDF concentration value are reported as the TDF assay cannot quantify the GGMO-AX substrate because oligosaccharides do not precipitate in 78% ethanol and, thus, are unable to be quantified. The TDF concentration values for diets were low except for the cellulose control treatment.
correct this problem, the dietary concentration of GGMO-AX substrate was added to the TDF (uncorrected) value to account for the GGMO-AX substrate not analyzed. After this correction was made, TDF concentrations increased and were similar among diets.

**Food Intake and Apparent Nutrient Digestibility**

Nutrient intakes were similar ($P = 0.45$) across treatments with dogs consuming between a mean of 248 and 288 g DM·d$^{-1}$ (Table 5.3). Uncorrected TDF intake values decreased ($P < 0.001$) linearly with increased supplementation of the GGMO-AX substrate. Total dietary fiber intake was corrected by multiplying the DM concentration of the GGMO-AX substrate by the dietary GGMO-AX substrate concentration. This value then was multiplied by the DM intake·d$^{-1}$ value and added to the TDF (uncorrected) concentration value. The correction increased TDF intake (g·d$^{-1}$) for each treatment and, after correction, TDF intakes were similar among treatments.

Fecal DM output decreased ($P = 0.006$) linearly as the GGMO-AX substrate concentration increased from 0 to 8% (63 to 45 g·d$^{-1}$ – DM basis, respectively; data not shown). Dry matter and OM digestibilities were greater ($P < 0.001$) for the 4 and 8% supplemental GGMO-AX treatments, while values for remaining treatments were lower but similar to each other. Crude protein digestibility decreased ($P < 0.001$) quadratically as dietary GGMO-AX substrate concentration increased. Fat digestibility was unaffected by treatment ($P = 0.43$). A corrected TDF digestibility could not be computed as there was no method to determine GGMO-AX substrate digestibility alone.

**Fermentation Metabolites**

Fecal concentrations of acetate, propionate, and total SCFA increased ($P < 0.001$) linearly as supplemental GGMO-AX concentration increased (Table 5.4), whereas butyrate concentration
decreased (P < 0.001) linearly. Fecal isobutyrate, isovalerate, and total BCFA concentrations were not different among treatments (average 5.44, 8.25, and 14.8 µmol·g⁻¹, respectively). A linear increase (P < 0.01) in valerate was noted as the dietary GGMO-AX substrate concentration increased.

Fecal pH decreased (P < 0.001) linearly as dietary GGMO-AX substrate concentration increased, whereas fecal score increased (P < 0.001) quadratically (Table 5.5). Fecal ammonia concentrations were similar among treatments (average 2.11 mg·g⁻¹). Fecal phenol (P < 0.05) and indole (P < 0.01) concentrations decreased linearly as dietary GGMO-AX concentration increased. Fecal biogenic amine concentrations were not different among treatments except for phenylethylamine and tryptamine, which decreased (P < 0.001 and P = 0.09, respectively) linearly as dietary GGMO-AX substrate concentration increased. Total biogenic amine concentrations were not different (P = 0.23) among treatments. Across treatments, agmatine and histamine were not detected in feces.

**Fecal Microbiota**

Fecal microbial concentrations of *E. coli*, *Lactobacillus* spp., and *Clostridium perfringens* were not different among treatments (P = 0.91, 0.78, and 0.82, respectively; Table 5.6). A quadratic increase (P < 0.01) was noted for *Bifidobacterium* spp. as supplemental GGMO-AX concentration increased.

**DISCUSSION**

The high DM concentration of the GGMO-AX test substrate is a result of the spray-drying process used to convert the molasses-like product into a powder form. The high OM concentration is due to the GGMO-AX substrate being composed mostly of carbohydrates, with
free sugars and hydrolyzed monosaccharides accounting for 86% of the OM. Crude protein and acid hydrolyzed fat concentrations were very low. Free arabinose concentration was much greater (3.5 times) than the next greatest sugar concentration (xylose). However, after hydrolysis, the concentration of arabinose was much lower compared to most other hydrolyzed monosaccharides. The concentration of mannose was very low in the free sugar form, but after hydrolysis, it was present in the highest concentration and was 2.22 times greater than the next highest sugar concentration (glucose). Mannose accounted for nearly half of the hydrolyzed monosaccharides present in the GGMO-AX substrate. Low molecular weight oligosaccharides accounted for 1.4% of the OM in GGMO-AX. Bound phenolic compounds accounted for 3.1% of the GGMO-AX substrate, and are likely derived from the lignin in the starting material. Some polyphenolic compounds may not have been accounted for due to the lack of a standard for some compounds. The GGMO-AX substrate also may contain acetyl groups and sugar alcohols; however, analysis of these compounds was not conducted.

The high concentrations of mannan, xylans, and glucans result from the cellulose and hemicelluloses present in the wood chips used for production of the GGMO-AX substrate. These carbohydrates resist hydrolytic digestion in the small intestine (Asano et al., 2003; Flickinger et al., 2000), but are partially fermented in the large bowel. Several in vitro and in vivo studies have reported that they exert beneficial effects in the large bowel by increasing production of SCFA’s, lowering pH, and modulating microbial populations (Djouzi and Andrieux, 1997; Flickinger et al., 2000; Smiricky-Tjardes et al., 2003; Swanson et al., 2002).

Dietary composition was similar among diets except for TDF concentration. Differences in TDF concentration were expected as the GGMO-AX substrate does not precipitate in 78% ethanol and, thus, is unable to be analyzed properly using the TDF procedure. Nutrient intakes
were high, with no significant differences noted among treatments except for TDF. However, when TDF intakes were corrected for supplemental GGMO-AX, values were similar among diets.

It is unusual that the highest concentration (8%) of a material such as GGMO-AX did not affect nutrient intake. In addition, dogs did not demonstrate any adverse effects such as emesis, signs of gastric distress, or severe diarrhea, to the higher dietary concentrations of GGMO-AX. However, fecal scores for dogs fed the 8% GGMO-AX treatment were unacceptably high, indicating production of a loose stool. This was not the case for dogs fed the remaining treatments. The ability of the dog to safely consume a diet with such a high concentration of fermentable substrate indicates the potential utility of the GGMO-AX. The 4 to 8% concentrations tested far exceed practical levels of dietary inclusion, but our intention was to conduct a study where tolerance could be assessed along with key nutritional/microbiological outcomes. Results indicate that concentrations of GGMO-AX (4 to 8%) are well-tolerated by dogs.

Digestibility coefficients were high for all nutrients, in part due to the high quality ingredients incorporated in the diet. Dry matter and OM digestibility differences were due mainly to the presence of cellulose, a 0% fermentable insoluble dietary fiber. This lack of fermentability increases DM and OM output in feces, thus decreasing DM and OM digestibility. Muir et al. (1996) found that adding Solka floc (7.5%) to diets decreased total tract DM and OM digestibilities in dogs. A similar response was reported by Middelbos et al. (2007b) when select fiber substrates were tested. The diet containing cellulose resulted in lower DM and OM digestibilities compared to those containing fermentable substrates (fructooligosaccharides, yeast cell wall, or their combination).
Crude protein digestibility decreased as the GGMO-AX concentration increased due perhaps to an increase in microbial biomass production in the large bowel. Increased fermentation in the large bowel would stimulate growth of microbiota, which would be excreted in feces in the form of microbial protein. Several studies have reported lower apparent CP digestibility due to inclusion of fermentable substrates such as pectin, galactooligosaccharides, mannanoligosacharides, and fructooligosaccharides (Silvio et al., 2000; Flickinger et al., 2000; Zentek et al., 2002; Middelbos et al., 2007b, respectively).

As the dietary concentration of GGMO-AX substrate increased, SCFA concentrations in feces increased, indicative of increased fermentation in the large bowel. However, butyrate concentration decreased overall as a result of GGMO-AX substrate addition to the diet. This decrease could be explained by the rapid fermentation of GGMO-AX in the large bowel, probably in the proximal colon, allowing butyrate, an energy substrate for colonocytes, to be absorbed during passage through the tract rather than be excreted in feces (Topping and Clifton, 2001). Swanson et al. (2002) noted no differences in butyrate concentrations after feeding fermentable fibers (fructooligosaccharides, 1 g; mannanoligosacharides, 1 g; and fructooligosaccharides and mannanoligosacharides, 1 g each) to dogs. The authors stated that this lack of difference could be due to rapid absorption of butyrate by colonocytes. Measurement of SCFA concentrations, particularly butyrate, in the proximal colon would have been useful but impractical in the in vivo dog model. Another possible explanation relates to the mixture of oligosaccharides affecting SCFA production. Englyst et al. (1987) demonstrated in vitro that fermentation of select oligosaccharides results in different quantities of SCFA produced. The oligosaccharides found in the GGMO-AX substrate could possibly ferment to predominantly acetate and propionate with less butyrate. This would explain the linear increase in acetate and
propionate concentrations, and linear decrease in butyrate concentration, as dietary GGMO-AX concentration increased. The linear decrease in fecal pH was due to the greater production of SCFA.

Peptides and amino acids entering the large bowel serve as potential fermentative substrates for the microbiota, especially when energy is limiting. If carbohydrate fermentation occurs rapidly, fermentation likely takes place in the proximal colon (Topping and Clifton, 2001). This leaves little carbohydrate to be fermented in the transverse and distal colon. Bacteria then must ferment peptides and amino acids for energy. End-products of amino acid fermentation include BCFA, phenol and indole compounds, and biogenic amines. Branched-chain fatty acids result from fermentation of branched-chain amino acids (valine, leucine, and isoleucine; Macfarlane et al., 1992). The addition of the dietary GGMO-AX substrate did not impact fecal ammonia or BCFA concentrations with the exception of valerate, which made up less than 8% of the total BCFA.

Phenolic compounds result from the fermentation of aromatic amino acids (phenylalanine, tyrosine, and tryptophan; Hughes et al., 2000). Fecal phenol and indole concentrations decreased linearly as dietary GGMO-AX concentration increased, indicative of a decrease in amino acid catabolism by colonic microbiota. Fecal samples were analyzed for eight different phenol and indole compounds (phenol, 4-methyl phenol, 4-ethyl phenol, indole, 7-methyl indole, 3-methyl indole, 2-methyl indole, and 2,3 dimethyl indole); however, only phenol and indole were detected. The decrease in phenol and indole concentrations could result from the GGMO-AX substrate providing sufficient fermentable energy throughout the large bowel for the microbiota, thus preventing amino acids from being needed as an energy source. Interestingly, concentrations of phenol in this experiment were much greater than values noted
by Middelbos et al. (2007b) who fed a diet containing cellulose (1% of diet),
fructooligosaccharides (0.9, 1.2, or 1.5% of diet), and yeast cell wall (0.3 or 0.6% of diet), a
source of mannanoligosaccharides, to dogs. But, phenol concentrations in the current study were
lower than those noted by Propst et al. (2003) who reported numerical increases in these
metabolites after addition of oligofructose or inulin (0.3, 0.6, or 0.9% of diet) to diets fed to
dogs. Swanson et al. (2002) noted a decrease in indole concentration with supplementation (1
g·dog⁻¹·d⁻¹) of fermentable substrates (fructooligosaccharides, mannanoligosaccharides, or
fructooligosaccharides + mannanoligosaccharides) to the diet. Middelbos et al. (2007b) reported
greater concentrations of indole compared to concentrations observed in the current study.
Indole concentration was not affected by addition of fructooligosaccharides plus yeast cell wall
to canine diets. Differences in phenol and indole concentrations among studies could be
attributed to the TDF concentration of the diets. This would alter the amount of fermentable
substrate entering the large bowel, and thus alter phenol and indole production.

No differences in fecal biogenic amine concentrations were noted among treatments,
except for phenylethylamine. This indicates that the supplemental GGMO-AX substrate does
not impact biogenic amine production by the colonic microbiota. Biogenic amine concentrations
in this study were greater than those noted by Middelbos et al. (2007b) and Swanson et al.
(2002), but were comparable, if not slightly lower, than concentration values noted by Propst et
al. (2002) who evaluated fermentable carbohydrates (fructooligosaccharides + yeast cell wall;
fructooligosaccharides, mannanoligosaccharides, and fructooligosaccharides +
mannanoligosaccharides; oligofructose and inulin, respectively) fed to dogs. In all studies, a
numerical increase in total biogenic amine concentration was noted when the dietary
concentration of fermentable substrate was increased. Overall, supplemental GGMO-AX did not
alter protein fermentation in the large bowel as indicated by a lack of change in fecal ammonia, BCFA, or biogenic amine concentrations.

Biogenic amines, such as putrescine, spermine, and spermidine are beneficial metabolites due to their ability to modulate apoptosis and cellular turnover (Chen et al., 2003; Guo et al., 2005; Seiler and Raul, 2005). Increases, or lack of change in amine concentration after dietary intervention, may be viewed as beneficial to colonic health.

One requirement of a fermentable substrate to be declared a prebiotic is that it must result in an increase in beneficial bacteria (e.g., *Bifidobacterium* spp. and *Lactobacillus* spp.), a decrease in harmful bacteria (e.g., *Escherichia coli* and *Clostridium perfringens*) concentrations, or appropriate changes in both (Roberfroid, 2007). Fecal microbial populations were unaffected by addition of the GGMO-AX substrate except for *Bifidobacterium* spp. whose concentration increased quadratically. A prebiotic effect often is characterized by a one log unit increase in concentration of a “beneficial” bacterium in the fermentative compartment (Roberfroid et al., 1998). An approximate 2 log unit increase was noted between the control and 8% GGMO-AX substrate treatment, indicating the prebiotic potential of the GGMO-AX substrate in the dog, but at an excessive dietary concentration. A 1 log unit decrease was noted when comparing the control and the 2% GGMO-AX treatment. Numerous factors exist that may influence changes in microbial populations such as pH, transit rate, fiber substrate composition, and microbial interactions (El Oufir et al., 2000; Fons et al., 2000; Scott et al., 2008). It is likely that a combination of these factors altered the large bowel environment to one that was not favorable for the growth of *Bifidobacterium* spp., thus the decrease in concentration at this level of supplementation.
Swanson et al. (2002) and Middelbos et al. (2007a) observed no change in fecal bacterial populations when dogs were fed 0.05 to 1% yeast cell wall, a source of mannanoligosaccharides. Strickling et al. (2000) noted numeric changes, less than 0.43 log CFU·g⁻¹ DM, in populations of \textit{C. perfringens, Bifidobacterium} spp., and \textit{E. coli} after dogs were fed 0.5% yeast cell wall and xylooligosaccharide. Authors noted that lactobacilli populations increased by 1.02 and 0.83 log CFU·g⁻¹ DM after dogs were fed yeast cell wall and xylooligosaccharide, respectively; however, these changes were considered insignificant. It is possible in these studies that the dose used was insufficient to elicit an effect on the microbial populations. The microbiological data from our study do not support use of the GGMO-AX substrate as an effective prebiotic substrate, especially at the concentrations that normally would be included in commercial diets (0.5 or 1.0%).

Increases in nutrient digestibility and fecal SCFA concentrations, in addition to decreased crude protein digestibility, digesta pH values, and phenol and indole concentrations, indicate an active large bowel fermentation when supplemental GGMO-AX is fed to dogs. Data presented here provide evidence of the positive nutritional properties, but not necessarily prebiotic potential, of supplemental GGMO-AX when incorporated in a high quality dog food. Due to a high concentration of mannan, continued research on its pathogen-binding capability and its potential as an immunomodulatory agent is necessary to determine its efficacy as a dietary supplement impacting canine health and well being.

**LITERATURE CITED**


Table 5.1. Ingredient and chemical composition of diets supplemented with the galactoglucomannan oligosaccharide (GGMO-AX) complex and fed to dogs.

<table>
<thead>
<tr>
<th>Item</th>
<th>Diet, % GGMO-AX substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.0</td>
</tr>
<tr>
<td>Ingredient, %</td>
<td></td>
</tr>
<tr>
<td>Poultry by-product meal</td>
<td>39.00</td>
</tr>
<tr>
<td>Brewer’s rice</td>
<td>27.35</td>
</tr>
<tr>
<td>Poultry fat</td>
<td>14.00</td>
</tr>
<tr>
<td>Ground corn</td>
<td>10.00</td>
</tr>
<tr>
<td>Solka floc&lt;sup&gt;1&lt;/sup&gt;</td>
<td>8.00</td>
</tr>
<tr>
<td>GGMO-AX (spray-dried)&lt;sup&gt;2&lt;/sup&gt;</td>
<td>0.00</td>
</tr>
<tr>
<td>Salt</td>
<td>0.65</td>
</tr>
<tr>
<td>Potassium chloride</td>
<td>0.50</td>
</tr>
<tr>
<td>Chromic oxide</td>
<td>0.20</td>
</tr>
<tr>
<td>Vitamin premix&lt;sup&gt;3&lt;/sup&gt;</td>
<td>0.10</td>
</tr>
<tr>
<td>Mineral premix&lt;sup&gt;4&lt;/sup&gt;</td>
<td>0.10</td>
</tr>
<tr>
<td>Choline chloride</td>
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</tr>
</tbody>
</table>

DM content and chemical composition (DM basis), %

<table>
<thead>
<tr>
<th>Item</th>
<th>0.0</th>
<th>0.5</th>
<th>1.0</th>
<th>2.0</th>
<th>4.0</th>
<th>8.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>DM, %</td>
<td>94.3</td>
<td>94.1</td>
<td>94.1</td>
<td>94.0</td>
<td>93.8</td>
<td>93.6</td>
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<td>OM</td>
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<td>93.4</td>
<td>93.5</td>
<td>93.0</td>
<td>93.1</td>
<td>93.0</td>
</tr>
<tr>
<td>CP</td>
<td>31.6</td>
<td>31.1</td>
<td>31.9</td>
<td>32.0</td>
<td>31.1</td>
<td>32.5</td>
</tr>
<tr>
<td>Acid hydrolyzed fat</td>
<td>21.6</td>
<td>22.5</td>
<td>20.9</td>
<td>21.4</td>
<td>22.3</td>
<td>20.9</td>
</tr>
<tr>
<td>Total dietary fiber – uncorrected&lt;sup&gt;5&lt;/sup&gt;</td>
<td>11.5</td>
<td>10.5</td>
<td>10.3</td>
<td>9.3</td>
<td>7.5</td>
<td>4.2</td>
</tr>
<tr>
<td>Total dietary fiber – corrected&lt;sup&gt;6&lt;/sup&gt;</td>
<td>11.5</td>
<td>11.0</td>
<td>11.3</td>
<td>11.3</td>
<td>11.5</td>
<td>12.2</td>
</tr>
<tr>
<td>GE, kcal·g&lt;sup&gt;1&lt;/sup&gt;</td>
<td>5.4</td>
<td>5.5</td>
<td>5.4</td>
<td>5.4</td>
<td>5.5</td>
<td>5.4</td>
</tr>
</tbody>
</table>

<sup>1</sup> Solka floc, International Fiber Corporation, North Tonawanda, NY 14120.
Galactoglucomannan oligosaccharide (Previda), Temple-Inland, Diboll, TX 75941.

Provided per kilogram of diet: vitamin A, 17,600 IU; vitamin D₃, 1760 IU; vitamin E, 180 kIU; vitamin K, 0.88 mg; thiamin, 4.40 mg; riboflavin, 5.72 mg; pantothenic acid, 22.00 mg; niacin, 39.60 mg; pyridoxine, 3.52 mg; biotin, 0.13 mg; folic acid, 0.44 mg; and vitamin B₁₂, 0.11 mg.

Provided per kilogram of diet: manganese (as MnSO₄), 66.00 mg; iron (as FeSO₄), 120 mg; copper (as CuSO₄), 18 mg; cobalt (as CoSO₄), 1.20 mg; zinc (as ZnSO₄), 240 mg; iodine (as KI), 1.8 mg; and selenium (as Na₂SeO₃), 0.24 mg.

These values were determined using the total dietary fiber assay (Prosky et al., 1984) that cannot quantify GGMO-AX.

These values were determined by adding the amount of GGMO-AX substrate present in each diet to the total dietary fiber (uncorrected) value.
Table 5.2. Chemical composition of the spray-dried galactoglucomannan oligosaccharide-arabinoxylan (GGMO-AX) complex.

<table>
<thead>
<tr>
<th>Item</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>DM, %</td>
<td>94.1</td>
</tr>
<tr>
<td>OM, %</td>
<td>95.9</td>
</tr>
<tr>
<td>CP, %</td>
<td>0.2</td>
</tr>
<tr>
<td>Acid hydrolyzed fat, %</td>
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</tr>
<tr>
<td>GE, kcal·g⁻¹</td>
<td>4.2</td>
</tr>
<tr>
<td>Free sugars, mg·g⁻¹</td>
<td></td>
</tr>
<tr>
<td>Fucose</td>
<td>1.25</td>
</tr>
<tr>
<td>Arabinose</td>
<td>50.76</td>
</tr>
<tr>
<td>Rhamnose</td>
<td>1.80</td>
</tr>
<tr>
<td>Galactose</td>
<td>11.88</td>
</tr>
<tr>
<td>Glucose</td>
<td>2.16</td>
</tr>
<tr>
<td>Sucrose</td>
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<tr>
<td>Xylose</td>
<td>14.47</td>
</tr>
<tr>
<td>Mannose</td>
<td>4.64</td>
</tr>
<tr>
<td>Fructose</td>
<td>0.97</td>
</tr>
<tr>
<td>Total</td>
<td>87.93</td>
</tr>
<tr>
<td>Hydrolyzed monosaccharides¹, mg·g⁻¹</td>
<td></td>
</tr>
<tr>
<td>Fucose</td>
<td>3.44</td>
</tr>
<tr>
<td>Arabinose</td>
<td>36.79</td>
</tr>
<tr>
<td>Rhamnose</td>
<td>5.55</td>
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<tr>
<td>Galactose</td>
<td>76.33</td>
</tr>
<tr>
<td>Glucose</td>
<td>159.19</td>
</tr>
<tr>
<td>Xylose</td>
<td>134.00</td>
</tr>
<tr>
<td>Mannose</td>
<td>353.73</td>
</tr>
<tr>
<td>Total</td>
<td>769.03</td>
</tr>
<tr>
<td>Oligosaccharides, mg·g⁻¹</td>
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</tr>
<tr>
<td>Cellobiose</td>
<td>1.64</td>
</tr>
<tr>
<td>Raffinose</td>
<td>2.28</td>
</tr>
<tr>
<td>Cellotriose</td>
<td>3.68</td>
</tr>
<tr>
<td>Maltotriose</td>
<td>0.43</td>
</tr>
<tr>
<td>Cellopentaose</td>
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<td>Maltotetraose</td>
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<td>2.09</td>
</tr>
<tr>
<td>Maltohexaose</td>
<td>1.12</td>
</tr>
<tr>
<td>Maltoheptaose</td>
<td>0.87</td>
</tr>
<tr>
<td>Total</td>
<td>13.22</td>
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<tr>
<td>Polyphenolics, mg·g⁻¹</td>
<td></td>
</tr>
<tr>
<td>m-Coumaric acid</td>
<td>0.08</td>
</tr>
<tr>
<td>p-Coumaric acid</td>
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</tr>
<tr>
<td>Ferulic acid</td>
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</tr>
<tr>
<td>p-Hydroxybenzoic acid</td>
<td>0.02</td>
</tr>
<tr>
<td>4’-Hydroxypropiophenone</td>
<td>0.01</td>
</tr>
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Table 5.2 (cont.).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
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<tbody>
<tr>
<td>Isovanillic acid</td>
<td>0.08</td>
</tr>
<tr>
<td>Sinapyl acid</td>
<td>0.92</td>
</tr>
<tr>
<td>Sinapyl alcohol</td>
<td>0.04</td>
</tr>
<tr>
<td>Sinapyl aldehyde</td>
<td>0.30</td>
</tr>
<tr>
<td>Vanillin</td>
<td>1.50</td>
</tr>
<tr>
<td>Total</td>
<td>3.11</td>
</tr>
</tbody>
</table>

*1Hydrolyzed monosaccharide concentrations were corrected for free sugar concentrations.*
Table 5.3. Nutrient intakes and digestibilities of diets supplemented with the galactoglucomannan oligosaccharide-arabinoxylan (GGMO-AX) complex and fed to dogs.

<table>
<thead>
<tr>
<th>Item</th>
<th>Diet, % GGMO-AX substrate</th>
<th>P-value</th>
<th>SEM</th>
<th>Linear</th>
<th>Quadratic</th>
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<tbody>
<tr>
<td></td>
<td>0.0</td>
<td>0.5</td>
<td>1.0</td>
<td>2.0</td>
<td>4.0</td>
</tr>
<tr>
<td>Intake, g·d⁻¹</td>
<td></td>
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<td></td>
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<tr>
<td>DM</td>
<td>288</td>
<td>258</td>
<td>253</td>
<td>264</td>
<td>280</td>
</tr>
<tr>
<td>OM</td>
<td>268</td>
<td>241</td>
<td>236</td>
<td>246</td>
<td>261</td>
</tr>
<tr>
<td>CP</td>
<td>91</td>
<td>80</td>
<td>81</td>
<td>84</td>
<td>87</td>
</tr>
<tr>
<td>Acid hydrolyzed fat</td>
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<td>58</td>
<td>53</td>
<td>57</td>
<td>63</td>
</tr>
<tr>
<td>Total dietary fiber - uncorrected¹</td>
<td>33</td>
<td>27</td>
<td>26</td>
<td>25</td>
<td>21</td>
</tr>
<tr>
<td>Total dietary fiber - corrected²</td>
<td>33</td>
<td>28</td>
<td>28</td>
<td>30</td>
<td>32</td>
</tr>
<tr>
<td>Apparent digestibility, %</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DM</td>
<td>78.0</td>
<td>78.7</td>
<td>77.9</td>
<td>79.5</td>
<td>82.0</td>
</tr>
<tr>
<td>OM</td>
<td>81.3</td>
<td>82.0</td>
<td>81.4</td>
<td>82.7</td>
<td>85.0</td>
</tr>
<tr>
<td>CP</td>
<td>84.2</td>
<td>83.7</td>
<td>82.7</td>
<td>82.7</td>
<td>81.5</td>
</tr>
<tr>
<td>Acid hydrolyzed fat</td>
<td>95.6</td>
<td>95.7</td>
<td>95.3</td>
<td>95.5</td>
<td>96.0</td>
</tr>
</tbody>
</table>

¹Intake values were determined using the total dietary fiber assay that cannot quantify the GGMO-AX substrate.

²Intake values were determined by multiplying DM concentration of the GGMO-AX substrate by the dietary GGMO-AX substrate concentration. This value then was multiplied by DM intake·d⁻¹ and added to the total dietary fiber (uncorrected) value.
Table 5.4. Concentrations (µmol·g⁻¹, DM basis) of fecal short-chain (SCFA) and branched-chain fatty acids (BCFA) for dogs fed diets containing the galactoglucomannan oligosaccharide-arabinoxylan (GGMO-AX) complex.

<table>
<thead>
<tr>
<th>Item</th>
<th>Diet, % GGMO-AX substrate</th>
<th>SEM</th>
<th>Linear</th>
<th>Quadratic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.0</td>
<td>0.5</td>
<td>1.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Acetate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Propionate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Butyrate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total SCFA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isobutyrate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isovalerate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Valerate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total BCFA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Diet, %  GGMO-AX substrate  0.0  0.5  1.0  2.0  4.0  8.0  SEM  Linear  Quadratic
P-value <0.001  0.003
<0.001  0.076
0.001  0.201
<0.001  0.014
<0.681  0.375
<0.284  0.975
<0.010  0.151
<0.538  0.632
Table 5.5. Fecal pH, fecal score, and concentrations of fecal ammonia, phenol, indole, and biogenic amines for dogs fed diets supplemented with the galactoglucomannan oligosaccharide-arabinoxylan (GGMO-AX) complex.

<table>
<thead>
<tr>
<th>Item</th>
<th>Diet, % GGMO-AX substrate</th>
<th>Contrast, P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.0-</td>
<td>0.5</td>
</tr>
<tr>
<td>pH</td>
<td>6.7</td>
<td>6.4</td>
</tr>
<tr>
<td>Fecal score1</td>
<td>2.4</td>
<td>2.4</td>
</tr>
<tr>
<td>Ammonia, mg·g⁻¹</td>
<td>2.3</td>
<td>2.3</td>
</tr>
<tr>
<td>Phenol, µg·g⁻¹</td>
<td>112.0</td>
<td>101.7</td>
</tr>
<tr>
<td>Indole, µg·g⁻¹</td>
<td>133.5</td>
<td>158.4</td>
</tr>
<tr>
<td>Biogenic amines, µmol·g⁻¹</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cadaverine</td>
<td>1.29</td>
<td>1.03</td>
</tr>
<tr>
<td>Phenylethylamine</td>
<td>0.07</td>
<td>0.07</td>
</tr>
<tr>
<td>Putrescine</td>
<td>4.45</td>
<td>3.57</td>
</tr>
<tr>
<td>Spermidine</td>
<td>1.37</td>
<td>1.43</td>
</tr>
<tr>
<td>Spermine</td>
<td>0.62</td>
<td>0.86</td>
</tr>
<tr>
<td>Tryptamine</td>
<td>0.52</td>
<td>0.49</td>
</tr>
<tr>
<td>Tyramine</td>
<td>0.08</td>
<td>0.06</td>
</tr>
<tr>
<td>Total</td>
<td>8.38</td>
<td>7.51</td>
</tr>
</tbody>
</table>

1Based on the 5-point scale with score 1 being hard, dry pellets, and small hard mass, and score 5 being watery, liquid that can be poured.
Table 5.6. Fecal microbial populations of dogs consuming diets supplemented with the galactoglucomannan oligosaccharide-arabinoxylan (GGMO-AX) complex.

<table>
<thead>
<tr>
<th>Item</th>
<th>% GGMO-AX substrate</th>
<th>SEM</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.0</td>
<td>0.5</td>
<td>1.0</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>10.0</td>
<td>9.9</td>
<td>10.2</td>
</tr>
<tr>
<td>Lactobacillus spp.</td>
<td>11.0</td>
<td>10.5</td>
<td>11.0</td>
</tr>
<tr>
<td>Bifidobacterium spp.</td>
<td>8.0</td>
<td>8.0</td>
<td>8.3</td>
</tr>
<tr>
<td>Clostridium perfringens</td>
<td>9.1</td>
<td>8.9</td>
<td>8.9</td>
</tr>
</tbody>
</table>

CFU, log10·g⁻¹ fecal DM
ABSTRACT: Fermentable carbohydrates may enhance the ability of the gastrointestinal tract to defend against pathogenic infection. We hypothesized that a mannose-rich, galactoglucomannan oligosaccharide-arabinoxylan (GGMO-AX) complex would positively impact immune status and prevent weight loss resulting from acute coccidiosis (Eimeria acervulina) infection of chicks. Using a completely randomized design, day-old commercial broiler chicks (n=160; 4 replications/treatment; 5 chicks/replication) were assigned to one of four corn-soybean meal-based diets containing supplemental GGMO-AX (0, 1, 2, or 4%) that replaced dietary cellulose. On d 9 post-hatch, an equal number of chicks on each diet were inoculated with either distilled water (sham control) or E. acervulina (1x10^6 oocysts). All birds were euthanized on d 7 post-inoculation (PI) for collection of cecal contents and duodenal tissue. Overall, body weight gain of chicks was not affected by diet PI, while infection decreased (P < 0.01) weight gain on d 3-7 and 0-7 PI. Feed intake was not affected by dietary treatment, but infection decreased (P < 0.01) feed intake on d 3-7 and 0-7 PI. Overall, infection, but not diet, decreased (P < 0.01) G:F on d 3-7 and 0-7 PI. Cecal propionate concentrations were independently affected by infection and diet, while butyrate concentrations were affected only by infection (P = 0.02). Cecal Bifidobacterium spp. populations were affected (P < 0.01) by diet, with the 2% GGMO-AX resulting in the highest CFU/g of cecal contents (on a DM basis). Messenger RNA expression of all duodenal cytokines evaluated was affected by infection status (P ≤ 0.02), but not by dietary treatment alone. Supple
inflammatory cytokine expression, while inhibiting anti-inflammatory cytokine expression, which indicates a more robust innate immune response. Despite decreasing performance, dietary supplementation with GGMO-AX improved select fermentation indices and the innate intestinal immune response to an acute *E. acervulina* infection.

**INTRODUCTION**

Avian coccidiosis is caused by several species of *Eimeria*, an infectious protozoan that penetrates and damages the epithelial cells of intestinal tissue, and is estimated to cost $3 billion U.S. in annual production losses worldwide (Williams, 1999; Shirley et al., 2004; Dalloul and Lillehoj, 2006). In particular, *Eimeria acervulina* invades the duodenal intestinal epithelium resulting in intestinal inflammation and, potentially, intestinal hemorrhaging (Lillehoj and Trout, 1996). The intestinal damage results in decreased animal growth and feed efficiency, as well as diarrhea and mortality, all of which have significant implications for the commercial poultry industry.

The avian intestinal tract possesses numerous defenses to help protect against and overcome infection, with cell-mediated, or innate, immunity serving as an important first line of defense for a coccidiosis infection. Cell-mediated immunity involves activation of specific cell types, including macrophages, natural killer cells, and antigen-specific cytotoxic T-lymphocytes, and occurs in immediate response to pathogen recognition. Macrophages, in particular, produce cytokines, which serve as cell-signaling molecules to elicit appropriate pro- or anti-inflammatory responses. In addition, certain cytokines, including IFN-γ and IL-1β, serve as important mediators during a coccidiosis challenge, as these proteins are able to decrease oocyst production and improve body weight gain during an *E. acervulina* infection (Lowenthal et al., 1997; Lillehoj
and Choi, 1998). Cytokine production of an appropriate magnitude and duration is crucial to quickly restore the animal to a healthy state, while minimizing expenditure of costly nutrient and energy resources to support immune defenses.

Research has shown a positive correlation between the effect of fermentable carbohydrates and improved intestinal health. Fiber fermentation in the distal segment of the intestinal tract may benefit the immune status of the entire gut by several potential mechanisms including increased mucin thickness, modulation of epithelial inflammation by short-chain fatty acids (SCFA), increased resistance to microbial colonization, and enhanced immune activity (Cavaglieri et al., 2003; Tedelind et al., 2007; Hedemann et al., 2009; Ito et al., 2009). Through these mechanisms, the detrimental effects of \textit{E. acervulina} infection may be limited or eliminated in the intestine.

Mannanoligosaccharides (MOS), a fermentable oligosaccharide mainly derived from yeast cell wall (YCW), has been extensively evaluated for its ability to improve weight gain and feed conversion and decrease mortality in chickens (Hooge, 2004). In addition, YCW has been shown to modulate the immune system of chicks (Gao et al., 2008; Janardhana et al., 2009). Perhaps more than any other compound, MOS have been repeatedly shown to elicit beneficial effects in \textit{Eimeria}-infected chicks (Elmusharaf et al., 2006, 2007; Gao et al., 2009). Chicks infected with either \textit{E. tenella} or \textit{E. acervulina} and fed diets containing 0.1 or 1% YCW, respectively, had reduced numbers of schizonts, an intermediate life stage during \textit{Eimeria} oocyst reproduction, in the lamina propria of the cecum and oocyte shedding in excreta (Elmusharaf et al., 2006, 2007). Gao et al. (2009) suggested that YCW supplementation (0.25 and 0.5%) promoted overall immune function in \textit{E. tenella}-infected chicks as indicated by improved responses of both the innate immune response and antibody production.
A novel source of MOS can be found in a galactoglucomannan oligosaccharide-arabinoxylan (GGMO-AX) complex carbohydrate (Price et al., 2011). The GGMO-AX complex, a co-product of the fiberboard manufacturing industry, is well fermented when evaluated both *in vitro* and *in vivo* (Faber et al., 2011a, b). It has yet to be determined, however, if the GGMO-AX complex can positively modulate the intestinal immune system. Thus, a coccidiosis-induced immune response may be an appropriate model to test the effects of this novel GGMO-AX complex.

The objective of this experiment was to determine the effects of a supplemental GGMO-AX complex in diets with emphasis on growth performance, fermentative effects, and immune indices in chicks challenged with an acute coccidial (*Eimeria acervulina*) infection.

**MATERIALS AND METHODS**

All animal care procedures were approved by the University of Illinois Institutional Animal Care and Use Committee and Institutional Biosafety Committee before initiation of the experiment.

*Galactoglucomannan Oligosaccharide Substrate*

Production of the GGMO-AX substrate involves use of wood chips, water, and pressure, but does not use strong acids or bases unlike other fiberboard production processes. This results in an ingredient better tailored for consumption by animals. During hydrolysis, hemicelluloses are depolymerized through hydronium ions from water and other compounds such as uronic, acetic, and phenolic acids (Garrote et al., 1999). The release of pressure on material in the ‘wood chip digester’ results in destruction primarily of cellulose, hemicelluloses, and lignin, which, in turn, releases soluble sugars into the surrounding water, along with polyphenolic compounds.
from lignin. The resulting water solution contains increased concentrations of sugars (3 to 4% w/w), a concentration unsafe for disposal into wastewater streams. Thus, the sugar solution is removed from the wood chips and further condensed into a viscous liquid (i.e., syrup) with a final sugar concentration of 30 to 54% (Michalka, 2007); in this final product, sugars are mostly found in the oligosaccharide form, and not free sugars.

To obtain a dry product, the GGMO-AX syrup was spray-dried (Valentine Chemicals, Lockport LA) with an inlet temperature approximately 155°C and exit temperature of 102°C; the starting substrate was adjusted to 53% solids before drying. Spray-drying allowed the substrate to be mixed in a mash diet matrix that was appropriate for young chicks.

**Chicks and Husbandry**

One-hundred sixty, 1-d-old male Ross x Ross broiler chicks were used in this experiment. Chicks were housed in thermostatically controlled starter batteries with raised wire floors in an environmentally controlled room with continuous lighting. At hatch, chicks were weighed, wing-banded, and assigned to treatment groups, so that the initial weight was similar among treatment groups. Four replicates of 5 chicks were assigned to each treatment in the experiment. Chicks were randomly assigned to both an infection treatment (sham-inoculated vs. *E. acervulina*-inoculated) and a dietary treatment that differed only in GGMO-AX concentration (0, 1, 2, or 4% of as-is diet); GGMO-AX supplementation occurred at the expense of cellulose (Solka-Floc; International Fiber Corporation, North Tonawanda, NY). All diets were formulated to meet or exceed NRC (1994) recommendations (Table 6.1), and feed and fresh water were offered ad libitum. Chicks and feeders were weighed on d 1, 3, and 6 post-hatch, and each day between d 9-16 post-hatch to determine weight gain and feed intake before and during coccidial infection.
**Inoculation**

Sporulated *E. acervulina* oocysts (Parasite Biology and Epidemiology Lab, USDA, Beltsville, MD) were diluted with distilled water immediately prior to inoculation. Chicks were administered an acute 0.5 ml oral dose of either $1 \times 10^6$ sporulated *E. acervulina* oocysts or distilled water on d 9 post-hatch using 1-cc syringes without needles.

**Sample Collection**

On d 16 post-hatch (d 7 post-inoculation, PI) chicks were euthanized by CO$_2$ inhalation. Chicks were immediately dissected and ceca were removed and contents pooled per replicate pen of chicks. An aliquot of cecal digesta was immediately transferred to a sterile cryogenic vial (Nalgene, Rochester, NY) and snap-frozen in liquid nitrogen. Additional aliquots were taken for measurement of pH, DM, SCFA, and branched-chain fatty acids (BCFA). Duodenal and spleen tissue were sampled from two randomly chosen chicks per replicate. Duodenal and splenic tissues were rinsed with PBS (pH 7.2), minced with a scalpel, placed in RNAlater® solution, and stored at -80°C pending analysis.

**Chemical Analyses**

Fresh cecal samples were analyzed for DM using AOAC (2006) methods. Cecal SCFA and BCFA concentrations were determined by gas chromatography according to Erwin et al. (1961) using a gas chromatograph (Hewlett-Packard 5890A series II, Palo Alto, CA) and a glass column (180 cmx 4 mm i.d.) packed with 10% SP-1200/1% H$_3$PO$_4$ on 80/100+ mesh Chromosorb WAW (Supelco Inc., Bellefonte, PA); nitrogen was used as the carrier gas with a flow rate of 75 mL·min$^{-1}$. Oven, detector, and injector temperatures were 125, 175, and 180°C, respectively.
**Microbial Analyses**

Cecal microbial populations were analyzed using methods described by Middelbos et al. (2007) with minor adaptations. Briefly, cecal DNA was extracted from freshly collected samples that had been stored at –80°C based on the repeated bead beater method described by Yu and Morrison (2004), followed by use of a DNA extraction kit (QIAamp DNA Stool Mini Kit, Qiagen, Valencia, CA) according to the manufacturer’s instructions. A TissueLyser apparatus (Qiagen, Valencia, CA) with a 5 mm steel bead (30 sec at 15 Hz) was used in lieu of the repeated bead beater. Extracted DNA was quantified using a spectrophotometer (NanoDrop ND-1000, Nano-Drop Technologies, Wilmington, DE). Quantitative PCR was performed using specific primers for *Bifidobacterium* spp. (Matsuki et al., 2002), *Lactobacillus* spp. (Collier et al., 2003), *Escherichia coli* (Malinen et al., 2003), and *Clostridium perfringens* (Wang et al., 1994). Amplification was performed according to DePlancke et al. (2002). Briefly, a 10-µL final volume contained 5 µL of 2 x SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), 15 pmol of the forward and reverse primers for the bacterium of interest, and 5 ng of extracted cecal DNA. Standard curves were obtained by harvesting pure cultures of the bacterium of interest in the log growth phase in triplicate, followed by serial dilution. Bacterial DNA was extracted from each dilution using a DNA extraction kit as stated above, and amplified with the cecal DNA to create triplicate standard curves (ABI PRISM 7900HT Sequence Detection System, Applied Biosystems, Foster City, CA). Colony-forming units in each dilution were determined by plating on specific agars; lactobacilli MRS (Difco, BD, Franklin Lakes, NJ) for lactobacilli, reinforced clostridial medium (bifidobacteria, *C. perfringens*), and Luria Bertani medium (*E. coli*). The calculated log cfu per milliliter of each serial dilution was plotted against the cycle threshold to create a linear equation to calculate cfu per gram of dry cecal digesta.
Inflammatory Cytokine Analyses

Total RNA was extracted from duodenal and spleen tissues using the Qiazol Lysis Reagent protocol (Qiagen, Valencia, CA) and the TissueLyser apparatus described above. Extracted RNA was quantified using a spectrophotometer (NanoDrop ND-1000, Nano-Drop Technologies, Wilmington, DE). Ribonucleic acid samples were subjected to a 7x gDNA Wipeout Buffer and then converted to complementary DNA (cDNA) using a QuantiTec® Reverse Transcription kit (Qiagen, Valencia, CA). Wipeout of gDNA and reverse transcription was performed in a thermocycler (model PTC-200, Biorad, Hercules, CA) with the program set for 2 min at 42°C, 2 min at 4°C, 15 min at 42°C, 3 min at 95 °C, and then cooled to 4 °C. The cDNA synthesized was stored at -20°C.

Quantitative real time PCR was performed using the Applied Biosystems (Foster, CA) TaqMan® Gene Expression Assay as previously described (Dilger and Johnson, 2010). The cDNA was amplified by PCR where a target cDNA (interferon-γ [IFN-γ], Y07922; interleukin [IL]-1β, Y15006; IL-6, AJ309540; IL-12β, NM_213571; and IL-15, AF139097) and reference cDNA (glyceraldehyde 3-phosphate dehydrogenase [GAPDH], K01458; Hong et al., 2006) were amplified using Taqman (Invitrogen, Carlsbad, CA), an oligonucleotide probe with a 5′ fluorescent reporter dye (6-FAM) and a 3′ non-fluorescent quencher dye (NFQ). Fluorescence was determined on an ABI PRISM 7900HT-sequence detection system (Applied Biosystems, Forest City, CA). To normalize gene expression, a parallel amplification of endogenous GAPDH was performed for each gene. Reactions with no reverse transcription and no template were included as negative controls. Data were analyzed using the comparative threshold cycle (Ct) method (Livak and Schmittgen, 2001) and results are expressed as fold-change relative to the control treatment.
Statistical Analyses

Data were analyzed by ANOVA with procedures appropriate for a completely randomized design. Data for continuous variables were analyzed by the MIXED procedure (SAS Inst., Cary, NC). The statistical model included the fixed main effects of infection and dietary treatment. Additionally, interactive effects were evaluated using polynomial contrasts (linear and quadratic) designed for the non-equally spaced levels of supplemental GGMO-AX vs. infection status. Least squares means were separated using least squares differences with a Tukey adjustment. Outlier data were removed from analysis after analyzing data using the UNIVARIATE procedure to produce a normal probability plot based on residual data and visual inspection of the raw data. Outlier data were defined as data points three or more standard deviations from the mean. Differences among treatment level least squares means with a probability of P < 0.05 were accepted as statistically significant, whereas mean differences with P-values ranging from 0.06 to 0.10 were accepted as trends.

RESULTS

Chick Growth and Performance

Overall, body weight gain of chicks was not affected by diet PI, while infection decreased (P < 0.01) weight gain on d 3-7 and 0-7 PI (Table 6.2). An interaction of infection by quadratic response to diet (Quad Dt; P < 0.01) affected weight gain on d 0-3 PI. On d 0-7 PI, an infection by linear response to diet (Lin Dt; P = 0.09) was noted. Overall, feed intake was not affected by dietary treatment. Numerically, uninfected chicks fed 4% GGMO-AX diet exhibited the lowest feed intake, while infected chicks fed the 4% GGMO-AX diet exhibited the greatest feed intake during all time periods. Infection decreased (P < 0.01) feed intake on d 3-7 and 0-7 PI. Overall,
infection, but not diet, decreased (P < 0.01) G:F on d 3-7 and 0-7 PI. An interaction of infection by Lin Dt (P < 0.01) was observed for G:F during d 3-7 and 0-7 PI.

Fermentation Metabolites

Cecal acetate and total SCFA concentrations were not affected by infection or diet (Table 6.3). Infection and diet affected (P < 0.01) propionate concentrations independently. As the dietary concentration of GGMO-AX increased, propionate concentrations decreased. Cecal butyrate concentrations were not affected by diet (P = 0.26), but were affected by infection (P = 0.02), with infected chicks having greater concentrations of butyrate than uninfected chicks. Cecal BCFA (isobutyrate, isovalerate, valerate, and total BCFA) concentrations were not affected by infection or dietary treatment.

Cecal pH

Cecal pH was greater (P < 0.01) in infected chicks than uninfected chicks (Fig. 6.1). In addition, diet affected (P < 0.01) cecal pH, regardless of infection status. As the dietary concentration of GGMO-AX increased, cecal pH linearly decreased (P < 0.01), but no interaction effects were noted.

Cecal Microbiota

Cecal Bifidobacterium spp. populations were affected (P < 0.01) by diet, with the 2% GGMO-AX resulting in the highest CFU/g of cecal contents (on a DM basis; Table 6.4). Cecal E. coli populations were increased (P = 0.01) due to infection. An infection by Ln Dt trend (P = 0.08) was noted, as dietary GGMO-AX concentration increased, E. coli populations increased in uninfected chicks while populations decreased in infected birds. Cecal Lactobacillus spp. populations were greater (P < 0.01) in infected chicks, regardless of diet.
Cytokine Expression

Messenger RNA expression of all duodenal cytokines evaluated was affected by infection status ($P \leq 0.02$; Fig. 6.2), but not by dietary treatment alone. An infection by Lin Dt and Quad Dt tended ($P = 0.10$ and $0.06$, respectively) to affect IL-15 expression. An infection by Quad Dt also tended ($P = 0.06$) to affect IL-12$\beta$ expression. Neither infection nor diet affected cytokine mRNA expression in the spleen (data not shown).

DISCUSSION

The intestinal and systemic health benefits of dietary fiber, particularly fermentable carbohydrates, in human and animal nutrition suggest fiber to be an important dietary ingredient. In addition, some oligosaccharides, particularly MOS, may directly prevent pathogenic bacteria from binding to epithelial cells in the small intestine and prevent colonization and proliferation in the intestinal tract (Grieshop et al., 2004; Gouveia et al., 2006; Baurhoo et al., 2009). We hypothesized that ingestion of a novel fermentable carbohydrate, GGMO-AX, could improve the immune response of growing chickens to an acute *E. acervulina* infection through fermentative changes, and possibly through an MOS-dependent mechanism, in the distal gastrointestinal tract. Results of our studies suggest that while the GGMO-AX complex can be fermented by the young chick, direct alteration of the immune profile was insufficient to provide significant protection from the acute coccidial infection that was induced.

Based on preliminary evidence from our laboratory, chicks inoculated with an acute dose of *E. acervulina* first exhibit illness-related symptoms on d 3 PI, characterized by a decrease in both weight gain and feed intake that persists through d 7 PI. Moreover, the greatest induction in duodenal cytokine mRNA expression occurs on d 7 PI. Therefore, based on these preliminary
findings, d 7 PI was deemed the time of ‘peak infection’ and served as the optimum day to collect tissue samples. This timeline also permitted sufficient time for chicks to adapt to the dietary treatments containing the fermentable GGMO-AX complex.

Overall, dietary GGMO-AX supplementation did not alter chick body weight gain either pre- or post-inoculation with *E. acervulina*. Results were similar to those of Jacobs (2011) who demonstrated that 21-d-old chicks fed a corn-soybean meal diet supplemented with liquid GGMO-AX up to 1% increased (P < 0.05) amino acid and ME digestibility without affecting weight gain, feed intake, or feed efficiency compared with control chicks. However, in a meta-analysis of 44 broiler trials evaluating MOS mainly derived from YCW, 79.5% of the trials reported improved weight gain due to provision of this carbohydrate alone (Hooge, 2004). Differences in substrate characteristics, including levels of β-glucans and crude protein, may help explain improved growth performance in chicks fed MOS directly, and it is possible that concentrations of MOS provided from GGMO-AX in our studies did not reach a minimum threshold to elicit this type of response.

Following inoculation with *E. acervulina*, clear differences in weight gain were expected as attributable to several factors. First, the observed decrease in feed intake was likely due to an increase in systemic cytokine expression. Select cytokines, particularly IL-1β and TNF-α, are known to decrease both feed intake and feed efficiency in animal models (Klasing et al., 1987; Beutler and Cerami, 1989; Johnson, 1997). However, in the current study, the greatest numerical differences in cytokine mRNA expression due to *E. acervulina* inoculation were observed in chicks receiving 4% dietary GGMO-AX complex, suggesting that dietary fermentation characteristics may directly affect an active immune response. Infected chicks fed more dietary GGMO-AX linearly decreased in feed efficiency, which suggests a maximum threshold for
fermentable carbohydrate ingestion by the chick. Secondly, chicks experience a decrease in digestibility of ME, glucose, fat, and amino acids, as indicated by both *in vitro* and *in vivo* experiments (Ruff and Wilkins, 1980; Adams et al., 1996; Persia et al., 2006; Jacobs, 2011). Preston-Mafham and Sykes (1970) showed that the decrease in feed intake due to infection accounts for approximately 30-70% of the decrease in weight gain, as determined in uninfected chicks that were pair-fed with infected chicks. Moreover, decreases in nutrient digestibility are known to occur concomitantly with changes in intestinal enzyme activity, which are associated with *Eimeria*-induced damage of duodenal tissue (Adams et al., 1996). Lastly, dietary energy and endogenous resources (e.g., amino acids) are required to mount an active immune response, which decreases available substrates for body weight gain and maintenance in the rapidly growing chick (Lochmiller and Deerenberg, 2000; Humphrey and Klasing, 2004).

Fermentation of substrates by commensal bacteria results in the production of SCFA, which lowers the pH of the fermentative segment of the gastrointestinal tract (i.e., the ceca in avian species). As concentration of fermentable substrate increases, colonic pH decreases (Roberfroid, 2007). Despite a decrease in cecal pH with increasing GGMO-AX concentrations, total SCFA concentrations were unaffected. In contrast, a linear increase in total fecal SCFA was observed in dogs receiving diets containing up to 8% GGMO-AX (Faber et al., 2011a), so lack of a similar response in chickens was perplexing. In other experiments evaluating fermentable carbohydrates (fructooligosaccharides [1%] and xylose [5%]) in chicks, total cecal SCFA concentrations were either unchanged or numerically lower (Ricke et al., 1982; Rehman et al., 2008a; Rehman et al., 2008b). But cecal SCFA concentration is only part of the story as total cecal volume is not an inherent property of this measurement. Fermentable substrates are known to increase cecal volume (Berggren et al., 1993; Campbell et al., 1997), which would effectively
serve to dilute SCFA concentrations. Thus, it is highly probably that differences in total SCFA production would have been observed if our data were expressed on total cecal volume basis compared with concentration alone (Berggren et al., 1993; Campbell et al., 1997; Knapp, 2011).

*Eimeria acervulina* infected chicks had a greater cecal pH compared to uninfected chicks, which is similar to results of Ruff et al. (1974) on d 7 PI. This may be the result of a change in the microbial populations as noted in the current study, since total SCFA concentrations were similar among treatments. Significant increases in *E. coli* and *Lactobacillus* spp. were noted in infected chicks, likely resulting in increased propionate and butyrate concentrations. An increase in the quantity and type of available fermentable substrate would modulate the microbiome, resulting in a shift in the profile of fermentative end-products being produced. For example, butyrate is mainly produced from fermentation of starch (Cummings, 1995). *Eimeria acervulina* decreases amylotic activity (Major and Ruff, 1978), which would increase the concentration of dietary starch entering the avian ceca and lead to greater cecal butyrate concentrations, as noted in the current study. Similar changes in *E. coli* and *Lactobacillus* spp. populations in the ceca of *E. acervulina*-infected chicks have been noted by Turk and Littlejohn (1987) and Jacobs (2011). Wang and Gibson (1993) demonstrated *in vitro* that lowering the pH of the medium limited *E. coli* growth. Therefore, populations of *E. coli* may have been enhanced in infected chicks due to higher cecal pH. To our knowledge, no other study has evaluated cecal SCFA concentrations in chicks inoculated with *E. acervulina*.

Branched-chain fatty acid concentrations were not affected by infection or dietary treatment. Production of BCFA results from fermentation of nitrogenous compounds in the ceca. Our data suggest little difference in the quantity of nitrogenous compounds entering the ceca of infected or non-infected chicks. This is important as *Eimeria* infection has been shown to
decrease amino acid utilization and thereby permit more of these nitrogen-containing compounds to enter the lower gastrointestinal tract (Preston-Mafham and Sykes, 1970; Persia et al., 2006; Jacobs, 2011).

*Bifidobacterium* spp. are indicators of improved intestinal health due to their association with prebiotic carbohydrates. Diets containing 2% GGMO-AX resulted in the greatest population of *Bifidobacterium* spp. in ceca, regardless of infection status. Populations were, on average, one log$_{10}$ CFU greater for the 2% GGMO-AX-fed chicks compared to the 0% GGMO-AX-fed chicks. Interestingly, 4% GGMO-AX supplementation resulted in lower *Bifidobacterium* spp. populations than 2% GGMO-AX-fed chicks, suggesting that dietary GGMO-AX included above 2% of the diet may inhibit bifidobacteria growth. In a related study, bifidobacteria populations were increased by 0.84 and 1.23 log$_{10}$ CFU/g DM on d 3 and d 7 post-hatch, respectively, in chicks receiving a corn-soybean meal diet containing 0.5% liquid GGMO-AX (Jacobs, 2011).

Messenger RNA cytokine expression is a common method to evaluate the immune response elicited by various tissues to an infectious organism. In our studies, infected chicks receiving 4% supplemental GGMO-AX had increased mRNA expression of pro-inflammatory cytokines (IFN-$\gamma$, IL-1$\beta$, IL-6, IL-12 $\beta$) and lower expression of anti-inflammatory cytokine IL-15 compared with infected chicks receiving other dietary treatments. Previous research suggested chicks that were genetically enhanced to elicit a strong innate immune response had reduced weight loss and fecal oocyst shedding with greater IL-1$\beta$, IL-6, and IL-8 expression after an *E. maxima* infection (Kim et al., 2008). In addition, the simultaneous administration of recombinant IFN-$\gamma$ and *E. acervulina* to chicks resulted in a decrease in oocyst excretion and improved body weight gain (Lowenthal et al., 1997; Lillehoj and Choi, 1998). However, in our
study, increased cytokine expression on d 7 PI was associated with lower weight gain, which highlights the complexity associated with cytokine signaling. Nonetheless, the more robust cytokine response observed may aid the chick in overcoming infection in less time, which would translate into direct benefits for production efficiency. Additionally, we speculate that a greater innate immune response may enhance the acquired immunity to subsequent infections, and thereby potentially ameliorate the negative effects of a secondary infection (Bendelac and Fearon, 1997; Medzhitov and Janeway, 1997; Parish and O’Neil, 1997; Iwasaki and Medzhitov, 2010).

Although the GGMO-AX substrate was fermented in the ceca, modulation of cytokine expression clearly occurred at proximal sites in the gastrointestinal tract (i.e., the duodenum). This suggests that the GGMO-AX substrate provided at 4% of the diet elicited a systemic alteration of the immune response to an intestinal infection. Although not evaluated in our study, the immune response in the cecal tonsils may have been enhanced during the *Eimeria* infection. Cecal tonsils are the major lymphoid tissue of the ceca, and represent the largest collection of gut associated lymphoid tissue in the chicken (Yun et al., 2000). Cytokine expression in the spleen was measured as an indicator of systemic immune activity, but no changes were noted in this tissue, suggesting that only the intestinal lymphatic system was responding to the infection and not the systemic lymphatic system.

Short-chain fatty acids *in vitro* and *in vivo* have been shown to modulate cytokine expression of lymphocytes and intestinal tissue (Milo et al., 2002; Cavaglieri et al., 2003; Pié et al., 2007). Newly weaned piglets fed a diet supplemented with beet pulp (5%), inulin (0.75%), and lactulose (0.1%), which resulted in increased SCFA concentrations, had elevated IL-6 expression in colon, but not ileum compared to piglets fed a control with no supplemental
fermentable carbohydrate inclusion (Pié et al., 2007). On the contrary, Tedelind et al. (2007) demonstrated *in vitro* that acetate, propionate, and butyrate independently-dosed into medium were able to suppress IL-6 and TNF-α expression in an inflamed colonic cell culture. In the current study, infected chicks receiving the 1% GGMO-AX treatment had the lowest cecal acetate, butyrate, and total SCFA concentrations, yet duodenal mRNA expression of IL-6 and IL-12β were elevated compared with chicks receiving other dietary treatments.

The benefits of fermentable fiber to digestive and host health are well accepted. Limited research has been conducted on the ability of fermentable fibers to positively improve intestinal health indices during an *E. acervulina* challenge. In this study, chicks infected with *E. acervulina* had decreased growth performance and increased fermentation and immune responses compared to uninfected chicks. Dietary supplementation with GGMO-AX decreased feed efficiency of infected chicks, but improved select cecal health indices. Supplementing 4% GGMO-AX consistently resulted in the greatest fold change in pro-inflammatory cytokine expression, while inhibiting anti-inflammatory cytokine expression, which indicates a more robust innate immune response. Despite decreasing growth performance, dietary supplementation with GGMO-AX improved the innate intestinal immune response to an acute *E. acervulina* infection.

**LITERATURE CITED**


Table 6.1. Ingredient composition of diets supplemented with a galactoglucomannan oligosaccharide-arabinoxylan (GGMO-AX) complex and fed to chicks.

<table>
<thead>
<tr>
<th>Ingredient</th>
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<th>2.0%</th>
<th>4.0%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn</td>
<td>456.9</td>
<td>456.9</td>
<td>456.9</td>
<td>456.9</td>
</tr>
<tr>
<td>Soybean meal</td>
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<td>397.1</td>
<td>397.1</td>
<td>397.1</td>
</tr>
<tr>
<td>Soybean oil</td>
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<td>50.0</td>
<td>50.0</td>
<td>50.0</td>
</tr>
<tr>
<td>Limestone</td>
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<td>12.0</td>
<td>12.0</td>
<td>12.0</td>
</tr>
<tr>
<td>Dicalcium phosphate</td>
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<td>20.2</td>
<td>20.2</td>
<td>20.2</td>
</tr>
<tr>
<td>Salt</td>
<td>4.0</td>
<td>4.0</td>
<td>4.0</td>
<td>4.0</td>
</tr>
<tr>
<td>Vitamin mix</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Mineral mix</td>
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<td>1.5</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>DL-Met</td>
<td>1.9</td>
<td>1.9</td>
<td>1.9</td>
<td>1.9</td>
</tr>
<tr>
<td>Choline</td>
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<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Dextrose</td>
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<td>13.4</td>
<td>13.4</td>
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<td>Solka floc</td>
<td>40.0</td>
<td>30.0</td>
<td>20.0</td>
<td>0.0</td>
</tr>
<tr>
<td>GGMO-AX</td>
<td>0.0</td>
<td>10.0</td>
<td>20.0</td>
<td>40.0</td>
</tr>
</tbody>
</table>

Calculated Analysis:

<table>
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<th>2.0%</th>
<th>4.0%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude protein, %</td>
<td>23.25</td>
<td>23.25</td>
<td>23.25</td>
<td>23.25</td>
</tr>
<tr>
<td>MEh, kcal/g</td>
<td>3.07</td>
<td>3.07</td>
<td>3.07</td>
<td>3.07</td>
</tr>
<tr>
<td>Calcium, %</td>
<td>1.02</td>
<td>1.02</td>
<td>1.02</td>
<td>1.02</td>
</tr>
<tr>
<td>Non-phytate phosphorus, %</td>
<td>0.50</td>
<td>0.50</td>
<td>0.50</td>
<td>0.50</td>
</tr>
</tbody>
</table>

1 Corn was ground through the following hammer mill screen sizes: 1.59 and 9.52 mm. This provided an average particle size of 557 and 1,387 μm, respectively.

2 Provided per kilogram of diet: retinyl acetate, 4,400 IU; cholecalciferol, 25 μg; DL-α-tocopheryl acetate, 11 IU; vitamin B12, 0.01 mg; riboflavin, 4.41 mg; D-Ca-pantothenate, 10 mg; niacin, 22 mg; menadione sodium bisulfite, 2.33 mg.

3 Provided as milligrams per kilogram of diet: Mn, 75 from MnO; Fe, 75 from FeSO4•7H2O; Zn, 75 from ZnO; Cu, 5 from CuSO4•5H2O; I, 0.75 from ethylene diamine dihydroiodide; Se, 0.1 from Na2SeO3.

4 Purified cellulose, International Fiber Corp., North Tonawanda, NY.
Table 6.2. Body weight gain (g), feed intake (g/chick), and gain:feed (g/kg) of broiler chicks fed a diet (Dt) supplemented with a galactoglucomannan oligosaccharide-arabinoxylan (GGMO-AX) complex and infected (Inf.) with *Eimeria acervulina* or uninfected on select days (d) post-inoculation (PI).

<table>
<thead>
<tr>
<th>Infection status</th>
<th>Uninfected</th>
<th><em>E. acervulina</em></th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>GGMO-AX concentration</td>
<td>0% 1% 2% 4%</td>
<td>0% 1% 2% 4%</td>
<td>PSEM Inf. Diet Inf x Lin Dt Inf x Quad Dt</td>
</tr>
<tr>
<td>Weight gain, g/chick</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PI d 0-3</td>
<td>69 79 80 78</td>
<td>83 70 80 86</td>
<td>3.7</td>
</tr>
<tr>
<td>PI d 3-7</td>
<td>157 163 164 157</td>
<td>68 54 45 46</td>
<td>7.8</td>
</tr>
<tr>
<td>PI d 0-7</td>
<td>226 242 244 235</td>
<td>151 136 126 131</td>
<td>9.3</td>
</tr>
<tr>
<td>Feed intake, g/chick</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PI d 0-3</td>
<td>110 109 116 105</td>
<td>113 106 119 123</td>
<td>6.2</td>
</tr>
<tr>
<td>PI d 3-7</td>
<td>205 203 205 192</td>
<td>142 124 125 136</td>
<td>14.2</td>
</tr>
<tr>
<td>PI d 0-7</td>
<td>315 312 321 297</td>
<td>256 230 244 262</td>
<td>18.1</td>
</tr>
<tr>
<td>Gain:feed, g/kg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PI d 0-3</td>
<td>639 726 695 741</td>
<td>736 666 676 694</td>
<td>35.0</td>
</tr>
<tr>
<td>PI d 3-7</td>
<td>768 804 801 819</td>
<td>480 420 336 317</td>
<td>35.5</td>
</tr>
<tr>
<td>PI d 0-7</td>
<td>723 777 763 791</td>
<td>593 568 498 490</td>
<td>31.5</td>
</tr>
</tbody>
</table>

1 Inf x Lin Dt = Interaction of infection by linear response to diet.

2 Inf x Quad Dt = Interaction of infection by quadratic response to diet.
Table 6.3. Short-chain fatty acid (SCFA) and branched-chain fatty acid (BCFA) concentrations (µmol/g) in ceca of commercial broiler chicks fed a diet (Dt) supplemented with a galactoglucomannan oligosaccharide-arabinoxylan (GGMO-AX) complex and infected (Inf.) with *Eimeria acervulina* or uninfected.

<table>
<thead>
<tr>
<th>Infection status</th>
<th>Uninfected</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th>E. acervulina</th>
<th></th>
<th></th>
<th></th>
<th>P-value</th>
<th>Inf x Lin Dt¹</th>
<th>Inf x Quad Dt²</th>
</tr>
</thead>
<tbody>
<tr>
<td>GGMO-AX concentration</td>
<td>0%</td>
<td>1%</td>
<td>2%</td>
<td>4%</td>
<td>0%</td>
<td>1%</td>
<td>2%</td>
<td>4%</td>
<td>PSEM</td>
<td>Inf.</td>
<td>Diet</td>
<td>Lin Dt¹</td>
</tr>
<tr>
<td>SCFA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetate</td>
<td>397.8</td>
<td>292.6</td>
<td>344.8</td>
<td>285.1</td>
<td>360.0</td>
<td>306.8</td>
<td>338.5</td>
<td>349.1</td>
<td>34.13</td>
<td>0.73</td>
<td>0.15</td>
<td>0.19</td>
</tr>
<tr>
<td>Propionate</td>
<td>16.3</td>
<td>18.8</td>
<td>6.9</td>
<td>7.8</td>
<td>22.8</td>
<td>19.4</td>
<td>16.7</td>
<td>17.2</td>
<td>2.26</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>0.25</td>
</tr>
<tr>
<td>Butyrate</td>
<td>69.8</td>
<td>52.9</td>
<td>62.6</td>
<td>47.3</td>
<td>77.0</td>
<td>66.3</td>
<td>75.5</td>
<td>71.0</td>
<td>8.25</td>
<td>0.02</td>
<td>0.26</td>
<td>0.35</td>
</tr>
<tr>
<td>Total SCFA</td>
<td>483.9</td>
<td>364.2</td>
<td>414.3</td>
<td>340.1</td>
<td>459.9</td>
<td>392.5</td>
<td>430.7</td>
<td>437.2</td>
<td>42.13</td>
<td>0.34</td>
<td>0.15</td>
<td>0.20</td>
</tr>
<tr>
<td>BCFA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isobutyrate</td>
<td>4.8</td>
<td>7.8</td>
<td>4.1</td>
<td>5.6</td>
<td>5.1</td>
<td>5.5</td>
<td>5.1</td>
<td>3.3</td>
<td>0.86</td>
<td>0.19</td>
<td>0.06</td>
<td>0.35</td>
</tr>
<tr>
<td>Isovalerate</td>
<td>2.0</td>
<td>4.1</td>
<td>1.7</td>
<td>3.6</td>
<td>3.1</td>
<td>2.2</td>
<td>3.0</td>
<td>2.3</td>
<td>0.86</td>
<td>0.72</td>
<td>0.77</td>
<td>0.39</td>
</tr>
<tr>
<td>Valerate</td>
<td>1.9</td>
<td>2.9</td>
<td>2.3</td>
<td>2.7</td>
<td>4.9</td>
<td>1.8</td>
<td>3.1</td>
<td>2.3</td>
<td>0.94</td>
<td>0.40</td>
<td>0.71</td>
<td>0.23</td>
</tr>
<tr>
<td>Total BCFA</td>
<td>8.7</td>
<td>14.9</td>
<td>8.1</td>
<td>11.9</td>
<td>13.0</td>
<td>9.5</td>
<td>11.1</td>
<td>7.9</td>
<td>2.27</td>
<td>0.77</td>
<td>0.65</td>
<td>0.24</td>
</tr>
</tbody>
</table>

¹ Inf x Lin Dt = Interaction of infection by linear response to diet.

² Inf x Quad Dt = Interaction of infection by quadratic response to diet.
Table 6.4. Cecal microbial populations (log$_{10}$ CFU/g of cecal content DM) in commercial broiler chicks fed a diet (Dt) supplemented with a galactoglucomannan oligosaccharide-arabinoxylan (GGMO-AX) complex and infected (Inf.) with *Eimeria acervulina* or uninfected.

<table>
<thead>
<tr>
<th>Infection status</th>
<th>GGMO-AX concentration</th>
<th>Uninfected</th>
<th>E. acervulina</th>
<th>P-value</th>
<th>Inf x Linear Dt$^1$</th>
<th>Inf x Quad Dt$^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0% 1% 2% 4%</td>
<td></td>
<td></td>
<td>PSEM</td>
<td>Inf.</td>
<td>Diet</td>
</tr>
<tr>
<td><em>Bifidobacterium</em> spp.</td>
<td>8.8 8.2 9.6 9.1</td>
<td>8.6 8.5 9.8 9.1</td>
<td>0.36</td>
<td>0.79</td>
<td>&lt;0.01</td>
<td>0.89</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>12.8 13.0 12.9 13.1</td>
<td>13.9 13.2 13.5 13.1</td>
<td>0.25</td>
<td>0.01</td>
<td>0.80</td>
<td>0.08</td>
</tr>
<tr>
<td><em>Lactobacillus</em> spp.</td>
<td>11.8 11.8 11.8 11.9</td>
<td>12.0 11.9 12.1 12.0</td>
<td>0.08</td>
<td>&lt;0.01</td>
<td>0.28</td>
<td>0.77</td>
</tr>
</tbody>
</table>

$^1$ Inf x Lin Dt = Interaction of infection by linear response to diet.

$^2$ Inf x Quad Dt = Interaction of infection by quadratic response to diet.
Figure 6.1. Cecal pH in commercial broiler chicks fed a diet (Dt) supplemented with a galactoglucomannan oligosaccharide-arabinoxylan (GGMO-AX) complex and infected (Inf.) with (black bars) *Eimeria acervulina* or uninfected (white bars).

![Bar chart showing cecal pH in commercial broiler chicks fed a diet supplemented with a galactoglucomannan oligosaccharide-arabinoxylan complex and infected with *Eimeria acervulina*.](chart)

<table>
<thead>
<tr>
<th>GGMO-AX concentration</th>
<th>Cecal pH</th>
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<td>0%</td>
<td>5.0</td>
</tr>
<tr>
<td>1%</td>
<td>5.5</td>
</tr>
<tr>
<td>2%</td>
<td>6.0</td>
</tr>
<tr>
<td>4%</td>
<td>6.5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>P-value</th>
<th>Inf. x Diet</th>
<th>Inf. x Lin Dt</th>
<th>Inf. x Quad Dt</th>
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</thead>
<tbody>
<tr>
<td>0.01</td>
<td>&lt;0.01</td>
<td>0.72</td>
<td>0.23</td>
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</table>

$^1$Inf x Lin Dt = Interaction of infection by linear response to diet.

$^2$Inf x Quad Dt = Interaction of infection by quadratic response to diet.
Figure 6.2. Duodenal cytokine expression (fold changes relative to uninfected control chicks) of interferon-γ (IFN-γ), interleukin (IL)-1β, IL-6, IL-15, IL-12β in commercial broiler chicks fed a diet (Dt) supplemented with a galactoglucomannan oligosaccharide/arabinoxylan (GGMO-AX) complex and infected (Inf.) with (black bars) *Eimeria acervulina* or uninfected (white bars).

<table>
<thead>
<tr>
<th></th>
<th>IFN-γ</th>
<th>IL-1β</th>
<th>IL-6</th>
<th>IL-15</th>
</tr>
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<tbody>
<tr>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diet</td>
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<td>0.17</td>
<td>0.02</td>
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<td>0.53</td>
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¹Inf x Lin Dt = Interaction of infection by linear response to diet
²Inf x Quad Dt = Interaction of infection by quadratic response to diet
CHAPTER 7: INGESTION OF A NOVEL GALACTOGLUCOMANNAN OLIGOSACCHARIDE-ARBINOXYLAN (GGMO-AX) COMPLEX AFFECTED GROWTH PERFORMANCE AND FERMENTATIVE AND IMMUNOLOGICAL CHARACTERISTICS OF BROILER CHICKS CHALLENGED WITH SALMONELLA TYPHIMURIUM

ABSTRACT: Fermentable carbohydrates may enhance the ability of the gastrointestinal tract to defend against a pathogenic infection. We hypothesized that a mannose-rich galactoglucomannan oligosaccharide-arabinoxylan (GGMO-AX) complex would positively impact immune status and prevent colonization and shedding in Salmonella typhimurium (ST)-infected chicks. Using a completely randomized design, one day old commercial broiler chicks (n=240; 4 replications/treatment; 5 chicks/replication) were assigned to 1 of 6 dietary treatments differing in concentration of GGMO-AX (0, 1, 2, or 4%) or containing 2% Safmannan (Saf) or 2% short-chain fructooligosaccharides (scFOS). Cellulose was used to make the diets iso-total dietary fiber (TDF). On d 10 post-hatch, an equal number of chicks on each diet were inoculated with either phosphate buffered saline (sham control) or ST (1x10^8 CFU). On d 1, 5, and 10 PI, excreta were collected from each pen for analysis of ST shedding. All birds were euthanized on d 10 post-inoculation (PI) for collection of intestinal contents and select tissues. Overall, body weight gain and feed intake of chicks was greater (P < 0.05) in infected chicks PI, except for weight gain on d 0-3 PI. Diet affected (P < 0.05) weight gain and feed intake from d 0-10 PI. Gain:feed was affected (P < 0.05) by diet, with Saf-fed chicks having the greatest G:F. The GGMO-AX substrate demonstrated prebiotic-like effects as indicated by increased cecal short-chain fatty acid (SCFA) concentrations, particularly butyrate, decreased cecal pH, and increased populations of Lactobacillus spp. and Bifidobacteria spp. as dietary GGMO-AX concentration
increased. Excreta ST populations on d 5 and 10 PI, and ileal and cecal ST populations, tended to be affected (P < 0.10) by diet. Messenger RNA expression of IFN-\(\gamma\) in the cecal tonsils was the only cytokine independently affected by infection and diet (P < 0.01). An interaction of infection and diet, and an infection by linear response to GGMO-AX inclusion, affected (P ≤ 0.03) IFN-\(\gamma\), IL-1\(\beta\), and IL-10 expression. Chicks fed 2 and 4% GGMO-AX had similar expression of IFN-\(\gamma\) and IL-1\(\beta\), regardless of infection, suggesting that virulence of ST was suppressed. Dietary supplementation with GGMO-AX resulted in prebiotic-like effects, but did not limit ST intestinal colonization or shedding, but possibly decreased the virulence of the ST within the digestive tract.

INTRODUCTION

_Salmonella_ spp. are common intestinal pathogens for most animals and one of the major causes of food-borne illness for humans in the United States with an estimated one million reported cases per year (Scallan et al., 2011). _Salmonella typhimurium_ (ST) and _Salmonella enteriditis_ (SE) infections result in gastroenteritis, diarrhea, and fever (Ohl and Miller, 2001). _Salmonella_ spp. utilize appendages, fimbriae, to attach to the mucosal surface of the intestine and colonize the digestive tract (Ofek et al., 1977). In particular, type-1 fimbriae recognize and adhere to mainly mannose and mannan moieties that are either free in the intestinal tract or bound to proteins (glycoproteins) or lipids (glycolipids; Mirelman and Ofek, 1986; Neeser et al., 1986).

Feeding D-mannose and mannanoligosaccharides (MOS) may prevent colonization of pathogenic bacteria in the digestive tract by competitive inhibition. Dietary mannose acts as a receptor analog by being structurally similar to glycoprotein receptors for type-1 fimbriae and, thus, is able to prevent attachment to the mucosal layer (Mirelman et al., 1980; Firon et al., 1984;
Studies evaluating MOS have been shown to decrease intestinal populations of type-1 fimbriae bacteria (Spring et al., 2000; Fernandez et al., 2002; Yang et al., 2008; Baurhoo et al., 2009). In addition, it may be possible to enhance the activity of the gut-associated lymphoid tissues (GALT) through dietary methods, particularly fermentable carbohydrate feeding. Research has shown a positive correlation between the effect of fermentable carbohydrates and improved gut health (Anderson et al., 2009; Roberfroid et al., 2010). Fiber fermentation in the large bowel may benefit the GALT through several potential mechanisms including modulation of the GALT and enhanced intestinal defenses such as increased mucin thickness, modulation of epithelial inflammation by SCFA, and quantity and type of microbiota limited bacterial translocation (Schley and Field, 2002; Cavaglieri et al., 2003; Tedelind et al., 2007; Stecher and Hardt, 2008; Hedemann et al., 2009; Ito et al. 2009).

A mannose-rich galactoglucamannan oligosaccharide-arabinoxylan (GGMO-AX) complex has been shown to be well fermented when evaluated using in vitro and in vivo methodologies (Faber et al., 2011a; Faber et al., 2011b). In addition, in chicks infected with *Eimeria acervulina*, a strain of coccidiosis, GGMO-AX supplementation resulted in increased fold changes in duodenal cytokine expression, an indication of an increased innate immune response (Faber et al., 2011c). Based on these data, GGMO-AX may be able to prevent ST colonization by preventing intestinal attachment and positively modulating the intestinal environment in an avian model.

The objective of this study was to evaluate the ability of GGMO-AX to prevent or limit ST colonization and improve intestinal immune response in broiler chicks challenged with an acute ST infection.
MATERIALS AND METHODS

All animal care procedures were approved by the University of Illinois Institutional Animal Care and Use Committee and Institutional Biosafety Committee before initiation of the experiment.

Galactoglucomannan Oligosaccharide Substrate

Production of the GGMO-AX substrate involves wood chips, water, and pressure, but does not use strong acids or bases unlike other fiberboard production processes. This results in an ingredient potentially safe for consumption by animals. During hydrolysis, hemicelluloses are depolymerized through hydronium ions from water and other compounds such as uronic, acetic, and phenolic acids (Garrote et al., 1999). The release of pressure on the "wood chip digester" results in destruction primarily of cellulose, hemicelluloses, and lignin that releases soluble sugars into the surrounding water, along with polyphenolic compounds from lignin. The resulting water solution contains increased concentrations of sugars (3 to 4%), a concentration unsafe for disposal into wastewater streams. Thus, the sugar solution is removed from the wood chips and further condensed into a syrup with a final sugar concentration of 30 to 54% (Michalka, 2007). Sugars are mostly in the form of oligosaccharides rather than free sugars.

To obtain a dry product, the GGMO-AX syrup is spray-dried (Valentine Chemicals, Lockport LA) with an inlet temperature approximately 155 °C and exit temperature of 102 °C; the starting substrate is adjusted to 53% solids before drying. Spray-drying allows the substrate to be mixed in a mash diet matrix appropriate for young chicks.

Chicks and Husbandry

Two-hundred forty, 1-d-old male Ross x Ross broiler chicks were used in this experiment. Chicks were housed in thermostatically controlled starter batteries with raised wire
floors in an environmentally controlled room with continuous lighting within separate high efficiency particle air–filtered units of the containment area. At hatch, chicks were weighed, wing-banded, and assigned to treatment groups so that the initial weight was similar among treatment groups. Four replicates of 5 chicks were assigned to each treatment in the experiment. Chicks were randomly assigned to 1 of 2 infection treatments (sham-inoculated or ST-inoculated) and 1 of 6 soy protein isolate-based semi-purified dietary treatments differing in concentration of GGMO-AX (0, 1, 2, or 4%), which replaced cellulose (Solka-Floc; International Fiber Corporation, North Tonawanda, NY) in the diet, or diets containing 2% Safmannan (Saf; Lesaffre Yeast Corp., Milwaukee, WI) plus 2% cellulose or 2% short-chain fructooligosaccharides (scFOS; GTC Nutrition, Golden, CO) plus 2% cellulose. All diets were formulated to meet or exceed NRC (1994) recommendations (Table 7.1). Feed and fresh water were offered to the chicks ad libitum. Chicks and feeders were weighed on chick age d 1 and 10-20 to determine weight gain and feed intake.

**Preparation of Bacterial Inoculum and Inoculation**

A primary poultry isolate of ST (ST-10, NVSL 95-1776; Southern Plains Agricultural Research Center, USDA, College Station, TX), resistant to novobiocin and nalidixic acid, was selected. A single bacterial colony was isolated on a trypticase soy agar plate and then cultured overnight in trypticase soy broth at 37°C. On day of inoculation, bacterial inoculum was diluted to 2 x 10⁸ CFU/ml using sterile phosphate-buffered saline. Final viable cell concentration was confirmed by serially diluting a sample of inoculum and plating on a trypticase soy agar plate. Plates were incubated overnight at 37°C and concentrations expressed as CFU/ml.
Cultured ST was diluted with phosphate buffered saline (PBS) immediately prior to inoculation. Chicks were administered an acute 0.5 ml oral dose of either $1 \times 10^8$ CFU ST /dose or PBS on d 10 of age using 1-cc syringes without needles.

**Sample Collection**

On d 0, 1, 5, and 10 post-inoculation (PI), excreta were collected from each pen. In the morning of the collection day, fresh paper was placed in the excreta collection pan located under each pen. Approximately 24 h later, excreta from each pen were collected and mixed and a sample was placed into a cryogenic vial and stored at -80°C until analysis. On d 20 of age (d 10 PI) chicks were euthanized by CO$_2$ inhalation. Chicks were immediately dissected and ileum and ceca were removed and contents pooled according to replicate. Ceca from all birds within replicate were weighed with cecal contents to determine total cecal weight. Cecal contents were then removed and all empty ceca were weighed to determine empty cecal weight. An aliquot of ileal and cecal digesta was immediately transferred to a sterile cryogenic vial (Nalgene, Rochsester, NY) and snap-frozen in liquid nitrogen. Additional aliquots of cecal digesta were taken for measurement of pH, DM, SCFA, and branched-chain fatty acids (BCFA). Ileum, cecal tonsil, and spleen tissue were collected from two random chicks per replicate and pooled. Tissues were rinsed with PBS (pH 7.2), minced with a scalpel, placed in RNAlater® solution, and stored at -80°C pending analysis.

**Chemical Analyses**

Fresh ileal and cecal samples were analyzed for DM using AOAC (2006) methods. Cecal SCFA and BCFA concentrations were determined by gas chromatography according to Erwin et al. (1961) using a gas chromatograph (Hewlett-Packard 5890A series II, Palo Alto, CA) and a glass column (180 cm x 4 mm i.d.) packed with 10% SP-1200/1% H$_3$PO$_4$ on 80/100+ mesh
Chromosorb WAW (Supelco Inc., Bellefonte, PA). Nitrogen was the carrier with a flow rate of 75 mL·min⁻¹. Oven, detector, and injector temperatures were 125, 175, and 180°C, respectively.

Microbial Analyses

Excreta, ileal, and cecal microbial populations were analyzed using methods described by Middelbos et al. (2007) with minor adaptations. Briefly, excreta or digesta DNA was extracted from freshly collected samples that had been stored at −80°C until analysis, using the repeated bead beater method described by Yu and Morrison (2004) with a DNA extraction kit (QIAamp DNA Stool Mini Kit, Qiagen, Valencia, CA) according to the manufacturer’s instructions. A TissueLyser (Qiagen, Valencia, CA) with a 5 mm steel bead (3 min at 15 Hz) was used in place of the repeated bead beater. Extracted DNA was quantified using a spectrophotometer (NanoDrop ND-1000, Nano-Drop Technologies, Wilmington, DE). Quantitative PCR of the DNA was performed using specific primers for *Salmonella typhimurium* (Csordas et al., 2004), *Bifidobacterium* spp. (Matsuki et al., 2002), *Lactobacillus* spp. (Collier et al., 2003), *Escherichia coli* (Malinen et al., 2003), and *Clostridium perfringens* (Wang et al., 1994), whereas for the total population of bacteria, a universal primer set (341F/534R; Applied Biosystems, Foster City, CA) was used. Amplification was performed according to DePlancke et al. (2002). Briefly, a 10-µL final volume contained 5 µL of 2 x SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), 15 pmol of the forward and reverse primers for the bacterium of interest, and 5 ng of extracted excreta or digesta DNA. Standard curves were obtained by harvesting pure cultures of the bacterium of interest in the log growth phase in triplicate, followed by serial dilution. Bacterial DNA was extracted from each dilution using a DNA extraction kit (Qiagen) and amplified with the excreta or digesta DNA to create triplicate standard curves (ABI PRISM 7900HT Sequence Detection System, Applied Biosystems, Foster City, CA). Colony-forming
units in each dilution were determined by plating on specific agars; tryptic soy agar (*Salmonella typhimurium*), lactobacilli MRS (Difco, BD, Franklin Lakes, NJ) for lactobacilli, reinforced clostridial medium (bifidobacteria, *C. perfringens*), and Luria Bertani medium (*E. coli*). The calculated log CFU per milliliter of each serial dilution was plotted against the cycle threshold to create a linear equation to calculate CFU per gram of excreta, ileal digesta, or cecal digesta (DMB).

**Inflammatory Cytokine Analyses**

Total RNA was extracted from ileum, cecal tonsil, and spleen tissues that had been stored at -80°C until analysis using a DNA/RNA extraction kit (AllPrep DNA/RNA Mini Kit, Qiagen, Valencia, CA) according to the manufacturer’s instructions and the TissueLyser apparatus described above. Extracted RNA was quantified using a spectrophotometer (NanoDrop ND-1000, Nano-Drop Technologies, Wilmington, DE). Extracted RNA from ileal, cecal tonsil, and spleen tissues were subjected to a 7x gDNA Wipeout Buffer and then converted to complementary DNA (cDNA) using a QuantiTec® Reverse Transcription kit (Qiagen, Valencia, CA). Wipeout of gDNA and reverse transcription was performed in a thermocycler (model PTC-200, Biorad, Hercules, CA) with the program set for 2 min at 42°C, 2 min at 4°C, 15 min at 42°C, 3 min at 95 °C, and then cooled to 4 °C. The cDNA synthesized was stored at -20°C.

Quantitative real time PCR was performed using the Applied Biosystems (Foster, CA) TaqMan® Gene Expression Assay as previously described (Dilger and Johnson, 2010). The cDNA was amplified by PCR where a target cDNA (interferon-γ [IFN-γ], Y07922; interleukin [IL]-1β, Y15006; IL-12β, NM_213571; and IL-10, AJ621614) and reference cDNA (glyceraldehyde 3-phosphate dehydrogenase [GAPDH], K01458; Hong et al., 2006) were amplified using Taqman (Invitrogen, Carlsbad, CA), an oligonucleotide probe with a 5′
fluorescent reporter dye (6-FAM) and a 3′ non-fluorescent quencher dye (NFQ). Fluorescence was determined on an ABI PRISM 7900HT-sequence detection system (Applied Biosystems, Forest City, CA). To normalize gene expression, a parallel amplification of endogenous GAPDH was performed for each gene. Reactions with no reverse transcription and no template were included as negative controls. Data were analyzed using the comparative threshold cycle (Ct) method (Livak and Schmittgen, 2001) and results are expressed as fold-change relative to the control treatment.

**Statistical Analyses**

Data were analyzed by ANOVA with procedures appropriate for a completely randomized design. Data for continuous variables were analyzed by the MIXED procedure (SAS Inst., Cary, NC). The statistical model included the fixed main effects of infection and dietary treatment. Interactive effects were evaluated using linear polynomial contrasts designed for the non-equally spaced levels of supplemental GGMO-AX vs. infection status. Additionally, interactive effects between dietary treatment (scFOS or Safmannan vs. 2% GGMO-AX) and infection status (uninfected vs. infected) were evaluated. Least squares means were separated using least squares differences with a Tukey adjustment. Outlier data were removed from analysis after analyzing data using the UNIVARIATE procedure to produce a normal probability plot based on residual data and visual inspection of the raw data. Outlier data were defined as data points three or more standard deviations from the mean. Differences among treatment level least squares means with a probability of $P \leq 0.05$ were accepted as statistically significant, whereas mean differences with P-values ranging from 0.06 to 0.10 were accepted as trends.
RESULTS

Chick Growth and Performance

Weight gain of chicks was not affected by infection or diet on d 0-3 PI (Table 7.2). Weight gain of Safmannan-fed infected chicks was greater (P = 0.01) than for 2% GGMO-AX-fed infected chicks only. On d 3-7 and 0-10 PI, the effect of diet and infection was significant (P < 0.05), with Safmannan-fed chicks having the greatest weight gain, regardless of infection status. Diet affected (P < 0.01) weight gain on d 7-10 PI, with Safmannan-fed chicks having the greatest weight gain. In addition, an interaction between dietary treatment (scFOS or Saf vs. 2% GGMO-AX) and infection status was noted in chicks on d 7-10 PI. Infection affected (P < 0.05) feed intake during all time periods, while diet affected (P < 0.05) feed intake during all time periods except d 0-3. An interaction between infection status and diet (P = 0.04) was noted on d 7-10 PI. An interaction of diet (Safmannan and 2% GGMO-AX) and infection status was observed for all time periods. Gain to feed ratio was affected (P < 0.05) by diet during all time periods.

Fermentation Metabolites

Cecal pH was affected by infection (P = 0.02), with infected chicks having a generally lower cecal pH than uninfected chicks (Figure 7.1). Diet affected (P < 0.01) cecal pH, with 4% GGMO-AX resulting in the lowest pH. Full and empty ceca weights were affected (P < 0.01) by diet (Table 7.3). Cecal propionate concentrations (µmol/g) were independently affected (P < 0.05) by both infection and diet. In addition, acetate and propionate concentrations (µmol/g) were affected (P < 0.05) by the interaction of infection and diet. Total SCFA concentrations (µmol/g) were affected (P < 0.01) by diet. When SCFA concentrations were expressed on a per ceca basis, acetate concentrations were affected by infection, diet, and the interaction of infection
by diet ($P < 0.05$), while propionate concentrations ($\mu$mol/ceca) were affected ($P = 0.04$) by infection. Infection status did not affect BCFA concentrations and only diet affected ($P = 0.04$) valerate concentrations ($\mu$mol/g). When BCFA concentrations were expressed on a per ceca basis, isobutyrate concentrations were increased ($P = 0.01$) by infection.

**Ileal and Cecal Microbiota**

Ileal *Bifidobacterium* spp. and *E. coli* populations were not affected by infection or diet (Table 7.4). *Lactobacillus* spp. populations were affected by diet ($P = 0.01$) and the interaction of infection and diet ($P < 0.01$). In addition, *Lactobacillus* spp. populations were affected ($P < 0.01$) by the interaction of diet (scFOS and 2% GGMO-AX) and infection, with infected 2% GGMO-AX-fed chicks having the greatest population. *Clostridium perfringens* populations were affected by diet ($P < 0.01$), with 4% GGMO-AX-fed chicks having the lowest concentration. Total microbial populations were affected ($P < 0.01$) by infection and the interaction of scFOS and 2% GGMO-AX and infection.

Cecal *Bifidobacterium* spp. populations were affected by both diet and the interaction of infection and diet ($P < 0.05$). *Lactobacillus* spp. populations were affected by infection ($P < 0.01$) and diet ($P = 0.04$). *Escherichia coli* populations were affected ($P < 0.05$) by infection, diet, and interaction of infection by linear response to GGMO-AX supplementation (Lin GGMO-AX). Total microbial populations were affected ($P < 0.01$) by infection and the interaction of scFOS and 2% GGMO-AX and infection.

**Digesta and Excreta Salmonella typhimurium Populations**

Ileal digesta ST populations were affected ($P = 0.05$) by diet (Table 7.5). *Salmonella typhimurium* was detected in ileal digesta in 1 of 4 replicates of chicks fed diets containing 0, 1,
and 4% GGMO-AX, while remaining treatments had 2 of 4 replicates with ileal colonization by ST. Cecal ST populations were not affected by diet, and all replications were colonized by ST.

On d 1 PI, 4 of 4 replicates containing 0 and 2% GGMO-AX- and Safmannan-fed chicks were shedding ST, while 2 of 4 replicates containing 1 and 4% GGMO-AX-fed chicks were shedding ST (Table 7.5). On d 5 PI, diet tended (P = 0.06) to affect ST populations in the excreta of replicates shedding ST, with scFOS-fed chicks having the lowest ST population. Replicates containing 1% GGMO-AX and scFOS-fed chicks had 3 of 4 replicates shedding ST, while 4 of 4 replicates of chicks fed the remaining dietary treatments were shedding ST. On d 10 PI, diet tended (P = 0.08) to affect ST populations in the excreta of replicates, with scFOS-fed chicks having the lowest ST population in excreta. Replicates containing chicks fed 0 and 1% GGMO-AX had the fewest numbers of replicates (2 of 4) shedding ST, while Safmannan had the greatest (4 of 4).

**Intestinal Cytokine Expression**

Messenger RNA expression of all cytokines in the ileum and spleen were not affected by diet or infection (data not shown). Messenger RNA IFN-γ expression in the cecal tonsils was affected (P < 0.01) by infection, diet, and the interaction of diet and infection (Figure 7.2). In addition, an interaction of infection by linear response to GGMO-AX supplementation (P = 0.03) was observed for IFN-γ expression. An interaction between scFOS and 2% GGMO-AX in uninfected and infected chicks was observed, with infected scFOS-fed chicks having a greater fold change in IFN-γ expression compared to other treatments. An interaction of infection and diet (P ≤ 0.05) and an interaction of infection by linear response to GGMO-AX supplementation (P = 0.01) was observed for IL-1β and IL-10 expression. An interaction (P = 0.01) between diet (scFOS and 2% GGMO-AX) and infection affected IL-10 expression.
DISCUSSION

The intestinal and systemic health benefits of dietary fiber, particularly fermentable carbohydrates, in human and animal nutrition suggest fiber to be an important dietary ingredient. In addition, some oligosaccharides, particularly MOS, may directly prevent pathogenic bacteria from binding to epithelial cells in the small intestine and prevent colonization and proliferation in the intestinal tract (Spring et al., 2000; Grieshop et al., 2004; Gouveia et al., 2006; Baurhoo et al., 2009). It has been shown in unpublished in vitro data that the particular strain of ST utilized in the current study will bind to GGMO-AX. Therefore, we hypothesized that ingestion of a novel fermentable carbohydrate, GGMO-AX, could decrease intestinal colonization of ST and improve the immune response of growing chicks to an acute ST infection through fermentative changes and/or through a MOS-dependent mechanism in the distal gastrointestinal tract. Results of our studies suggest that while the GGMO-AX complex can be fermented by the young chick, ST colonization and shedding were not reduced, but GGMO-AX was able to modulate cytokine expression of cecal tonsils in infected chicks in response to an acute infection with ST.

A semi-purified diet was utilized to limit the amount of fermentable fiber from dietary ingredients other than the test substrates. Two positive control diets (scFOS and Saf) were used to help determine the possible mode of action of the GGMO-AX substrate in the chick. Short-chain FOS has been shown to improve intestinal health and limit ST colonization through fermentative effects such as lowered cecal pH and SCFA production (Bailey et al., 1991 Chambers et al., 1997). Safmannan, a MOS source derived from yeast-cell wall (YCW), also has shown to limit ST colonization through fermentative effects, but also through the oligosaccharide-binding mechanism in which ST binds to the MOS instead of intestinal epithelial cells (Spring et al., 2000; Fernandez et al., 2002; Yang et al., 2008; Baurhoo et al., 2009). The
GGMO-AX substrate is unique in that it elicits a prebiotic-like response in chicks, but also contains a high concentration of MOS (354, mg·g⁻¹ DMB) to which ST is capable of binding.

Unexpectedly, chick body weight gain and feed intake were greater for infected chicks. Before inoculation, chicks assigned to the infected treatment had a greater (P < 0.01) weight gain than chicks assigned to the uninfected treatment (9.7 vs. 8.7 g/chick, respectively; data not shown). Chicks were housed in separate rooms with minimal differences in room temperature and humidity. Despite this, it appears that a room effect occurred. It previously has been shown that ST infection either does not affect or decreases growth and feed intake depending on ST strain and dose (Fasina et al., 2010; Hegazy and Adachi, 2000; Nakamura et al., 2002; Chalghoumi et al., 2009; Vandeplas et al., 2009; Marcq et al., 2011). The virulence of this ST strain obviously was not great enough to elicit a decrease in chick performance.

For most time intervals PI, Saf resulted in the greatest G:F ratio of all dietary treatments. The beneficial effects of MOS extracted from YCW on animal performance has been well established in poultry and swine (Hooge, 2004; Miguel et al., 2004). It is hypothesized that the combined effect of substrate fermentation and bacterial binding to the MOS results in an antibiotic-like effect, thus enhancing growth. However, in the current study, GGMO-AX, a fermentable substrate with a greater concentration of MOS, did not improve growth and performance to the same extent as Saf. This suggests that another component of Saf (e.g. beta-glucans, nucleotides, live yeast cells) maybe eliciting the beneficial effect.

Cecal total SCFA concentrations (umol/ceca) were numerically greater for infected (76.9) compared with uninfected chicks (52.1). This, in turn, caused cecal pH to be lower for infected chicks, as pH followed the same trend as total SCFA concentrations. A lower cecal pH in infected chicks was surprising since ST prefer a more alkaline environment to grow and colonize.
In addition, ST infection has been shown to increase cecal pH (Bohnhoff et al., 1964). In an acidic environment, the pH balance of the ST cell is disrupted due to the lower pH altering the dissociation of SCFA. The undissociated lipophilic form of the acid is capable of penetrating the bacterial wall (Meynell, 1963). In the current study, there was no apparent correlation between pH and cecal ST concentration, replicates colonized, or shedding. Corrier et al. (1995) did not reduce cecal ST populations in chicks until a cecal pH lower than 5 was achieved by feeding 10% lactose. This indicates that the decrease in cecal pH was not sufficient to achieve a significant reduction in cecal ST populations.

Cecal butyrate and total SCFA concentrations (µmol/ceca) were well correlated (P < 0.01) with empty ceca weight (r = 0.46 and 0.48, respectively) and full ceca weight (r = 0.66 and 0.72, respectively). For GGMO-AX-fed chicks, cecal weights increased as the concentration of dietary GGMO-AX increased. Short-chain fatty acids are the main energy source for intestinal epithelial cells and stimulate cell growth, which, in turn, increases intestinal weight (Sakata and Yajima, 1984; Kripke et al., 1989; Frankel et al., 1994). This effect of fermentable carbohydrate inclusion also is apparent in rats as regards cecum weight (Weaver et al., 2010; Knapp, 2011).

In the current study, butyrate and cecal \textit{Bifidobacterium} spp. and \textit{Lactobacillus} spp. populations were increased as dietary GGMO-AX concentration increased, suggesting a prebiotic-effect. In uninfected 4% GGMO-AX-fed chicks, \textit{Bifidobacterium} spp. concentrations were similar to those for chicks fed scFOS, a proven prebiotic. In infected chicks fed GGMO-AX, \textit{Bifidobacterium} spp. populations were greater compared to those of scFOS-fed chicks.

Uninfected chicks fed 0 and 1% GGMO-AX or scFOS had the lowest ileal and cecal \textit{E. coli} concentrations; however, infected chicks fed the same diets exhibited the largest difference relative to uninfected chicks in \textit{E. coli} population (≥ 0.4 CFU/g). This response due to infection
was not observed for other dietary treatments. Select strains of *E. coli* are considered pathogenic, so a reduction in overall *E. coli* population is considered beneficial. Overall, ST infection resulted in the proliferation of cecal *E. coli*, possibly due to both bacteria thriving in similar conditions. The greatest fold changes in IFN-γ and IL-1β expression was noted for chicks fed 0 and 1% GGMO-AX and scFOS, suggesting that inflammation promoted growth of *E. coli*. In a mouse colitis model, ST colonization was greatly enhanced due to intestinal inflammation reducing the commensal bacterial population, allowing overgrowth of ST (Stecher et al., 2007).

Diet tended to affect ileal and cecal ST populations. Surprisingly, the 0% GGMO-AX (4% cellulose) diet resulted in the fewest replicates colonized and one of the lowest ileal and cecal ST populations on d 10 PI despite having the highest cecal pH and the lowest total SCFA concentrations. The lack of fermentable substrate and the lower amount of cecal contents may have limited colonization due to lack of an energy source, a limited cecal pool, and (or) increased transit rate. Cao et al. (2003) observed that chicks fed a 3.5% cellulose diet experienced an increased transit rate and decreased total microbial concentration compared to 0% cellulose-fed chicks. Chicks fed 1% GGMO-AX (3% cellulose) had the greatest ileal and cecal ST concentrations, suggesting that the cellulose effect is not consistent.

A reduction in ST shedding is desirable since it lessens the spread of ST through feces to other animals and is important for food safety. In addition, bacterial shedding by chicks has been shown to strongly correlate (r = 0.84) with intestinal colonization with other species of bacteria such as *Clostridium jejuni* (Achen et al., 1998). On d 1 PI, 4% GGMO-AX resulted in the lowest ST concentration in excreta, and had the fewest replicates shedding, suggesting a lower incidence of initial cecal colonization. By d 5 PI, this effect was ameliorated and these chicks
had the greatest ST concentration among dietary treatments. It appears that the 4% GGMO-AX treatment is able to limit the initial colonization of ST, but unable to prevent later proliferation. On d 5 PI, scFOS-fed chicks experienced the lowest ST concentration in excreta of replicates shedding ST. Short-chain FOS continued to prevent ST colonization as indicated by the low d 10 PI ileal digesta and excreta concentrations, and only 2 of 4 replicates shedding. On d 10 PI, Saf-fed chicks had the highest ST concentration the excreta, with all 4 replicates shedding ST, whereas all other dietary treatments had 3 or fewer replicates shedding, suggesting that YCW was unable to limit ST colonization or decrease shedding.

The detection of salmonella in epithelial cells by macrophages and phagocytes initiates a cell-mediated response resulting in release of cytokines (e.g., IFN-γ, IL-6, and IL-1β) from macrophages (Trebichavský, 1999). Additional cytokines, such as tumor necrosis factor (TNF)-α, IFN-γ, and IL-12β, initiate intestinal inflammation and recruitment of macrophages to destroy the pathogen and suppress ST growth (Mastroeni et al., 2000). Clearance of a ST infection involves IFN-γ-mediated Th1 responses that increase activity of lymphocytes and macrophages, which then destroy and clear ST from the body (Mastroeni and Menager, 2003; Withanage et al., 2005).

As the chick ages, immune responses to ST infection are reduced as illustrated with IL-1β expression in chicks where expression was drastically reduced in 7-d-old chicks compared to 1-d-old chicks (Withanage et al., 2004; 2005). In the current study, chicks were 20-d-old at the time of tissue collection, which likely resulted in the lack of fold changes in cytokine expression, particularly by the ileum and spleen in the current study. One objective of the current study was to evaluate the fermentative effect on prevention of growth and colonization of ST in the lower intestinal tract. It has been reported that not until d 10 of age does the cecal microbial population
begin to stabilize and the cecal tonsils develop (Jeurissen et al., 2005; Amit-Romach et al., 2004). Therefore, inoculation was withheld until d 10 of age.

Cecal tonsils are the major lymphoid tissue of the ceca and represent the largest collection of GALT in the chicken (Yun et al., 2000). Results from several studies have demonstrated that cecal tonsils play an important role in the immune response to ST infections as indicated by increased expression of pro-inflammatory and anti-inflammatory cytokines (Beal et al., 2004; Withanage et al., 2004; Haghighi et al., 2008). Chicks fed 0 and 1% GGMO-AX and scFOS had the greatest expression of IFN-γ, IL-1β, and IL-10 which related to the lowest ST concentration in excreta on d 10 PI, but not to the cecal population. There was no relationship of cytokine expression and cecal ST populations, suggesting that the virulence of ST, but not the concentration, resulted in the inflammatory response.

The interaction of infection by linear response to GGMO-AX on cecal tonsil pro-inflammatory (IFN-γ and IL-1β) and anti-inflammatory (IL-10) cytokine expression suggests that GGMO-AX fed to unchallenged chicks stimulates the intestinal immune system, but also is capable of suppressing inflammation associated with ST. Unlike scFOS, the lack of statistical difference between the Saf and 2% GGMO-AX treatment indicates that they utilized a similar biological mechanism to suppress inflammation associated with ST, perhaps related to the MOS found in the complex. These results are contradictory to those of Janardhana et al. (2009) where unchallenged day-old chicks fed scFOS and MOS for 25 d exhibited no difference in mRNA expression of IFN-γ or IL-10 in cecal tonsils.

Cecal tonsil fold changes in expression of IFN-γ, IL-1β, and IL-10 in infected 2 and 4% GGMO-AX-fed chicks were similar between the two diets, suggesting a threshold for GGMO-AX supplementation and its ability to suppress cytokine expression. In addition, fold changes in
expression of IFN-γ and IL-10 were similar between uninfected 2 and 4% GGMO-AX-fed chicks, again suggesting a threshold for the effectiveness of dietary GGMO-AX to enhance or suppress the immune system compared to 0% GGMO-AX-fed chicks.

The increased expression of innate immune response cytokines in uninfected chicks suggest that GGMO-AX supplementation stimulates the GALT of the lower intestinal tract, which would allow a more rapid response to a pathogen. When the intestinal tract is compromised by a pathogen, the GALT is ready to respond and lessen the detrimental effects. This theory is demonstrated in uninfected and infected 2 and 4% GGMO-AX-fed chicks and the similar expression of IFN-γ and IL-1β in 2% GGMO-AX-fed chicks.

The decrease in IL-10 expression of 2 and 4% GGMO-AX-fed chicks suggests that the intestinal immune system was suppressing anti-inflammatory cytokines and potentially playing a role in the hypothesized decreased virulence. Studies with mouse and rat models have indicated that during a bacterial infection, both pro- and anti-inflammatory cytokine expression is elevated, as was the case in the current study (Pie et al., 1996; VanCott et al., 1996). In mice, IL-10 did not suppress IFN-γ expression during an ST infection, and elevated IL-10 expression was related to increased bacterial multiplication and decreases in host defenses (Arai et al., 1995; Pie et al., 1996). Therefore, a suppression of IL-10 expression due to infection in infected chicks fed 2 and 4% GGMO-AX may have improved the intestinal defenses and decreased the virulence of ST.

The effects of fermentable fibers on microbial colonization, populations, and concentrations particularly of pathogenic bacteria, have been well studied. In this study, increasing dietary GGMO-AX resulted in a prebiotic response in chicks, regardless of infection status. Dietary GGMO-AX supplementation did not decrease ileal and cecal ST colonization to the same extent as scFOS, an established prebiotic. However, greater dietary GGMO-AX
concentrations (2 and 4%) and Saf were able to limit the immune response in the cecal tonsils of infected chicks as indicated by similar cytokine expression as uninfected chicks, suggesting a MOS-dependent mechanism. Overall, dietary concentrations of at least 2% GGMO-AX are able to elicit a prebiotic response and limit intestinal immune responses to an acute ST infection in chicks.

**LITERATURE CITED**


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systemic antibody responses by T helper cell subsets, macrophages, and derived
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Table 7.1. Ingredient composition of diets supplemented with galactoglucomannan oligosaccharide-arabinoxylan (GGMO-AX) complex, short-chain fructooligosaccharides (scFOS), or Safmannan (Saf) and fed to chicks infected (Inf.) with *Salmonella typhimurium* or uninfected.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>0.0%</th>
<th>1.0%</th>
<th>2.0%</th>
<th>4.0%</th>
<th>scFOS</th>
<th>Saf</th>
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<td>Soy protein isolate$^1$</td>
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<td>272.00</td>
<td>272.00</td>
<td>272.00</td>
<td>272.00</td>
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<td>294.88</td>
<td>294.88</td>
<td>294.88</td>
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<td>50.00</td>
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<tr>
<td>NaHCO$_3$</td>
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<td>5.00</td>
<td>5.00</td>
<td>5.00</td>
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<tr>
<td>Purified mineral mix$^2$</td>
<td>53.70</td>
<td>53.70</td>
<td>53.70</td>
<td>53.70</td>
<td>53.70</td>
<td>53.70</td>
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<tr>
<td>Purified vitamin mix$^3$</td>
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<td>2.00</td>
<td>2.00</td>
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<td>DL-$\alpha$-tocopherol acetate</td>
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<td>DL-Methionine</td>
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<td>2.90</td>
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<td>Solka Floc$^4$</td>
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<td>GGMO-AX$^5$</td>
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<td>2.00</td>
<td>4.00</td>
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<tr>
<td>Short-chain FOS$^6$</td>
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<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>20.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Safmannan$^7$</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
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Calculated analysis:

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<th></th>
<th>Crude protein, %</th>
<th>MEn, kcal/g</th>
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<td></td>
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<td>3.55</td>
</tr>
<tr>
<td></td>
<td>23.01</td>
<td>3.55</td>
</tr>
</tbody>
</table>

$^1$ ADM Ardex® AF.

$^2$ Provided per kilogram of diet: CaCO$_3$, 3 g; Ca$_3$(PO$_4$)$_2$, 28 g; K$_2$HPO$_4$, 9 g; NaCl, 8.8 g; CuSO$_4$·5H$_2$O, 20 mg; ZnCO$_3$, 100 mg; MgSO$_4$·7H$_2$O, 3.5 g; Fe$_6$C$_6$O$_7$·H$_2$O, 500 mg; MnSO$_4$·H$_2$O, 650 mg; H$_3$BO$_3$, 9 mg; NaMoO$_4$·2H$_2$O, 9 mg; KI, 40 mg; CoSO$_4$·7H$_2$O, 1 mg; and Na$_2$SeO$_3$, 215 μg.

$^3$ Provided per kilogram of diet: retinyl acetate, 1,789 μg; cholecalciferol, 15 μg; DL-$\alpha$-tocopheryl acetate, 20 mg; menadione dimethylpyrimidinol bisulfite, 2 mg; ascorbic acid, 250 mg; thiamin·HCl, 20 mg; niacin, 50 mg; riboflavin, 10 mg; D-calcium pantothenate, 30 mg; vitamin B$_{12}$, 40 μg; pyridoxine·HCl, 6 mg; D-biotin, 600 μg; folic acid, 4 mg; and ethoxyquin, 125 mg.

$^4$ Purified cellulose, International Fiber Corp., North Tonawanda, NY.

$^5$ Galactoglucomannan oligosaccharide-arabinoxylan complex, Temple-Inland, Diboll, TX.

$^6$ Short-chain fructooligosaccharide, NutraFlora P-95 short-chain fructooligosaccharides, GTC Nutrition, Golden, CO.

$^7$ Safmannan, Lesaffre Yeast Corp., Milwaukee, WI.
Table 7.2. Body weight gain (g), feed intake (g/chick), and gain:feed (g/kg) of broiler chicks fed a diet supplemented with galactoglucomannan oligosaccharide-arabinoxylan (GGMO-AX) complex, short-chain fructooligosaccharides (scFOS), or Safmannan (Saf) and fed to chicks infected (Inf.) with *Salmonella typhimurium* or uninfected on select days (d) post-inoculation (PI).

<table>
<thead>
<tr>
<th>Diet</th>
<th>Uninfected</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th>S. typhimurium</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0%</td>
<td>1%</td>
<td>2%</td>
<td>4%</td>
<td>scFOS</td>
<td>Saf</td>
<td></td>
<td>0%</td>
<td>1%</td>
<td>2%</td>
<td>4%</td>
<td>scFOS</td>
<td>Saf</td>
<td></td>
<td>PSEM</td>
</tr>
<tr>
<td>Weight gain (g/chick/d)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PI d 0-3&lt;sup&gt;5&lt;/sup&gt;</td>
<td>10.1</td>
<td>10.4</td>
<td>11.0</td>
<td>9.3</td>
<td>10.5</td>
<td>10.7</td>
<td></td>
<td>11.1</td>
<td>10.7</td>
<td>10.2</td>
<td>9.9</td>
<td>11.0</td>
<td>12.6</td>
<td>0.64</td>
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<td>15.7</td>
<td>15.6</td>
<td>15.2</td>
<td>13.0</td>
<td>15.9</td>
<td>20.2</td>
<td></td>
<td>16.5</td>
<td>17.7</td>
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<td>17.4</td>
<td>19.9</td>
<td>0.70</td>
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</tr>
<tr>
<td>PI d 7-10&lt;sup&gt;1,4,5&lt;/sup&gt;</td>
<td>19.4</td>
<td>17.7</td>
<td>20.2</td>
<td>17.3</td>
<td>19.3</td>
<td>23.1</td>
<td></td>
<td>19.5</td>
<td>18.9</td>
<td>18.0</td>
<td>20.3</td>
<td>21.1</td>
<td>26.0</td>
<td>0.96</td>
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<tr>
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<td>17.9</td>
<td>17.4</td>
<td>18.2</td>
<td>15.6</td>
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<td>21.5</td>
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<td>16.9</td>
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<td>19.6</td>
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<tr>
<td>Feed intake (g/chick/d)</td>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PI d 0-3&lt;sup&gt;1,5&lt;/sup&gt;</td>
<td>13.4</td>
<td>14.1</td>
<td>14.3</td>
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<td>13.5</td>
<td>14.3</td>
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<td>15.4</td>
<td>0.66</td>
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<td>20.5</td>
<td>20.2</td>
<td>17.3</td>
<td>20.1</td>
<td>21.8</td>
<td></td>
<td>20.9</td>
<td>22.4</td>
<td>19.2</td>
<td>20.8</td>
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<td>22.8</td>
<td>23.9</td>
<td>20.8</td>
<td>23.3</td>
<td>25.8</td>
<td></td>
<td>23.4</td>
<td>24.4</td>
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<td>24.0</td>
<td>24.4</td>
<td>29.6</td>
<td>0.96</td>
<td></td>
</tr>
<tr>
<td>PI d 0-10&lt;sup&gt;1,2,5&lt;/sup&gt;</td>
<td>22.3</td>
<td>22.7</td>
<td>23.0</td>
<td>19.9</td>
<td>22.7</td>
<td>23.9</td>
<td></td>
<td>23.1</td>
<td>24.6</td>
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<td>23.3</td>
<td>23.8</td>
<td>27.5</td>
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<tr>
<td>Gain:Feed (g/kg)</td>
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</tr>
<tr>
<td>PI d 0-3&lt;sup&gt;2&lt;/sup&gt;</td>
<td>751.1</td>
<td>739.9</td>
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<td>745.0</td>
<td>751.9</td>
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<td>814.3</td>
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<td>763.1</td>
<td>755.9</td>
<td>751.0</td>
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<td>823.0</td>
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</tbody>
</table>
1 Main effect of infection is significant at P < 0.05.

2 Main effect of diet is significant at P < 0.05.

3 Interaction of infection and diet is significant at P < 0.05.

4 Interaction between scFOS vs. 2% GGMO-AX and infection status (uninfected vs. infected) is significant at P < 0.05.

5 Interaction between Safmannan vs. 2% GGMO-AX and infection status (uninfected vs. infected) is significant at P < 0.05.
Table 7.3. Total full and empty ceca weight (g/chick) and short-chain fatty acid (SCFA) and branched-chain fatty acid (BCFA) concentrations of commercial broiler chicks fed a diet supplemented with galactoglucomannan oligosaccharide-arabinoxylan (GGMO-AX) complex, short-chain fructooligosaccharides (scFOS), or Safmannan (Saf) fed to chicks infected (Inf.) with *Salmonella typhimurium* or uninfected.

<table>
<thead>
<tr>
<th>Diet</th>
<th>0%</th>
<th>1%</th>
<th>2%</th>
<th>4%</th>
<th>scFOS</th>
<th>Saf</th>
<th>0%</th>
<th>1%</th>
<th>2%</th>
<th>4%</th>
<th>scFOS</th>
<th>Saf</th>
<th>PSEM</th>
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<tbody>
<tr>
<td>Full ceca weight, % of BW²</td>
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<td>0.55</td>
<td>0.64</td>
<td>0.85</td>
<td>0.61</td>
<td>0.64</td>
<td>0.59</td>
<td>0.63</td>
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<td>0.89</td>
<td>0.64</td>
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<tr>
<td>Empty ceca weight, % of BW²</td>
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<td>0.43</td>
<td>0.45</td>
<td>0.54</td>
<td>0.40</td>
<td>0.44</td>
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<td>0.43</td>
<td>0.027</td>
</tr>
<tr>
<td>SCFA, µmol/g³</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetate³</td>
<td>161.4</td>
<td>110.4</td>
<td>211.4</td>
<td>136.7</td>
<td>149.9</td>
<td>163.4</td>
<td>138.1</td>
<td>162.2</td>
<td>179.8</td>
<td>225.5</td>
<td>176.7</td>
<td>159.0</td>
<td>19.91</td>
</tr>
<tr>
<td>Propionate¹,²,³</td>
<td>69.5</td>
<td>36.3</td>
<td>54.6</td>
<td>39.6</td>
<td>49.4</td>
<td>69.1</td>
<td>58.6</td>
<td>68.2</td>
<td>53.2</td>
<td>61.2</td>
<td>108.4</td>
<td>84.0</td>
<td>10.14</td>
</tr>
<tr>
<td>Butyrate</td>
<td>99.3</td>
<td>80.8</td>
<td>139.3</td>
<td>108.6</td>
<td>134.5</td>
<td>96.4</td>
<td>63.1</td>
<td>89.3</td>
<td>121.9</td>
<td>131.8</td>
<td>161.6</td>
<td>106.1</td>
<td>24.11</td>
</tr>
<tr>
<td>Total SCFA²</td>
<td>291.2</td>
<td>227.5</td>
<td>405.3</td>
<td>284.9</td>
<td>418.0</td>
<td>341.4</td>
<td>260.1</td>
<td>319.8</td>
<td>354.8</td>
<td>418.5</td>
<td>416.9</td>
<td>349.0</td>
<td>36.90</td>
</tr>
</tbody>
</table>

SCFA, µmol/ceca³

| Acetate¹,²,³ | 20.4 | 11.1 | 36.2 | 30.7 | 23.9 | 24.2 | 15.2 | 26.1 | 35.1 | 84.1 | 36.3 | 34.2 | 6.68 |
| Propionate¹ | 11.8 | 3.6  | 8.6  | 9.0  | 10.1 | 12.6 | 6.6  | 10.7 | 10.3 | 17.5 | 15.2 | 18.1 | 3.01 |
| Butyrate | 13.3 | 8.5  | 25.5 | 24.3 | 15.0 | 13.6 | 7.0  | 15.4 | 24.1 | 49.3 | 32.5 | 22.3 | 5.54 |
| Total SCFA | 45.6 | 23.2 | 70.4 | 64.0 | 59.3 | 50.4 | 28.7 | 52.1 | 69.5 | 152.4 | 84.1 | 74.6 | 14.62 |
Table 7.3 (con’t.).

<table>
<thead>
<tr>
<th></th>
<th>µmol/g&lt;sup&gt;a&lt;/sup&gt;</th>
<th></th>
<th>µmol/ceca&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Isobutyrate</td>
<td>Isovalerate</td>
<td>Valerate&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>19.1</td>
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<td>38.5</td>
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<td>28.5</td>
<td>38.7</td>
<td>36.2</td>
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<tr>
<td></td>
<td>22.0</td>
<td>18.7</td>
<td>21.9</td>
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<td>28.8</td>
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<td>33.0</td>
<td>28.6</td>
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<td>24.6</td>
<td>21.6</td>
<td>66.9</td>
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<td>6.88</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>Isobutyrate&lt;sup&gt;1&lt;/sup&gt;</td>
<td>Isovalerate</td>
<td>Valerate</td>
</tr>
<tr>
<td></td>
<td>2.7</td>
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<td>3.9</td>
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<td>3.6</td>
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<tr>
<td></td>
<td>1.07</td>
<td>1.86</td>
<td>2.53</td>
</tr>
</tbody>
</table>

<sup>a</sup>Values are expressed on a dry matter basis.

<sup>1</sup>Main effect of infection is significant at P < 0.05.

<sup>2</sup>Main effect of diet is significant at P < 0.05.

<sup>3</sup>Interaction of infection and diet is significant at P < 0.05.
Table 7.4. Ileal and cecal microbial populations (log$_{10}$ CFU/g of sample DM) of commercial broiler chicks fed a diet supplemented with galactoglucomannan oligosaccharide-arabinoxylan (GGMO-AX) complex, short-chain fructooligosaccharides (scFOS), or Safmannan (Saf) fed to chicks and infected (Inf.) with *Salmonella typhimurium* or uninfected.

<table>
<thead>
<tr>
<th>Inf.</th>
<th>Uninfected</th>
<th>S. typhimurium</th>
<th>PSEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diet</td>
<td>0% 1% 2% 4% scFOS SAF</td>
<td>0% 1% 2% 4% scFOS SAF</td>
<td></td>
</tr>
<tr>
<td>Ileum</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bifidobacterium spp.</td>
<td>6.8 7.2 6.8 6.9 6.9 6.7</td>
<td>6.8 6.7 6.9 7.1 6.8 6.9</td>
<td>0.18</td>
</tr>
<tr>
<td>Lactobacillus spp. $^{2,3,4}$</td>
<td>11.6 11.7 11.7 11.4 11.8 11.7</td>
<td>11.0 11.5 12.5 12.3 9.2 12.4</td>
<td>0.46</td>
</tr>
<tr>
<td>Clostridium perfringens $^2$</td>
<td>8.3 8.5 8.7 7.9 8.0 9.0</td>
<td>8.5 8.5 8.7 7.8 8.3 8.8</td>
<td>0.20</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>10.2 10.3 10.4 11.0 10.7 10.8</td>
<td>10.9 10.7 10.4 10.7 10.5 10.8</td>
<td>0.28</td>
</tr>
<tr>
<td>Total population, AU$^{a,1,4}$</td>
<td>12.0 12.1 12.3 11.8 12.6 12.0</td>
<td>12.5 12.4 12.9 12.8 12.1 12.9</td>
<td>0.27</td>
</tr>
<tr>
<td>Ceca</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bifidobacterium spp. $^2$</td>
<td>8.4 9.1 9.4 9.7 9.7 8.4</td>
<td>9.2 9.1 9.4 9.9 8.7 8.8</td>
<td>0.26</td>
</tr>
<tr>
<td>Lactobacillus spp. $^{1,2}$</td>
<td>12.2 12.4 12.7 12.5 12.5 12.5</td>
<td>12.6 12.8 12.8 12.7 12.6 12.7</td>
<td>0.10</td>
</tr>
<tr>
<td>Clostridium perfringens</td>
<td>9.7 9.4 9.5 10.4 10.1 10.8</td>
<td>10.0 9.8 9.9 10.0 10.2 10.3</td>
<td>0.32</td>
</tr>
<tr>
<td>Escherichia coli $^{1,3,5}$</td>
<td>8.9 9.3 9.7 9.4 9.2 9.6</td>
<td>10.0 10.2 10.1 10.0 10.0 10.2</td>
<td>0.11</td>
</tr>
<tr>
<td>Total population, AU$^{a,1,4}$</td>
<td>13.5 13.6 13.7 13.6 13.5 13.5</td>
<td>13.9 14.2 13.7 13.9 14.1 13.8</td>
<td>0.13</td>
</tr>
</tbody>
</table>

$^a$ AU = arbitrary units.

1 Main effect of infection is significant at P < 0.05.

2 Main effect of diet is significant at P < 0.05.

3 Interaction of infection by diet is significant at P < 0.05.

4 Interaction between dietary treatment (scFOS vs. 2% GGMO-AX) and infection status (uninfected vs. infected) (P < 0.05).

5 Infection by linear response to GGMO-AX supplementation is significant at P < 0.05.
Table 7.5. Populations of *Salmonella typhimurium* in digesta on d 10 post-inoculation (PI) and excreta on select days PI (log$_{10}$ CFU/g of excreta DM) in commercial broiler chicks fed a diet supplemented with galactoglucomannan oligosaccharide-arabinoxylan (GGMO-AX) complex, short-chain fructooligosaccharides (scFOS), or Safmannan (Saf). Values in parentheses indicate numbers of replicates with detectable populations of *Salmonella typhimurium*.

<table>
<thead>
<tr>
<th>Diet</th>
<th>0%</th>
<th>1%</th>
<th>2%</th>
<th>4%</th>
<th>scFOS</th>
<th>Saf</th>
<th>PSEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Digesta</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ileal$^1$</td>
<td>8.0 (1/4)</td>
<td>8.7 (1/4)</td>
<td>8.6 (2/4)</td>
<td>8.3 (1/4)</td>
<td>7.8 (2/4)</td>
<td>8.5 (2/4)</td>
<td>0.13</td>
</tr>
<tr>
<td>Ceca$^1$</td>
<td>10.7 (4/4)</td>
<td>11.3 (4/4)</td>
<td>10.9 (4/4)</td>
<td>10.9 (4/4)</td>
<td>10.9 (4/4)</td>
<td>10.7 (4/4)</td>
<td>0.14</td>
</tr>
<tr>
<td>Excreta</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PI d 1</td>
<td>9.8 (4/4)</td>
<td>10.1 (2/4)</td>
<td>10.1 (4/4)</td>
<td>9.7 (2/4)</td>
<td>10.1 (3/4)</td>
<td>10.0 (4/4)</td>
<td>0.29</td>
</tr>
<tr>
<td>PI d 10$^1$</td>
<td>9.3 (1/4)</td>
<td>8.6 (2/4)</td>
<td>9.3 (1/4)</td>
<td>8.7 (3/4)</td>
<td>8.3 (2/4)</td>
<td>9.9 (4/4)</td>
<td>0.42</td>
</tr>
</tbody>
</table>

$^1$ Main effect of diet is significant at P < 0.10.
Figure 7.1. Cecal pH of commercial broiler chicks fed a diet with galactoglucomannan oligosaccharide-arabinoxylan (GGMO-AX) complex, short-chain fructooligosaccharides (scFOS), or Safmannan (Saf) and fed to chicks and infected with (black bars) or without (white bars) *Salmonella typhimurium*.

<table>
<thead>
<tr>
<th>Diet</th>
<th>pH</th>
<th>0%</th>
<th>1%</th>
<th>2%</th>
<th>4%</th>
<th>scFOS</th>
<th>Saf</th>
</tr>
</thead>
<tbody>
<tr>
<td>scFOS and GGMO-AX</td>
<td>5.0</td>
<td>5.5</td>
<td>6.0</td>
<td>6.5</td>
<td>7.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SAF and GGMO-AX</td>
<td>5.0</td>
<td>5.5</td>
<td>6.0</td>
<td>6.5</td>
<td>7.0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>P-value</th>
<th>Inf.</th>
<th>Diet</th>
<th>Inf. x Dt</th>
<th>Inf. x Lin</th>
<th>scFOS and GGMO-AX</th>
<th>SAF and GGMO-AX x Inf.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inf.</td>
<td>0.02</td>
<td>&lt;0.01</td>
<td>0.82</td>
<td>0.52</td>
<td>0.90</td>
<td>0.39</td>
</tr>
</tbody>
</table>

1 Infection by linear response to GGMO-AX supplementation.

2 Interaction between scFOS vs. 2% GGMO-AX and infection status (uninfected vs. infected) (P < 0.05).

3 Interaction between Safmannan vs. 2% GGMO-AX and infection status (uninfected vs. infected) (P < 0.05).
Figure 7.2. Cecal tonsil cytokine expression (fold changes relative to uninfected control chicks) of interferon-γ (IFN-γ), interleukin (IL)-1β, IL-10, IL-12β in commercial broiler chicks fed a diet supplemented with galactoglucomannan oligosaccharide-arabinoxylan (GGMO-AX) complex, short-chain fructooligosaccharides (scFOS), or Safmannan (Saf) and fed to chicks infected (Inf.) with (black bars) *Salmonella typhimurium* or uninfected (white bars).

<table>
<thead>
<tr>
<th></th>
<th>Inf.</th>
<th>Diet</th>
<th>Inf. x Diet</th>
<th>Inf. x Ln Diet</th>
<th>scFOS and GGMOMO-AX x Inf.</th>
<th>Saf and GGMOMO-AX x Inf.</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-γ</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>0.03</td>
<td>0.01</td>
<td>0.50</td>
</tr>
<tr>
<td>IL-12β</td>
<td>0.28</td>
<td>0.69</td>
<td>0.94</td>
<td>0.99</td>
<td>0.77</td>
<td>0.30</td>
</tr>
<tr>
<td>IL-1β</td>
<td>0.68</td>
<td>0.86</td>
<td>0.05</td>
<td>0.01</td>
<td>0.31</td>
<td>0.27</td>
</tr>
<tr>
<td>IL-10</td>
<td>0.70</td>
<td>0.95</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
<td>0.68</td>
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</tbody>
</table>

1 Infection by linear response to GGMO-AX supplementation.
Interaction between scFOS vs. 2% GGMO-AX and infection status (uninfected vs. infected) (P < 0.05).

Interaction between Safmannan vs. 2% GGMO-AX and infection status (uninfected vs. infected) (P < 0.05).
CHAPTER 8: SUMMARY AND CONCLUSIONS

Functional ingredient inclusion, particularly prebiotic fibers, in petfoods has gained popularity due to their perceived ability to improve animal health. This stems from research indicating that dietary fiber inclusion may improve laxation, attenuate glycemic response and, perhaps, improve intestinal health (Dietary Reference Intakes, 2006; Anderson et al., 2009).

The health and potential immune benefits of dietary fibers stem from the effects of fermentation in the distal part of the digestive tract. Carbohydrate fermentation results in production of fermentative end-product (short-chain fatty acids [SCFA], gases, and branched-chain fatty acids), a decrease in intestinal pH, and modulation of the microbiome, all of which have been shown to directly or indirectly modulate intestinal health (Topping and Clifton, 2001; Roberfroid et al., 2010; Slavin, 2010).

Many novel dietary fibers exist. One such novel fiber is galactoglucomannan oligosaccharide-arabinoxylan (GGMO-AX) complex, a co-product of fiberboard manufacturing. Up to this point, in vitro and in vivo research on this novel substrate is lacking.

Evidence for the potential health and immune benefits associated with dietary fiber consumption include SCFA modulation of cytokine release and inhibition of pathogen colonization in the intestine (Cavaglieri et al., 2003; Van Immerseel et al., 2004; Pié et al., 2007). In particular, prebiotic fibers enhance the population of perceived beneficial microbiota such as Lactobacillus and Bifidobacterium spp., which are associated with improved intestinal health and are able to counter the negative effects of pathogenic bacteria (Servin, 2004; Cocconnier et al., 2005; Fayol-Messaoudi et al., 2005). In addition, prebiotics may increase overall population density of bacteria, which may prevent enteropathogenic colonization and prevent a systemic bacterial infection (Roberfroid et al., 2010). Commensal microbiota of the gastrointestinal tract
contribute greatly to the development and activity of the gut associated lymphoid tissues (GALT) and the defense against pathogenic organisms (Cebra, 1999). Dietary mannose acts as a receptor analog by being structurally similar to glycoprotein receptors for type-1 fimbriae and, thus, able to prevent attachment to the mucosal layer (Mirelman et al., 1980; Firon et al., 1984; Ofek et al., 2003).

The overall objectives of this research were to determine the effects of the GGMO-AX complex on fermentation and intestinal immune responses. Chemical composition and structure, simulated hydrolytic digestion, nutrient digestibility effects, fermentative characteristics, and intestinal immune responses were evaluated using laboratory analyses and in vitro, canine, and avian models.

The objective of the first study was to determine the detailed structural and chemical composition of GGMO-AX and select fractions. Such information allows a better understanding of the mechanism of action upon testing in biological systems. The GGMO-AX substrate contained free monosaccharides, predominately arabinose and xylose. After hydrolysis with trifluoroacetic acid, predominant monosaccharides were mannose (56.5%), glucose (13.5%), and xylose (10.7%) with a DP between 2 and 16 units. Limited acid hydrolysis hydrolyzed the arabinoxylan fraction and released a series of galactoglucomannan oligosaccharides (GGMOs) with a DP of 4 to 13, with the major component being DP 5 to 8. The structure of these oligosaccharides is a β-1,4-linked backbone of mannose and glucose residues, with occasional α-1,6 branching by single galactosyl units. The purified GGMO fraction was subjected to size exclusion chromatography to produce three fractions that had their own unique DP range. These were later evaluated in vitro (study 2).
In summary, this study revealed that the predominant oligosaccharides in the GGMO-AX complex were GGMO and AX that had a DP range of 2 to 16 units. The data further suggest that the oligosaccharides would be rapidly fermented based on the monosaccharide linkages and low DP. In addition, upon mild acid hydrolysis, the arabinoxylan fraction could be removed, leaving a relatively pure GGMO substrate.

Study 2 evaluated the hydrolytic digestibility, fermentative capacity, and microbiota-modulating properties of GGMO-AX molasses, four hydrolyzed fractions of GGMO-AX molasses, short-chain fructooligosaccharides (scFOS), and a yeast cell wall preparation (Safmannan). Substrates resisted in vitro hydrolytic digestion but were fermented in vitro by dog fecal inoculum. All GGMO-AX substrates decreased pH of the medium by at least 0.64 units, which was greater than for Safmannan but not for scFOS. Safmannan fermentation resulted in the overall lowest concentrations of acetate, propionate, and total SCFA among substrates, while scFOS fermentation resulted in lower concentrations of total SCFA, acetate, propionate, and butyrate compared to the GGMO-AX substrates. Among GGMO-AX substrates, those with the lowest DP resulted in the greatest butyrate concentrations, while the GGMO-AX molasses substrate resulted in the lowest butyrate production. Total SCFA production was greatest for the purified GGMO oligosaccharides and lowest for Safmannan. The GGMO-AX substrates resulted in higher or equal Bifidobacterium spp. concentrations compared to scFOS.

After 12 h of fermentation, GGMO-AX substrates were well fermented compared to control substrates, scFOS, or Safmannan, as indicated by pH change data and total SCFA production data. The GGMO-AX substrates were able to clearly modify the bifidobacteria population as indicated by a greater bidobacteria concentration (0.9 log units) compared with Safmannan. The GGMO-AX substrates resulted in either an equal or significantly greater
bifidobacteria population compared to the proven prebiotic, scFOS. Overall, GGMO-AX had the strongest prebiotic effect based on SCFA production data, promotion of bifidobacteria and lactobacilli growth, and inhibition of \( E. \ coli \) growth.

Study 3 evaluated the nutritional effects and prebiotic potential of spray-dried GGMO-AX when added to canine diets, each containing a different concentration of supplemental GGMO-AX (0, 0.5, 1, 2, 4, and 8%) that replaced dietary cellulose, and that were tested in a dose-response experiment in dogs. Total tract DM and OM apparent digestibilities increased linearly, while total tract CP apparent digestibility decreased linearly, as dietary GGMO-AX substrate concentration increased. Based on fecal analyses, GGMO-AX was well fermented as indicated by linear increases in fecal acetate, propionate, and total SCFA, whereas butyrate concentration and pH decreased linearly with increasing dietary concentrations of GGMO-AX. Fecal phenol and indole concentrations decreased linearly while biogenic amines concentrations were not different with GGMO-AX supplementation. Fecal microbial concentrations of \( E. \ coli \), \textit{Lactobacillus} spp., and \textit{Clostridium perfringens} were not different among treatments, but a quadratic increase was noted for \textit{Bifidobacterium} spp. as dietary GGMO-AX substrate concentration increased.

Data suggest positive nutritional properties of supplemental GGMO-AX when incorporated in a high quality dog food. The effects on nutrient digestibility were expected as an insoluble, low fermentable fiber (cellulose) was replaced with a soluble, highly fermentable fiber (GGMO-AX) that decreased fecal dry matter and organic matter output. Linear increases in SCFA were a result of increased fermentable substrate entering the large bowel, which resulted in the decreased fecal pH. The decrease in fecal butyrate concentrations could be explained by GGMO-AX being fermented in the proximal colon with butyrate being absorbed by colonocytes.
as the digesta moved through the large bowel. Swanson et al. (2002) also noted no differences in butyrate concentration after feeding fructooligosaccharides and MOS. Only *Bifidobacterium* spp. populations were modulated by GGMO-AX supplementation, not *Lactobacillus* spp. No adverse effects of GGMO-AX supplementation were noted except for production of loose stools at dietary concentrations greater than 4%. These data indicate that GGMO-AX supplementation results in positive nutritional properties, but the prebiotic effect was not as strong as had been demonstrated in *in vitro* and an avian model. Reasons for this include: sampling site (fecal vs. *in vitro* media or cecal content), experimental model, and (or) possibly diet matrix effects.

The objective of study 4 was to determine the effects of GGMO-AX in diets with emphasis on growth performance, fermentative effects, and immune indices in an avian model challenged with an acute coccidial (*Eimeria acervulina*) infection and fed one of four dietary concentrations of GGMO-AX (0, 1, 2, or 4%) that replaced dietary cellulose. Body weight gain, feed intake, and G:F of chicks were not affected by diet post-inoculation (PI), while infection decreased weight gain, feed intake, and G:F on d 3-7 and 0-7 PI. Cecal propionate concentrations were independently affected by infection and diet, while butyrate concentrations were affected only by infection. Cecal *Bifidobacterium* spp. populations were affected by diet, with the 2% GGMO-AX treatment resulting in the highest CFU/g of cecal contents (expressed on a DM basis). Messenger RNA expression of duodenal cytokines was affected by infection status, but not by dietary treatment alone. Supplementing 4% GGMO-AX consistently resulted in the greatest fold change in pro-inflammatory cytokine expression, while inhibiting anti-inflammatory cytokine expression.

Dietary GGMO-AX was unable to ameliorate the depression in animal performance associated with the coccidiosis infection. The decreased performance was likely a result of a
decrease in feed intake caused by elevated cytokine expression in 4% GGMO-AX-fed chicks. Based on total SCFA concentration (µmol/g), GGMO-AX was not fermented in the ceca; however, a linear decrease in cecal pH was noted as GGMO-AX concentration increased, suggesting increased SCFA production. It is likely that if the data were expressed on a per cecal pool basis, differences in SCFA concentrations would have been noted. Supplementing 4% GGMO-AX consistently resulted in the greatest fold change in pro-inflammatory cytokine expression, while inhibiting anti-inflammatory cytokine expression, indicating a more robust innate immune response that would enhance acquired immunity to subsequent infections and thereby potentially ameliorate the negative effects of a secondary infection.

The objective of study 5 was to evaluate the ability of GGMO-AX to prevent or limit Salmonella typhimurium (ST) colonization and improve intestinal immune response in an avian model challenged with an acute ST infection and fed one of six dietary treatments differing in concentration of GGMO-AX (0, 1, 2, or 4%), which replaced dietary cellulose, or diets containing 2% Safmannan (Saf) plus 2% cellulose or 2% short-chain fructooligosaccharides (scFOS) plus 2% cellulose. Infection resulted in greater weight gain and feed intake in chicks throughout the study, while diet affected only weight gain and feed intake on d 0-10 PI, with Safmannan-fed chicks experiencing the greatest weight gain. The GGMO-AX substrate demonstrated prebiotic-like effects as indicated by increased cecal SCFA concentrations, particularly butyrate, a decrease in cecal pH, and increased populations of Lactobacillus spp. and Bifidobacteria spp. as dietary GGMO-AX concentration increased. Excreta ST populations on d 5 and 10 PI and ileal and cecal ST populations had a tendency to be affected by diet. Messenger RNA expression of IFN-γ in the cecal tonsils was the only cytokine independently affected by infection and diet. An interaction between infection and diet and an infection by linear response
to GGMO-AX inclusion affected IFN-γ, IL-1β, and IL-10 expression. Chicks fed 2 and 4% GGMO-AX had similar expressions of IFN-γ and IL-1β regardless of infection, suggesting that virulence of ST was suppressed.

Growth performance of chicks fed GGMO-AX was less than that of chicks fed Saf, the mannanoligosaccharide control substrate, suggesting that another component of Saf (e.g., beta-glucans, live yeast cells) may have elicited the beneficial effect. Dietary GGMO-AX supplementation resulted in similar fermentative responses as scFOS, a proven prebiotic substrate, suggesting that GGMO-AX may be a bonafide prebiotic. Overall, no dietary treatment, except for scFOS, resulted in a reduction in ST shedding or intestinal colonization. The interaction between infection and GGMO-AX concentration on cecal tonsil pro-inflammatory (IFN-γ and IL-1β) and anti-inflammatory (IL-10) cytokine expression suggested that GGMO-AX fed to unchallenged chicks stimulated the intestinal immune system and was capable of suppressing inflammation associated with ST. Comparing cytokine expression data among diets, it appears that dietary concentrations greater than 2% were able to suppress virulence of ST. Overall, dietary concentrations of at least 2% GGMO-AX were able to elicit a prebiotic response and limited intestinal immune responses to an acute ST infection in chicks.

In conclusion, the research presented here clarifies that GGMO-AX is well fermented by in vitro and select animal models and is able to elicit a prebiotic response in in vitro and an avian model. In addition, GGMO-AX, particularly at dietary concentrations greater than 2%, is able to modulate the intestinal immune response during a challenge by an intestinal pathogen. This research offers insight into the chemical structure and composition of GGMO-AX and shows how these characteristics influence fermentation in vitro and in vivo, and how GGMO-AX supplementation affects immunological responses by the intestine in avian disease-challenged
model. The data presented in this dissertation provide valuable information about a novel fermentable carbohydrate that may be incorporated into diets of livestock, poultry, and (or) pet animals. In addition, these data expand the knowledge base in the area of fermentable carbohydrates, particularly mannanoligosaccharides, and their impacts on intestinal health. Further research is necessary to verify that GGMO-AX may be a consistent prebiotic substrate and to investigate its effects on intestinal health in both healthy and immune-challenged animal models.

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