EFFECTS OF POTATO FIBER AND SOLUBLE CORN FIBER ON THE FECAL MICROBIOME OF DOGS WITH IMPLICATIONS TO INFLAMMATION

BY

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DISSERTATION

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Abstract

Dietary fiber provides numerous benefits to the health of both companion animals and humans including improved digestive health (increases stool weight and promotes normal laxation) and systemic health (promotes satiety and decreases incidences of obesity and type II diabetes). Novel dietary fibers often are evaluated for their ability to induce prebiotic effects. Prebiotics are defined as non-digestible food ingredients that, when consumed in sufficient amounts, are fermented and selectively stimulate the growth, activity, or both, of one or a limited number of microbial genera or species in the gut microbiota that ultimately benefits health of the host. Not all dietary fibers are the same in terms of their physico-chemical properties, which ultimately affects their fermentability. The overarching objectives of this thesis were to assess two novel dietary fiber sources, potato fiber (PF) and soluble corn fiber (SCF), for their inclusion in commercial dog diets, and ultimately, their prebiotic potential and implications for host health.

Specifically, PF and SCF were evaluated for their chemical composition, *in vitro* fermentability, *in vivo* characteristics (nutrient digestibility and fecal fermentation characteristics), and ability to change fecal microbiota concentrations. Previous research in our laboratory found that feeding graded concentrations of dietary PF to dogs elicited linear increases ($P < 0.05$) in fecal acetate, propionate, butyrate, and total short-chain fatty acids (SCFA) without causing detriments to nutrient digestibility. We investigated this further by studying the prebiotic potential of PF by analyzing the fecal microbiome. We predicted that our previous results on fecal fermentation characteristics would be associated with shifts in the fecal microbiome. Based on previous studies involving dietary fiber in pets and humans, we expected that at the phylum level, an increase in fecal Firmicutes and a decrease in fecal Fusobacteria would occur. More specifically, we also

predicted increases in bifidobacteria and other SCFA-producing genera (e.g., *Blautia*, *Lachnospira*, and *Faecalibacterium* spp.) with increasing dietary PF concentrations.

As anticipated, with increasing concentrations of dietary PF, there were significant ($P < 0.05$) changes in fecal concentrations of key SCFA-producing bacteria. Butyrate-producing genera, such as *Blautia* and *Faecalibacterium* spp. increased ($P < 0.05$) with increasing dietary PF concentrations, which was confirmed by qPCR. Our sequencing data also confirmed an increase ($P < 0.05$) in Firmicutes and a decrease in Fusobacteria ($P < 0.05$) with increased dietary PF consumption. Prebiotics often are evaluated by their ability to produce butyrate (a butyrogenic effect) or to stimulate the growth of bifidobacteria (a bifidogenic effect); however, the definition of a prebiotic is not limited to this genus. We found that with ingestion of PF, *Bifidobacterium* spp. were positively correlated with butyrate ($R = 0.49$; $P < 0.05$) and lactobacilli ($R = 0.82$; $P < 0.05$) concentrations. Bifidobacteria's main carbohydrate fermentation products are acetate and lactate when substrate is available, while lactobacilli produce lactic acid. This has previously been explained by bacterial cross-feeding where primary fermenters of carbohydrate will produce secondary metabolites (acetate and lactate) or hydrolyzed substrate that can be utilized by other bacteria. Overall, this research suggests to us that PF is beneficial to gut health and should be investigated as a novel functional ingredient in dog diets.

Soluble corn fiber (NUTRIOSE®) also was evaluated for its efficacy as a fiber source in dog foods; however, the objective was to determine a minimum dose to elicit a prebiotic effect. We found that SCF was highly fermentable *in vitro* showing increases ($P < 0.05$) in acetate, propionate, and butyrate concentrations over 12 h. When tested *in vivo* from 0-1.25% of the diet, we found no detrimental effects on nutrient digestibility or fecal consistency; however, no changes were shown in fecal fermentation characteristics. Furthermore, this translated into no appreciable

differences in the fecal microbiome. Overall, SCF was fermentable *in vitro*; however, more research is needed to evaluate a more effective dose to elicit *in vivo* effects.

The changes we observed with increasing dietary PF in the dog presented the most intriguing results. *Faecalibacterium,* and more specifically, *Faecalibacterium prausnitzii,* a well-characterized butyrate-producing species, was increased by feeding graded dietary levels of PF to dogs. This bacteria is often found in low concentrations in humans and dogs with inflammatory bowel diseases (IBD). These previous results led us to investigate whether PF, with its *in vivo* fermentation characteristics and ability to modulate the microbiome, would be efficacious in attenuating the inflammatory response in the dextran-sodium sulfate (DSS)-induced colitis mouse model. We hypothesized that a moderately fermentable PF would be more effective at attenuating the symptoms and inflammatory response in DSS colitis than non-fermentable cellulose (Cell). Mice provided the PF/DSS treatment exhibited decreased ($P < 0.05$) symptoms of DSS colitis by showing a delayed loss in body weight compared with mice provided the Cell/DSS treatment. Furthermore, fermentation of PF during DSS colitis was found to be anti-inflammatory by showing suppression ($P < 0.05$) of distal colon gene expression of inflammatory cytokines (TNF-α, IL-1β, IL-6, and IL-17A).

What was particularly interesting were the changes in distal colon gene expression of CXCL1, which is a neutrophil chemoattractant. Mice provided non-fermentable Cell had a 7-fold increase ($P < 0.05$) in CXCL1 expression compared with all other treatment groups. Pathology scores from an independent observer also showed decreases ($P < 0.05$) in leukocyte infiltration scores in distal colon. In this acute-colitis model, neutrophils would be the predominant innate immune cell type at the site of inflammation. Therefore, this suggests that fermentation of PF to produce SCFA may be a driving force in controlling leukocyte recruitment to the site of

inflammation. As a potential mechanism linking SCFA concentrations and leukocyte recruitment, we analyzed free fatty acid receptor 2 (FFAR2) expression in distal colon. Leukocytes, and in particular neutrophils, have FFAR2 on their cell surface. Short-chain fatty acids can bind to this receptor, causing changes in chemotaxis and reactive oxygen species concentrations. We found that DSS caused an increase ($P < 0.05$) in FFAR2 expression in distal colon; however, there was no main effect of diet or an interactive effect. One alternative mechanism proposed was the role of SCFA suppressing innate T-helper 17 (Th17) cells, which recruit neutrophils and monocytes through the concentrations of IL-17A and chemokines. Short-chain fatty acids have been found to induce both effector and regulatory T cells by suppression of histone deacetylases that is independent of FFAR2 or 3. Inhibition of histone deacetylases by SCFA and, in particular, acetate, has been shown to regulate the mTOR pathway required for the generation of Th17 cells. Collectively, these data show that moderately fermentable fiber intake during DSS colitis is anti-inflammatory, which could potentially be due to SCFA decreasing leukocyte recruitment.

Overall, the ability of a dietary fiber to elicit positive effects on fermentation and health benefits is due to the physico-chemical properties of the fiber. Here, we found that ingestion of a moderately fermentable fiber elicited increases in fecal SCFA concentrations, and modulated the microbiome. This dietary fiber elicited anti-inflammatory effects in the DSS-model, which may have been a result of SCFA affecting leukocyte recruitment to the site of inflammation. These data show that ingestion of a moderately fermentable prebiotic fiber could affect the acute inflammatory response through controlling leukocyte recruitment. More studies are needed to assess the importance of moderately fermentable fibers in the diet during in controlling inflammatory bouts noted in IBD.

To my dad, Michael Panasevich

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CHAPTER 1: LITERATURE REVIEW

Introduction

The interest in dietary fiber improving health has increased with new insights about the beneficial effects of the gut microbiome and their fermentation end-products (i.e. short-chain fatty acids (SCFA)). The human colonic microbes and their metabolic end-products have been associated with diseases such as diabetes mellitus (Larsen et al., 2010) inflammatory bowel diseases (IBD), Crohn's disease, and ulcerative colitis (Kranich et al., 2011), obesity (Turnbaugh et al., 2009), and others. Furthermore, companion animals also are subject to these diseases, making this area of research applicable to dogs and cats as well. The most apparent link between colonic microbial populations and their role in health and disease is SCFA concentrations and, in particular, concentrations of acetate, propionate, and butyrate (Ganapathy et al., 2013). Dietary fermentable fiber, resistant starch, and other indigestible carbohydrates are foods for the colonic microbes, which will, in turn, produce SCFA. The capacity of dietary fibers to produce SCFA is due to a number of factors including the physico-chemical properties of the fiber, as well as the resident microbial populations present in the colon. It is well established that dietary fiber is important in both companion animal and human diets to improve intestinal and host health. These health benefits include improved stool characteristics, ease of laxation, and modulation of the intestinal microbiota concentrations. Specifically understanding how dietary fermentable fiber and changes in the colonic microbiome relate to the health of the host could provide more insight about the beneficial effects of dietary fiber in both companion animal and human diets. Through the use of animal models and modern day techniques, it has become possible to integrate the quantification of microbial populations with products of their metabolism and, in turn, the effects of the microbiome on host immunity in both healthy and diseased states. The underlying objective of this

thesis is to evaluate the efficacy of two novel fibers in the dog diet and how they change the microbiome. Furthermore, investigated how these changes relate to the health of the host using a mouse model.

Dietary fiber

Dietary fiber is the edible part of plants or analogous carbohydrates resistant to digestion and absorption in the small intestine, with complete or partial fermentation in the large intestine (Prosky, 2000). It includes polysaccharides, oligosaccharides, lignin, and associated plant substances. Fiber is indigestible to mammalian enzymes due to the unique structural and chemical aspects of the plant cell wall and some cell contents, as well as β - glycosidic bonds between monosaccharide units that make fiber indigestible to mammalian enzymes (Buxton and Redfearn, 1997). Pancreatic amylase and isomaltase hydrolyze only α-1,4 and α-1,6 glycosidic bonds found primarily in starches (Coombe and Siddons, 1973). Bacterial enzymes in the hindgut, such as β-galactosidase, α-glucosidase, β-glucosidase, and β-glucuronidase, hydrolyze polysaccharides with β-glycosidic bonds (Tan et al., 2010).

Potato fiber and soluble corn fiber

Polysaccharides in the plant cell wall can be separated into two categories: soluble fiber, which may be viscous and highly fermentable, and insoluble fiber, which is generally non-viscous and partially- to non-fermentable. Potato fiber (PF) is a co-product of potato starch isolation that is widely used in the beef industry (Nelson, 2010). Briefly, potato starch processing involves rupturing the starch granules and subsequent gelatinization. The rupturing process disrupts the cells to free the starch granule (Nigam and Singh, 1995). This mash then is diluted and placed into a centrisieve to separate the skin and other cell contents from the starch. The skin and cell fragments are referred to as the pulp or the fiber component that makes up PF (Klingspohn et al.,

1993). The carbohydrate fractions of PF include pectins, resistant and digestible starch, cellulose, and hemicelluloses (Klingspohn et al., 1993). Potato fiber has the potential to be a high quality fiber source in pet foods due to its considerable amount of insoluble and soluble fiber, as well as its resistant starch content. Soluble fiber sources are thought to benefit gut health due their fermentability and modulation of the microbial ecology of the colon.

Very little research has been conducted on the nutritional value of PF. Laerke et al. (2007) found that the soluble fiber fraction of PF resulted in higher rat cecal SCFA concentrations of 173.5 µmole compared to 83.0 µmoles for cellulose controls. Furthermore, solubilized PF decreased postprandial plasma triacylglycerol from 2.79 mmol/L in the cellulose control diet to 1.82 mmol/L in the solubilized PF diet. Human data showed that consumption of enzymatically-solubilized potato fibers induced a 200% increase in breath H_2 analysis compared to oat bran, which was interpreted as increased fermentation (Olesen et al. 1998). Thomassen et al. (2010) solubilized potato pulp and conducted an in vitro fermentation study with human fecal inoculum to evaluate prebiotic effects. Solubilized potato pulp was three times more bifidogenic than FOS by showing a 300% increase in fecal *Bifidobacterium* spp. DNA concentrations compared with FOS. Furthermore, it was concluded that soluble fiber fractions of greater than 100 kDa had the greatest effect. These studies focused on the soluble fiber fractions of potato pulp, but they did not evaluate potato pulp in its native form that contains significant amounts of both insoluble and soluble fibers. Furthermore, the application of potato pulp as a dietary fiber source in high quality dog foods has not been investigated.

Another fiber source more commonly found in the human food industry is soluble corn fiber (NUTRIOSE®FM). NUTRIOSE is a soluble fiber that is produced from the dextrinization of corn, tapioca, or wheat starch. It is a glucose polymer (degree of polymerization 12-25) of mixed

beta and some alpha linkages (Pouillart et al., 2010). Soluble corn fiber is a novel, low digestible carbohydrate derived from hydrolysis of corn starch by heat and acid. Upon cooling, reformation of mixed β-glycosidic linkages resistant to mammalian enzymatic hydrolysis occurs. Soluble corn fiber is commonly used in the human food industry to aid in colonic health and as a low glycemic food additive (Knapp et al., 2010). Previous research found it to be fermentable and have positive effects on changing the colonic microbiome of humans and rats; however, there is limited research on its use in dog foods. The previous *in vivo* studies in humans and rodents have shown the potential health benefits of SCF as a fiber source at higher dietary concentrations. Dietary SCF concentrations of 5% or higher in rats have been found to improve cecal and colonic fermentation characteristics, as well as indices of gut health (i.e., increased crypt depth, goblet cell numbers, and acidic mucins) (Guerin-Deremaux et al., 2010; Knapp et al., 2013). Similarly, in humans, consumption of SCF at 20 g/d resulted in a decrease in colonic pH, suggesting increased fermentative activity (Lefranc-Millot et al., 2012).

In our laboratory, both PF and SCF have been investigated for their chemical composition, *in vitro* fermentability, as well as *in vivo* effects on nutrient digestibility, fecal consistency and fermentability (Panasevich et al., 2013; Panasevich et al. 2015). Dietary fiber sources act differently within the colon depending on chemical composition and the resident microbial populations and their metabolic capabilities. Panasevich et al. (2013) evaluated PF for its chemical composition and 12 h *in vitro* fermentation, and *in vivo* effects on nutrient digestibility, fecal fermentation end-products, and fecal consistency. This study revealed that PF contained 55% TDF with 32% insoluble fiber and 23% soluble fiber. Along with having relatively high amounts of TDF, PF also contained digestible starch and resistant starch, along with low amounts of crude protein, free sugars, and acid hydrolyzed starch. This translated into favorable effects in a 12 h *in*

vitro fermentation by showing increases in acetate, propionate, and butyrate concentrations through 9 h (Panasevich et al., 2013).

The *in vivo* experiment revealed that dogs ingesting graded concentrations of PF up to 6% exhibited linear increases in fecal acetate, propionate, butyrate, and total SCFA. It was concluded from both *in vitro* and *in vivo* data that PF is a moderately fermentable fiber that could be a functional ingredient in commercial dog food. Like PF, our lab also evaluated SCF for its chemical composition, 12 h *in vitro* fermentation, *in vivo* effects on nutrient digestibility, and fecal fermentation characteristics and consistency in dogs (Panasevich et al., 2015). The SCF substrate had 23% TDF, with a majority of that portion being low molecular weight soluble fiber (degree of polymerization greater than 3 and less than 10). The substrate also had high concentrations of free glucose. The 12 h *in vitro* fermentation indicated that SCF was more fermentable than cellulose, but less fermentable than pectin. Overall, SCF resulted in increased concentrations of acetate, propionate, and butyrate throughout the *in vitro* experiment.

The *in vivo* experiment with SCF did not show the anticipated results as indicated from the *in vitro* experiment. The objective of the *in vivo* experiment was to determine the lowest concentration of NU in the diet that will elicit increases in fecal SCFA and prebiotic effects. With increasing concentrations of NU in the diet up to 1.25%, no changes in nutrient digestibility or fecal SCFA concentrations were noted. The *in vitro* experiment suggested that SCF would be highly fermentable *in vivo* in the dog. It was concluded that the amount of SCF could have been too low to elicit changes in fermentability.

The colonic microbiota

The gut microbiome is involved in a symbiotic relationship with the host and influences host health (Kamada et al., 2013). It has been estimated that there are over a thousand different

species of bacteria that have numerous implications on host health and disease (Hooper and Gordon, 2001). A principal function of the commensal microbiota is to protect against invasion of pathogens into the colon (Buffie and Pamer, 2013). These pathogens will compete for nutrients with the resident microbes and disrupt the normal microbiota populations that can have a negative effect on the immune system (Kamada et al., 2013). The colonic microbiome has been found to have an effect in acute and chronic inflammatory diseases such as inflammatory bowel diseases (IBD), Crohn's disease, ulcerative colitis, and obesity (Guinane and Cotter, 2013; Turnbaugh et al., 2009). Furthermore, modulation of the microbiome through nutrition has grown interest in helping to manage these diseases.

The canine fecal microbiota

The canine gut microbiota has been characterized and reviewed extensively in both the pre sequencing and sequencing eras (Hooda et al., 2012). The predominant genera of bacteria that have been cultured from the large intestine of dogs include *Bifidobacterium*, *Bacteroides*, *Fusobacterium*, *Peptostreptococcus*, *Eubacterium*, *Clostridium*, *Peptococcus*, and *Lactobacillus* (Davis et al., 1977). Middelbos et al., (2010), was the first to use 16S rRNA gene pyrosequencing to characterize the healthy canine fecal microbiome. Results from this study indicated that the predominant phyla included Firmicutes (15-28%), Bacteroidetes (32-34%), Fusobacteria (24-40%), Actinobacteria (0.8 – 1.4%), and Proteobacteria (5-6%) (Middelbos et al., 2010). Overall, this suggested that the canine feces mainly consist of Firmicutes, Bacteroides, and Fusobacteria; however, shotgun sequencing identified the same phyla only in difference in abundance (Bacteroides and Firmicutes, ~35%; Fusobacteria, 7-8%) (Swanson et al., 2011).

Amplification of the variable regions (V) 1 – V3 found the most abundant bacterial families include Ruminococcaceae, Clostridiaceae, Lachnospiraceae, and Erysipelotrichaceae (Handl et al.,

2011; Garcia-Mazcorro et al., 2011). Within the clostridia class, *Clostridium*, *Ruminococcus*, *Dorea*, and *Roseburia* are prevalent genera in dog feces. Furthermore, within *Clostridium*, clusters XIVa and XI were most dominant and have been linked to GI health outcomes by their role in concentrations of SCFA (Hooda et al., 2012).

Amplification of 16S rRNA gene pyrosequencing has provided a more comprehensive understanding of the dog fecal microbiota populations. Some inconsistencies exist with past studies however, due to DNA extraction methods and primer biases that result in over or under representation of some microbial genera (Hooda et al., 2012). Along with characterizing the microbiota populations, it is equally important to also establish the metabolic capabilities of the microbiota. Swanson et al., (2011) reported that the primary functional categories of the fecal microbiota in healthy dogs include associations with carbohydrate metabolism (12.5-13% of total sequences), protein metabolism (8.1-9.1%), DNA metabolism (7.1-7.4%), cell wall and capsule (7.0-7.6%), amino acids and derivatives (6.8-6.9), virulence (6.2-7.2%), and cofactors, vitamins, prosthetic groups and pigments (5.7-6.0%). Understanding microbial dysbiosis and host-microbe interactions is important in characterizing pathogenesis of diseases. For instance, a recent study compared the fecal microbiota and serum metabolite profiles between healthy dogs and dogs with inflammatory bowel disease (IBD) before and after 3 weeks of medical therapy (Minamoto et al., 2015). Specifically, dogs with IBD exhibited lower bacterial diversity and higher amounts of Gammproteobacteria and revealed overrepresentation of bacterial secretion system and underrepresentation of amino acid metabolism (Minamoto et al., 2015). Overall, this reveals the importance of understanding both the microbial populations and their implications to host health.

The microbiome and inflammatory bowel disease

Inflammatory bowel disease includes both Crohn's disease and ulcerative colitis. Overall, IBD is an economic burden in the United States. In 2008, direct treatment costs for patients with IBD were estimated to be greater than $6.3 billion and indirect costs, such as missed work opportunities, estimated to cost an additional $5.5 billion (Center for Disease Control, 2012). Currently, there is no cure, and the disease requires constant pharmacological management (Center for Disease Control, 2012). Recently, more research is focused on investigating the role of the colonic microbiota in the pathogenesis of IBD. With new insights into microbial populations and their metabolic implications through the use of next-generation high-throughput sequencing techniques, we can more accurately assess the role of the microbes in the pathogenesis of IBD. In general, IBD patients are thought to maintain a diverse colonic microbiota, but evidence suggests that Crohn's disease patients may have higher proportions of *Enterobacteria* compared to healthy controls, with up to 30% of their dominant microbiome belonging to undefined phylogenetic groups (Seksik et al., 2003). However, the microbial populations of these two diseases are quite different (Bibiloni et al., 2006). Specifically, Crohn's disease patients had higher proportions of unclassified members of Bacteroides than ulcerative colitis patients (Bibiloni et al., 2006).

More recent studies have identified specific microbial genera and species that are involved in IBD. Patients with IBD tend to have a microbiome that is more indicative of proteolytic fermentation and less carbohydrate fermentation. Specifically, IBD patients tend to have higher fecal gamma-proteobacteria (Li et al., 2012), *Enterobacteraceae* (Garrett et al., 2010) and *Clostridium* spp. and lower Firmicutes, Bacteroidetes (Frank et al., 2007), SCFA-producing super-family *Lachnospiracheae*, *Faecalibacterium* (*F.prausnitzii*), *Roseburia*, and *Phascolarctobacterium* (Li et al., 2012; Guinane et al., 2013). Similarly, dogs with idiopathic inflammatory bowel disease and acute diarrhea have lower proportions of fecal *Blautia* and

Faecalibacterium compared to healthy dogs (Suchodolski et al., 2012). Honneffer at al. (2014) reviewed intestinal dysbiosis in dogs and cats and compared human IBD with IBD in dogs and cats. Specifically compared with the dog, humans with IBD show similar shifts of dysbiosis by exhibiting lower Firmicutes, *Ruminococcaceae*, and *Lachnospiraceae*. Collectively, these results give insight into the potential role that nutrition and, in particular, dietary fermentable fiber could help to control the inflammatory response through modulation of the microbiome.

Ingestion of fermentable fibers can modulate the microbiome that may be favorable in suppressing inflammation in IBD in humans and companion animals. Having diversity within the bacterial phylum, Firmicutes, is suggests increased SCFA concentrations and an overall reduced risk of certain bowel diseases (Hooda et al., 2012; Li et al., 2012; Frank et al., 2007). Specifically in humans, ingestion of fermentable polydextrose and soluble corn fiber has been found to increase *Faecalibacterium* and, in particular, *F. prausnitzii*, a known butyrate producer (Hooda et al., 2012). Similarly in healthy dogs, there was an increase in *Faecalibacterium* and, in particular, *F. prausnitzii*, with increased concentrations of dietary potato fiber (Panasevich et al., 2015). In dogs and humans, low concentrations of *Faecalibacterium* have been associated with acute and non-hemorrhagic diarrhea and poor gut health (Suchodolski et al., 2012; Sokol et al., 2008). The presence of *F. prausnitzii* in the hindgut has been associated with suppressing the onset of Crohn's disease in humans (Sokol et al., 2008). These results have suggested *F. prausnitzii* as a potential probiotic for the suppression of Crohn's disease (Hooda et al., 2012; Sokol et al., 2008) and IBD prevention in general (Hooda et al., 2012).

Prebiotics

Prebiotics are a common food additive in the human food industry and are increasingly popular in companion animal diets. Prebiotics are defined as non-digestible food ingredients that,

when consumed in sufficient amounts, selectively stimulate the growth, activity, or both, of one or a limited number of microbial genera or species in the colonic microbiota that ultimately benefits health of the host (Tremaroli and Backhed, 2012). Ingestion of a substrate by the host, such as a soluble fiber, could be used by bacteria like *Bifidobacterium* and *Lactobacillus* spp. to promote growth of their numbers while simultaneously decreasing harmful bacteria like *Clostridium perfringens* and *Escherichia coli*. Common prebiotics used in human foods also are frequently used in pet foods and include inulin, and fructooligosaccharides (FOS) (Tomasik and Tomasik, 2003). Certain prebiotic fibers can influence the colonic microbiota differently by their selectively influencing certain genera or species of bacteria. For example, inulin and trans–galactooligosaccharides both are considered bifidogenic prebiotics due to their ability to selectively stimulate the growth of bifidobacteria. (Roberfroid et al., 2010). Chicory root, artichokes, garlic, onion, bananas, rye, wheat, oats, and soybean are other common sources of naturally-occurring prebiotic oligosaccharides (Yen, 2003).

Potato fiber and SCF also were evaluated for their prebiotic potential (Panasevich et al. 2014; Panasevich et al. 2015). The increased concentrations in fecal SCFA when dogs were fed graded levels of dietary PF corresponded with what was noticed in the fecal microbiome. With increasing concentrations of PF in the diet, dogs exhibited linear increases in SCFA-producing bacteria at the genus and species levels. *Faecalibacterium,* and specifically *F. prausnitzii,* increased in dog feces when dogs were fed graded concentrations of PF. *Clostridium*, and in particular *C. hiranonis,* was lowest when dogs were fed 6% PF. *C. hiranonis* has been known to be involved in 7α-dehydroxylation activity, which results in 7α-dehydroxylation of primary and secondary bile salts (Kitahara et al., 2001). Secondary bile salts have been associated with an increased risk of colon cancer in humans (Reddy et al., 1996).

Other microbial genera and species in the Lachnospiraceae family, which is a known SCFA-producing bacterial family, increased with PF intake. In humans, ingestion of prebiotics has been found beneficial in the inflammatory bowel diseases, Crohn's and ulcerative colitis. Specifically, males ingesting polydextrose had increases in fecal SCFA with concomitant increases in *Faecalibacterium prausnitzii*, which is a butyrate producer found in lower concentrations in patients with Crohn's disease (Hooda et al., 2012). Similarly, dogs with idiopathic inflammatory bowel disease and acute diarrhea have lower proportions of fecal *Blautia* and *Faecalibacterium* compared to healthy dogs (Suchodolski et al., 2012). Soluble corn fiber also was evaluated for prebiotic effects but overall, there was very little modulation of the microbiome. Our lab concluded that SCF maintained the resident microbial populations. Overall, an increase in *Faecalibacterium* and a decrease in *Clostridium* genera with increasing concentrations of PF are believed to be beneficial to gut health. These results agreed with the fermentation characteristics reported by Panasevich et al. (2013).

After evaluation of both PF and SCF, it is clear that different dietary fibers act differently within the gastrointestinal tract. The degree of fermentability is due to the chemical composition of the substrate, the amount of that substrate entering the large bowel, and the resident microbes ability to ferment the substrate. Recently, there is growing interest in how changes in the colonic microbiome can influence health of the host. Ingestion of dietary PF elicited increases in SCFA concentrations, as well as beneficial changes in the fecal microbiome in the dog. More research is needed in finding how the PF substrate could affect host health through modulation of the microbiome and concentrations of SCFA.

The inflammatory response

Chronic inflammation

The chronic inflammatory response is characterized as a prolonged duration (weeks to years) of continuing inflammation, tissue injury, and healing, often fibrosis, and proceeding simultaneously (Robbins basic pathology) (Kumar et al., 2013). Different sets of reactions define the chronic inflammatory response as opposed to the acute inflammatory response. The three main steps of mounting chronic inflammation include infiltration of mononuclear cells, including plasma cells, lymphocytes, and macrophages, tissue destruction by products of inflammatory cells, and repair (Kumar et al., 2013). Acute inflammation could lead to chronic inflammation if unresolved. The most dominant cells of chronic inflammation are macrophages, which are together classified as the mononuclear phagocyte system. Two subsets of macrophages are involved in inflammation and then anti-inflammation. Classical activation involves macrophage type 1, which produce lysosomal enzymes, nitric oxide, and reactive oxygen species to kill a foreign antigen. Alternative activation involves macrophage type 2 that, in turn, is anti-inflammatory (Kumar et al., 2013). These macrophage types are induced by interleukin (IL)-4 and IL-13, which are cytokines produced by T lymphocytes, mast cells, and eosinophils, to help with tissue repair.

Lymphocytes also are major drivers in mounting the chronic inflammatory response. Both T and B lymphocytes will migrate to inflammatory sites and recruit other leukocytes (Kumar et al., 2013). Development of cluster of differentiation (CD) 4^+ T lymphocytes will secrete cytokines and influence the nature of the inflammatory status, while B lymphocytes will transition to plasma cells that secrete antibodies to the infection. There are subsets of $CD4^+$ helper T cells that promote different types of inflammation. T-helper 1 (T_H1) activate macrophage type 1 cell types to promote inflammation and produce interferon-gamma (IFNγ). T-helper-2 (T_H2) cells recruit and activate eosinophils and secrete IL-4, IL-13, and IL-5. Macrophage type 2 are activated by T_H2 to promote tissue repair. T-helper 17 (T_H17) recruit neutrophils and monocytes into the site of inflammation

through secretion of IL-17 and other chemokines (Kumar et al., 2013). Eosinophils and mast cells are also involved in chronic inflammatory responses by secreting cytokines and present antibodies to combat infections (Kumar et al., 2013).

Acute inflammation

The acute inflammatory response is defined as the rapid delivery of leukocytes and plasma proteins to the site of inflammation/injury (Kumar et al., 2013). The recruited leukocytes will begin to digest and remove necrotic tissue. The two major components of the acute inflammatory response include vascular and cellular changes. The onset of inflammation will promote vasodilation, vascular permeability to permit plasma proteins to leave circulation, and endothelial signaling to result in increased adhesion and migration of leukocytes (Kumar et al., 2013). Cellular recruitment of leukocytes allows for the first defense to a foreign antigen. Neutrophils, also referred to as polymorphonuclear cells, are the principal cell types to initiate the inflammatory response and are considered the first responders to the site of injury (Kumar et al., 2013).

The recruitment of leukocytes to the site of inflammation in an acute injury include margination and rolling, adhesion to the endothelium, transmigration between endothelial cells, and migration in interstitial tissues towards a chemoattractant. A series of lectins, selectins, and integrins on endothelial cells and leukocytes mediate the rolling, adhesion, and transmigration processes. These leukocyte and endothelial molecules are expressed more during an inflammatory insult and are critical in leukocyte recruitment. After extravasating from the blood, leukocytes will migrate toward a site of inflammation along a chemical gradient in a process called chemotaxis. There are a number of exogenous and endogenous chemoattractants for leukocytes including cytokines/chemokines, leukotriene B4, bacterial peptides with N-formylmethionine termini, and, more recently, SCFA (in the case of neutrophils) (Kumar et al., 2013).

The cell types that are recruited in an acute inflammatory response depend on the duration of inflammation. Neutrophils are the first responders to the site of inflammation (first 6 to 24 hours) and then are replaced by monocytes (24 to 48 hours) (Kumar et al., 2013). After two days, monocytes and macrophages will begin to be active in the inflammatory response. After being recruited to the site of inflammation, leukocytes will be activated to destroy the foreign substance. They phagocytize particles and destroy microbes and dead cells within the phagosome by producing reactive oxygen species, lysosomal enzymes, and nitrogen species (Kumar et al., 2013). Neutrophils also will create extracellular traps that can destroy extracellular microbes and dead cells, as well as recruit other leukocytes to amplify the inflammatory response (Kumar et al., 2013).

Dextran sodium sulfate (DSS)-induced colitis

The most widely used mouse model for colitis research uses DSS dissolved in the rodent's water (Chassaing et al., 2014). It is a water-soluble, poly-anionic, sulfated compound that has a highly variable molecular weight (5-1400 kDa). The most effective way of inducing colitis is administration of 40-50 kDa DSS into drinking water. The mechanism by which DSS induces colitis is unclear; however, one underlying mechanism is that it causes damage to the epithelial monolayer in the large bowel, making the gut more penetrable to the microbiota and intestinal contents. Some of the major advantages of the DSS-rodent model is that it is rapidly induced, easy to control, reproducible, and simple to prepare. In IBD, there are intermittent bouts of inflammation, which can be easily achieved by modifying the concentration and frequency at which DSS is provided.

One of the major pitfalls to DSS colitis is that it does not require a T or B cell response in order to cause inflammation (Chassaing et al., 2014). Therefore, the model is best when used to study the innate immune response. Despite this shortcoming, DSS has offered the most reliable

simulation of ulcerative colitis in humans. Ulcerative colitis is driven more by a Th2 response by showing resulting in concentrations of IL-4, IL-10, IL-5, and IL-13. It has been shown in a chronic colitis model of DSS (multiple longer bouts of DSS) that IL-4 secretion is upregulated (Stevceva et al., 2001). Furthermore, IL-12 knockout mouse models have been found to be protective against DSS colitis, whereas IL-18 knockouts have an exacerbated inflammatory response to DSS (Takagi et al., 2003). Both IL-12 and IL-18 strongly induce interferon-gamma (IFN-γ), and IL-18 and IL-2 in combination induce IL-4, which is a strong Th2 cytokine (Dinarello, 1999; Yoshimoto et al., 1999; Hoshino et al., 2000).

Dietary fiber and DSS colitis

Diet has been known to affect the severity of IBD. In particular, diets high in total fat, omega-6 fatty acids, meat, and total polyunsaturated fatty acids have been associated with increased risk of ulcerative colitis (Hou et al., 2011). Evidence from a DSS-induced colitis rat model suggested a variety of bacteria (e.g., *Bacteroides* spp. and others) may be involved in initiating the underlying inflammatory response (Seksik et al., 2003). Dietary fiber is a nutrient of interest in controlling the inflammatory response in IBD. Previous studies testing prebiotic fibers in DSS-colitis rodent models have typically elicited anti-inflammatory effects. Ingestion of dietary fibers such as rice bran, resistant starch, and hydrolyzed plant polysaccharide (Ambrotose® Complex and Advanced Ambrotose® Complex) were found to reduce mucosal damage and have anti-inflammatory properties (Komiyama et al., 2011; Moreau et al., 2003; Naito et al., 2006). Conversely, dietary flaxseed, which contains anti-inflammatory bioactive components such as fermentable fiber, phenolic compounds, and the polyunsaturated fatty acid, α-linolenic acid, was found to exacerbate colonic damage and inflammatory cytokines even with increases in cecal SCFA concentrations (Zarepor et al., 2014). Consumption of highly fermentable FOS also has

produced mixed results in reducing DSS-induced colonic damage (Winkler et al., 2007; Goto et al., 2010). A study investigating the fiber enriched vs. low-fiber germinated barley foodstuff in DSS-colitis found that the fiber component was anti-inflammatory (Kanauchi et al., 2001). Collectively, these studies suggest that moderately fermentable and butyrogenic dietary fibers are the most effective at attenuating DSS-colitis.

Dietary fiber and other models of colitis

Research involving dietary fermentable fiber and control of the inflammatory response has been shown mainly in mice, rats, and guinea pigs. Rats induced with trinitrobenzenesulfonic acid (TNBS) colitis and given a resistant starch diet were found to have less sickness behavior and increased proliferation of intestinal epithelium compared with rats fed no resistant starch (Jacobasch et al., 1999). Similarly, rats induced with TNBS colitis and fed 5% Plantago ovata seeds had decreases in myeloperoxidase (MPO) and nitric oxide activities, as well as a reduction in colonic tumor necrosis factor-alpha (TNF-α) compared to rats fed no fiber (Rodriguez-Cabezas et al., 2002). Another study in a more translational model to humans suggests that TNBS-induced colitis pigs ingesting soluble fiber dextran had a decreased inflammatory response, more beneficial microbiota (*Peptostreptococcus* and *Bifidobacterium*), and less weight loss compared to a no fiber group (Pouillart et al., 2010). Collectively, this suggests that with ingestion of dietary fermentable fiber and the concentrations of SCFA, the inflammatory response could be lessened in IBD. However, it is still crucial to elucidate the mechanisms that integrate dietary fermentable fiber modulating the microbiome that is indicative of SCFA concentrations and, specifically, how that could control inflammation.

Short-chain fatty acids and inflammation

The mechanisms by which dietary fermentable fiber could control inflammation are complex. The link between microbial metabolism and host health is mostly attributed to SCFA concentrations (Ganapathy et al., 2013). The inflammatory response is induced in a number of chronic physiological conditions such as kidney disease, IBD, diabetes mellitus, and obesity. Consumption of diets low in fiber and high in refined carbohydrates has been found to increase plasma pro-inflammatory markers IL-6, TNF-α, and IL-18, even in otherwise healthy individuals (Giugliano et al., 2006). Dietary fiber has been found to decrease the inflammatory response in diseases such as chronic kidney disease, diabetes mellitus, and obesity (Fujii et al., 2013; Slavin, 2005). One of the mechanisms that could explain a decrease in the inflammatory response is the role of microbial-derived SCFA in controlling the inflammatory response.

The three main SCFA produced in the colon from carbohydrate fermentation are acetate, propionate, and butyrate. The concentration of these compounds varies over the length of the gastrointestinal tract. Generally speaking, the proximal colon has a higher concentration of SCFA (70 to 140 mM) in all mammalian species due to greater availability of carbohydrate substrates compared with the distal colon (20 to 70 mM) where most of the carbohydrates have already been fermented (Slavin, 2005). In terms of inflammation and overall gut health, butyrate has been found to be the most beneficial in controlling intestinal inflammation. It is the preferred energy source for colonocytes, with only low concentrations of butyrate available to the liver. In dogs and humans, butyrate spares colonic oxidation of glucose and glutamine that can be used for energy in other tissues (Beaulieu et al., 2002; Topping et al., 2001). Evidence from recent cell culture studies investigating IBD and SCFA suggests butyrate reduces the inflammatory response by modulating antioxidant signaling pathways. Specifically, butyrate has been found to decrease inflammation

through inhibition of nuclear factor kappa-B (NFκB) (Segain et al., 2000; Russo et al., 2012), a master regulatory switch closely involved in inflammatory processes, and increased PPARγ in colonic epithelial cells (Kinoshita et al., 2002). Recently, there has been interest in how SCFA control inflammation through signaling leukocytes to the site of inflammation.

Short-chain fatty acids controlling leukocyte recruitment

One of the mechanisms by which dietary fermentable fiber could control the inflammatory response is through recruitment of leukocytes to the site of inflammation (Vinolo et al., 2009). Neutrophils, macrophages, $CD4^{+}$T lymphocytes, and monocytes are at the forefront of this recruitment effort as each of these cells plays a critical role in the innate immune response. The colon is a primary site where foreign pathogens can gain access to systemic circulation via endothelial cells. Prevailing hypotheses suggest SCFA produced by the colonic microbiome could control leukocyte recruitment by two main mechanisms: (1) activation of G-protein coupled receptors (GPR41 and 43) or free fatty acid receptors 3 and 2 (FFAR3 and FFAR2), and (2) inhibition of histone deacetylase (Vinolo et al., 2011).

The acute inflammatory response recruits leukocytes to the site of inflammation in a multi-step process that involves concentrations of chemokines and activation of adhesion molecules that interact with endothelial cells (Vinolo et al., 2011). The concentrations of SCFA may modify the recruitment of leukocytes to the inflammatory site; however, this was more pronounced *in vitro* than *in vivo* (Vinolo et al., 2009; Vinolo et al., 2011). The *in vivo* study in rats indicated that oral administration of the pro-drug of butyrate, tributyrin, reduced migration of leukocytes to the peritoneum in response to an inflammatory stimulus (Vinolo et al., 2011). The differences between *in vitro* and *in vivo* responses could be explained, in part, by the different properties of SCFA to

induce cell migration and decrease concentrations of pro-inflammatory cytokines by other immune cell types (i.e. macrophages) (Vinolo et al., 2009; Le Poul et al., 2003; Park et al., 2007).

Neutrophil recruitment to the site of inflammation has been of recent interest in terms of controlling the inflammatory response. Specifically, SCFA have been found to bind members of the FFAR2 family, which are highly expressed on neutrophils, monocytes, and adipocytes. Since SCFA are binding agonists for FFAR2, they serve to activate several intracellular pathways such as protein kinase C, mitogen-activated protein kinases (MAPKs), and other transcriptional factors involved with the chemotactic movement of neutrophils (Vinolo et al., 2011). Although SCFA are a chemoattractant for neutrophils *in vitro,* there are limited studies that investigate their *in vivo* effects, as well as the effects of a SCFA/FFAR2 chemotactic interaction in the presence of other agonists such as formyl-methionine-leucine-phenylalanine, chemokines, and leukotriene B4 (Vinolo et al., 2011). Recent evidence from a DSS-colitis-induced model involving FFAR2-knockout mice suggested that ingestion of solubilized acetate in the presence of colonic microbiota ameliorated the acute inflammatory response (Maslowski et al., 2009). Furthermore, the ingestion of SCFA also had a systemic effect on inflammation by reducing inflammation in mice with asthma (Maslowski et al., 2009). The *in vitro* and *in vivo* studies clearly show an effect of SCFA on mounting a neutrophil chemotactic response; however, these results imply ingestion of SCFA, not intake of dietary fermentable fiber with subsequent concentrations of SCFA by the gut microbiota.

Research involving dietary fermentable fiber and control of the inflammatory response has been shown mainly in mice, rats, and guinea pigs. Rats induced with trinitrobenzenesulfonic acid (TNBS) colitis and given a resistant starch diet were found to have improved sickness behavior and increased proliferation of intestinal epithelium compared with rats fed no resistant starch

(Jacobasch et al., 1999). Similarly, rats induced with TNBS colitis and fed 5% dietary fiber had decreases in MPO and nitric oxide activities, as well as a reduction in colonic TNF-alpha compared to rats fed no fiber (Rodriguez-Cabezas et al., 2002). Another study in a more translational model suggest that TNBS-induced colitis pigs ingesting soluble fiber dextran had a decreased inflammatory response, more beneficial microbiota, and less weight loss compared to a no fiber group (Pouillart et al., 2010). Collectively, this suggests that with ingestion of dietary fermentable fiber and the concentrations of SCFA, the inflammatory response could be protective in IBD. However, it is still crucial to elucidate the mechanisms of how prebiotic effects of certain dietary fibers change immune cell recruitment through increased concentrations of SCFA.

Conclusions

Dietary fiber is important in both companion animal and human health. In particular, dietary fermentable fiber has been found to be beneficial in suppressing inflammatory diseases such as IBD and obesity. The anti-inflammatory effects of dietary fermentable fiber are attributed to beneficial shifts in the colonic microbiome that promote the increased concentrations of SCFA. With new high-throughput sequencing and analytical techniques, the fields of metagenomics and metabolomics are able to be combined to enable a more thorough understanding of how the microbiome can be altered via dietary fermentable fiber to ultimately benefit systemic health of both companion animals and humans.

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CHAPTER 2: MODULATION OF THE FECAL MICROBIOME OF HEALTHY ADULT DOGS BY INCLUSION OF POTATO FIBER IN THE DIET[1]

Abstract

Inclusion of fermentable fibers in the diet can impact the hindgut microbiome and provide numerous health benefits to the host. Potato fiber (PF), a co-product of potato starch isolation, has a favorable chemical composition of pectins, resistant and digestible starch, cellulose, and hemicelluloses. The objective of this study was to evaluate the effect of increasing dietary PF concentrations on the fecal microbiome of healthy adult dogs. Fresh fecal samples were collected from ten female dogs with hound bloodlines (6.13 ± 0.17 yr; 22.0 ± 2.1 kg) fed five test diets containing graded concentrations of PF (0, 1.5, 3, 4.5, or 6% as-fed; Roquette Frères, Lestrem, France) in a replicated 5 x 5 Latin square design. Extraction of DNA was followed by amplification of the V4-V6 variable region of the 16S rRNA gene using barcoded primers. Sequences were classified into taxonomic levels using BLASTn against a curated GreenGenes database. Inclusion of PF increased ($P < 0.05$) fecal proportions of Firmicutes, while Fusobacteria decreased ($P < 0.05$). Similar shifts were noted at the genus level and were confirmed by qPCR analysis. With increasing concentrations of PF, fecal *Faecalibacterium* increased ($P < 0.05$). Post-hoc Pearson's correlative analysis showed positive ($P < 0.05$) correlations with *Bifidobacterium* spp. and butyrate concentrations and *Lactobacillus* spp.

[1] Panasevich, M. R., K. R. Kerr, R. N. Dilger, G. C. Fahey, Jr., L. Guérin-Deremaux, G. L. Lynch, D. Wils, S. E. Dowd, and K. S. Swanson. 2014. Modulation of the faecal microbiome of healthy adult dogs by inclusion of potato fibre in the diet. Br. J. Nutr. 113: 125-133.

Overall, increases in *Faecalibacterium* (not *Lactobacillus*/*Bifidobacterium*; as confirmed by qPCR analysis) with increasing dietary PF along with increasing fecal short-chain fatty acid concentrations suggests that PF is a possible prebiotic fiber.

Introduction

The gastrointestinal tract of humans and animals is occupied with a dense and diverse population of bacteria that are known to influence health of the host. Changes in the host's microbiome are affected by a number of factors including genetics, age, geographic location, and especially diet (Khachatryan et al., 2008; Mariat et al., 2009; Yatsunenko et al., 2012; Hooda et al., 2012). In particular, fermentable fibers have been known to favorably modulate the hindgut microbiome of both humans and animals (Hooda et al., 2012; Middelbos et al., 2010). However, the effects of dietary inclusion of graded concentrations of fermentable dietary potato fiber on the hindgut microbiome have not been investigated.

Potato fiber is a co-product of potato starch manufacture with potential to beneficially modulate the hindgut microbiome. It is high in fibers such as hemicelluloses, cellulose, and pectin, and non-fiber components such as starch, oligopeptides, and free amino acids (Mayer, 1998). The monomeric composition of destarched PF consists mainly of galactose, galacturonic acid, arabinose, and rhamnose, which indicates that the cell wall material is made up primarily of homogalacturonan and rhamnogalacturonan I with long galactan side chains (Thomassen et al., 2011). Limited research has investigated the use of PF as a dietary fiber source in dogs and humans. *In vitro* data utilizing human fecal inoculum indicates that solubilized PF fractions were fermentable and increased bifidobacteria and lactobacillinumbers more than fructo-oligosaccharides (Thomassen et al., 2011; Olesen et al., 1998; Laerke et al. 2007)

The current research is being reported in two parts: (1) effects of PF on *in vitro* fermentation, and *in vivo* effects on nutrient digestibility, fecal fermentation end-products, and consistency, and (2) assessing the modulatory effects of dietary PF on the fecal microbiome. Our laboratory previously published data from part 1, which concluded that PF was a moderately fermentable fiber both *in vitro* and *in vivo*, and had no detrimental effects on total nutrient digestibility or fecal consistency (Panasevich et al., 2013). Part 2 is presented here to investigate the effects of graded dietary PF concentrations on modulation of the dog fecal microbiome using 16S rRNA gene pyrosequencing. We predicted that our previous results (i.e. increases in fecal acetate, propionate, and butyrate and a decrease in fecal pH) would be associated with shifts in the fecal microbiome (Panasevich et al., 2013). Based on previous studies involving dietary fiber in pets and humans, we predicted that at the phylum level, an increase in fecal Firmicutes and a decrease in fecal Fusobacteria would occur (Hooda et al., 2012; Middelbos et al., 2010). More specifically, we also predicted increases in *Bifidobacterium* and other SCFA producing genera (e.g. *Blautia*, *Lachnospira*, and *Faecalibacterium*) with increasing dietary PF concentrations.

Materials and methods

Animals and diets

The animal protocol was approved by the University of Illinois Animal Care and Use Committee and the study was conducted at the Edward R. Madigan Laboratory at the University of Illinois. Ten female dogs with hound bloodlines (6.13 ± 0.17 yr; 22.0 ± 2.1 kg) were utilized in a replicated 5 x 5 Latin square design to test five diets in five time periods. Dogs were housed individually (2.4 x 1.2 m kennels) in two temperature- and light-controlled rooms. Fresh water was available *ad libitum*.

Five diets containing graded concentrations of PF (0, 1.5, 3, 4.5, or 6% as-is; Roquette Frères, Lestrem, France) were formulated to exceed nutrient recommended allowance values of adult dogs (Table 2.1), with target concentrations of approximately 26% crude protein and 15% crude fat (as-is basis) (NRC, 2006). To achieve similar dietary fiber and protein concentrations among diets, PF was added at the expense of cellulose (Solka Floc, International Fiber Corp., North Tonawanda, NY) and brewer's rice. The remainder of the dry, extruded, kibble diet was composed of: low-ash poultry by-product meal, poultry fat, brewer's rice, ground corn, and vitamin and mineral premixes. Diets were mixed and extruded at the Kansas State University Bioprocessing and Industrial Value-Added Program facility (Manhattan, KS) under the supervision of Pet Food and Ingredient Technology, Inc. (Topeka, KS).

Each of the five, 14-d periods consisted of a 10-d dietary adaptation phase followed by 4-d of fecal collection. Dogs were offered 155 g diet twice daily (0800 and 1700 h) to meet the recommended energy needs of the dog based on the estimated metabolizable energy content of the diet. Fresh fecal samples were collected at the same time as was reported previously (Panasevich et al., 2013). On d 11, a fresh sub-sample of fecal was collected for analysis of microbial populations. Sub-samples were collected in 2-mL cryovials within 15 min of defecation, snap frozen in liquid nitrogen, and stored at -80° C until DNA extraction. Fecal butyrate determination was conducted according to previous methods (Panasevich et al., 2013). Briefly, one aliquot of ~2 g was collected and placed in ~2 mL of 2N hydrochloric acid and analyzed by gas chromatography (Hewlett-Packard 5890A Series II, Palo Alto, CA). and a glass column (180 cm × 4 mm i.d.), packed with 10% SP-1200/1% H_3PO_4 on 80/100 + mesh Chromosorb WAW (Supelco Inc., Bellefonte, PA). Nitrogen was the carrier gas with a flow rate of 75 mL/min. Oven, detector, and injector temperatures were 125, 175, and 180°C, respectively.

Fecal DNA extraction and 454 pyrosequencing

Bacterial DNA was extracted (McInnes and Cutting, 2010) using the MO BIO PowerSoil™ Kit (MO BIO Laboratories, Carlsbad, CA). Concentration of extracted DNA was quantified using a NanoDrop ND–1000 spectrophotometer (Nano-Drop Technologies, Wilmington, DE) and diluted to 10 ng/mL.

Quality of DNA was assessed by electrophoresis using precast agarose gels (E-Gel® EX Gel 1%, Invitrogen, Grand Island, NY). Amplification of a 600-bp sequence of the V4–V6 variable region of the 16S rRNA gene was done using barcoded primers (Cephas et al., 2011). Amplicons from PCR then were purified further utilizing AMPure XP beads (Beckman-Coulter Inc., Indianapolis, IN). Amplicons were combined in equimolar ratios to create a DNA pool that was used for pyrosequencing. Quality of DNA from amplicon pools was assessed before pyrosequencing using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Pyrosequencing was performed at the W. M. Keck Center for Biotechnology at the University of Illinois utilizing a 454 Genome Sequencer and FLX titanium reagents (Roche Applied Science, Indianapolis, IN).

Bioinformatics

High quality (quality value > 25) sequence data derived from the sequencing process was processed using a proprietary analysis pipeline (www.mrdnalab.com) and as described previously (Dowd et al., 2008a; Dowd et al., 2008b; Edgar 2010; Capone et al., 2011; Dowd et al., 2011; Eren et al., 2011; Swanson et al., 2011). Briefly, sequences were depleted of barcodes and primers, short sequences (< 200 bp), sequences with ambiguous base calls, and sequences with homopolymer runs exceeding 6 bp. Sequences then were denoised and chimeras were removed. Operational taxonomic units (OTU) were defined after removal of singleton sequences and clustering at 3%

divergence (97% similarity). Then, OTUs were taxonomically classified using BLASTn against a curated GreenGenes database and compiled into each taxonomic level into both “counts” and “percentage” files (Desantis et al., 2006).

Quantitative PCR (qPCR)

Quantitative PCR was performed at Texas A&M University according to previous methods (Rossi et al., 2014; Malinen et al., 2005). Briefly, primers were targeting group for universal bacteria, genus for *Lactobacillus, Bifidobacterium, Blautia, Faecalibacterium* and *Fusobacterium,* and species for *Eschericia coli* and *Clostridium perfringens*. Real-time PCR conditions and primers used for *Lactobacillus* spp., *Fusobacteria* spp., *Blautia* spp., *Faecalibacterium* spp., *Bifidobacterium* spp., and *Clostridium perfringens* have been described previously (Rossi et al., 2014). Primers for *Eschericia coli* were Forward: 5'-GTTAATACCTTTGCTCATTGA-3' and R: 5'-ACCAGGGTATCTAATCCTGTT-3' (Malinen et al., 2005) with a Tm: 55°C. Standard curves were generated using DNA (ranging from 2 ng to 0.2 pg) from lyophilized bacterial species of the genera (*Faecalibacterium prausnitzii* (ATCC 27766); *Lactobacillus rhamnosus* GG (ATCC 53103); *Bifidobacterium bifidum* (ATCC 11863); *Blautia coccoides* (ATCC 29236); *Fusobacterium nucleatum* (ATCC 25586)), clinical isolates from *E. coli* and *C. perfringens,* and canine fecal community DNA for universal bacteria. The qPCR data was expressed as log amount of DNA (fg) for each particular bacterial group per 10 ng of isolated total DNA (Suchodolski et al., 2012).

Statisitcal analyses

Sequence percentages at each taxonomic level were analyzed using the Mixed models procedure of SAS (version 9·2; SAS Institute, Inc. Cary, NC). The fixed effect of treatment was tested, and dog and period were considered random effects. ProcUnivariate was used to test for

homogeneity of variance. For data with non-homogeneous variance, observed values were reported, and these data were transformed using the log function prior to statistical analysis. Means were separated for treatments using a Fisher-protected least significant difference with Tukey's adjustment. Chao 1 values were calculated and rarefaction curves were created to compare microbial diversity and species richness among samples. Principal components analysis was calculated to determine clustering effects among diets. Results are reported as least-squares means with $P \leq 0.05$ defined as significant. Means that were above or below 3 standard deviations were considered outliers and removed. Post-hoc Pearson's correlations on the qPCR, sequence, and SCFA data were analyzed by the ProcCorr procedure in SAS. Additionally, orthogonal contrasts were used to test linear and quadratic effects of providing graded concentrations of dietary PF, and all PF treatments (1.5 to 6%) were compared to the 0% PF control using a single degree of freedom contrast.

Results

Pyrosequencing of 16S rRNA gene barcoded amplicons resulted in a total of 1,040,107 sequences, with an average of 20,802 reads (range: 10,647 to 56,247) per sample. Samples had an average read length of 542 base pairs. According to Chao 1 values and rarefaction curves (data not shown), microbial diversity and species richness was similar among treatments. Principal component analysis revealed no clustering amongst dietary treatments (data not shown). Regardless of dietary treatment, Firmicutes (22.63 to 94.21% of all sequences) was the predominant bacterial phylum in dog fecal, followed by Fusobacteria (0.53% to 58.18% of all sequences) and Tenericutes (1.78 to 40.96% of all sequences; Table 2.2). Actinobacteria (0 to 3.47% of all sequences), Bacteroidetes (0 to 9.06% of all sequences), and Proteobacteria (0 to 12.53% of all sequences) also were present. Inclusion of graded PF concentrations in canine diets

altered the proportions of bacterial phyla in the canine fecal microbiome. With increasing PF concentrations, the proportion of Firmicutes exhibited a positive quadratic response ($P < 0.05$), with the highest concentration observed in dogs fed the 4·5% PF diet. Fusobacteria exhibited a negative quadratic response ($P < 0.05$), with the lowest proportion observed in dogs fed the 4·5% PF diet. Tenericutes decreased linearly ($P < 0.05$) with increasing dietary PF concentrations.

Fusobacterium (0.53% to 58.18% of all sequences), *Clostridium* (3.21 to 61.35% of all sequences), *Blautia* (1.77 to 26.38% of all sequences), and *Faecalibacterium* (0.20 to 27.99% of all sequences) were the predominant genera in dog fecal (Table 2.2). The proportion of *Fusobacterium* exhibited a negative quadratic response ($P < 0.05$), with the lowest concentrations observed when dogs were fed the 4.5% PF diet. Within the phylum of Firmicutes, *Clostridium* was the predominant genera for dogs fed 0 to 4.5% PF. For dogs fed 6% PF, the proportion of *Clostridium* was lower ($P < 0.05$) than that for dogs fed the 0, 3, or 4.5% PF diets, while *Faecalibacterium* was greater ($P < 0.05$) than that for dogs fed the 0, 1.5, or 3% PF diets. The proportion of *Clostridium* exhibited a quadratic response ($P < 0.05$) with increasing dietary PF concentrations, with dogs fed 6% PF having lower proportions ($P < 0.05$) compared to dogs fed 0% PF. The proportions of *Faecalibacterium* and *Lachnospira* were greater ($P < 0.05$) for dogs fed all diets containing PF compared to those fed 0% PF. *Faecalibacterium* increased linearly ($P < 0.05$), while *Lachnospira* increased quadratically ($P < 0.05$), with increasing dietary PF. The proportions of *Blautia* increased quadratically ($P < 0.05$), with peak proportions observed when dogs were fed the 4.5% PF diet. For fecal *Coprobacillus*, there was also a quadratic increase ($P < 0.05$), with peak proportions observed when dogs were fed the 1.5 and 3% PF diets. The proportions of *Prevotella* (member of Bacteroidetes) and *Succinivibrio* (member of Proteobacteria) were lower ($P < 0.05$) for dogs fed all PF-containing diets compared with dogs fed

the 0% PF diet, and also exhibited a linear decrease ($P < 0.05$) with increasing dietary PF concentration. Bacterial species also were quantified and are presented as a supplementary table (Table 2.3).

Quantitative PCR was conducted on select groups of bacteria (universal bacteria), genera and species. Figure 2.1 shows the scatter plots of sequence and qPCR data with median values, and Pearson's correlation scatter plots of qPCR vs. 454-pyrosequencing for *Faecalibacterium* spp., *Fusobacterium* spp., and *Blautia* spp. Fecal *Faecalibacterium*, *Fusobacterium,* and *Blautia* spp. showed no significant diet effects with increasing concentrations of PF in the diet ($P = 0.10$, 0.07, and 0.32, respectively; means are not shown). A single degree of freedom contrast of the qPCR data calculating the difference between 0% PF vs all other diets containing PF revealed that fecal *Faecalibacterium* spp. were higher ($P < 0.05$) in dogs fed PF-containing diets compared to the 0% PF diet. Fecal *Fusobacterium* spp. were lower ($P < 0.05$) for dogs fed PF-containing diets compared to the 0% PF diet. There was a positive correlation between the sequence and qPCR data for *Faecalibacterium* spp. ($R = 0.64$; $P < 0.01$) and *Blautia* spp. ($R = 0.69$; $P < 0.01$). There was no correlation between sequence and qPCR data for *Fusobacterium* spp. ($R = 0.10$; $P < 0.44$).

Scatter plots for qPCR of *Bifidobacterium* and *Lactobacillus* spp. with median values and Pearson's correlation plots of *Bifidobacterium* spp. and butyrate and *Lactobacillus* spp. and *Bifidobacterium* spp. are shown in Figure 2.2. There were no differences in mean values (data not shown) for *Bifidobacterium* spp. and *Lactobacillus* spp. among dietary treatments; however, there were significant correlations between *Bifidobacterium* spp. and butyrate ($R = 0.49$; $P < 0.01$) and *Lactobacillus* and *Bifidobacterium* spp. ($R = 0.82$; $P < 0.01$). Scatter plots for Escherichia coli and *Clostridium perfringens* 16S are presented as a supplementary figure (data not shown).

Discussion

Part 1 of this study investigated the viability of PF as a potential dietary fiber source in dog foods by evaluating: (1) the fermentability of PF *in vitro* and (2) nutrient digestibility, fecal consistency, and fermentability of graded concentrations of dietary PF *in vivo* (Panasevich et al., 2013). Results from part 1 indicated that PF was fermentable through 9 h of *in vitro* fermentation, and showed linear increases in fecal acetate, propionate, and butyrate with increasing dietary PF concentrations (Panasevich et al., 2013). It was concluded that PF was moderately fermentable and showed no detrimental effects on total tract nutrient digestibilities or fecal consistency (Panasevich et al., 2013). The increased concentration of fecal SCFA, and in particular, butyrate, as well as decreases in fecal pH, were noted as being a benefit to host health. It is well known that SCFA have numerous benefits to the host, including improved gut health, satiety promotion, and attenuated inflammation (Wong et al., 2005; Tohurst et al., 2012; Vinolo et al., 2011). Furthermore, a decrease in fecal pH with increasing concentrations of dietary PF suggests carbohydrate fermentation throughout the entire length of the colon. After observing these beneficial changes in part 1, our objective in part 2 was to identify microbial taxa altered by the graded concentrations of dietary PF.

There is limited research investigating the use of PF as a dietary fiber source. The fiber fraction of potato fiber consists of 55% total dietary fiber, with 32% insoluble fiber and 23% soluble fiber. Specifically, the insoluble fiber fraction is predominantly cellulose (16%) and hemicellulose (9%). Pectin is the main component of the soluble fiber fraction of potato fiber. A study in rats reported that the soluble fiber fraction of PF resulted in higher cecal short-chain fatty acid concentrations compared to rats fed a non-fermentable cellulose control (Laerke et al., 2007). Research in humans indicated that the consumption of enzymatically-solubilized PF elicited

increased fermentation by increasing breath H_2 compared to oat bran (Olesen et al., 1998). Furthermore, *in vitro* data using human fecal inoculum indicated that solubilized PF that was minimally treated enzymatically was more bifidogenic than fructo-oligosaccharide (Thomassen et al., 2011). The chemical composition, *in vitro* fermentation, and *in vivo* responses, suggest PF has prebiotic potential; however, the effects of graded concentrations of dietary PF on the hindgut microbiome have yet to be studied.

Previous studies have investigated the modulatory effects of fiber on the hindgut microbiome in both dogs and humans (Hooda et al., 2012; Middelbos et al., 2010). We concluded from our previous work that PF is moderately fermentable *in vitro* and *in vivo* (Panasevich et al., 2013). Therefore, our diet formulation sought to keep total dietary fiber concentrations constant amongst all diets, while only changing the amount of PF at the expense of an inert fiber, cellulose. With this unique diet formulation, this study is the first to utilize high-throughput 16S rRNA gene-based pyrosequencing to determine how increasing concentrations of a moderately fermentable fiber modulates the hindgut microbiota of dogs.

Previous data indicated that a healthy dog fecal microbiome consists of approximately 14 to 52.5% Firmicutes and 7 to 50% Fusobacteria (Middelbos et al., 2010; Swanson et al., 2011; Handl et al., 2013; Suchodolski et al., 2008). The predominant bacterial phyla present in fecal of dogs fed all diets were Firmicutes and Fusobacteria. The order of predominant fecal bacterial phyla found in this study was different compared to other studies investigating the dog fecal microbiome. These differences could be explained by the variable regions of the 16S rRNA being amplified. For example, Middelbos et al. (2010) found healthy dogs to have predominantly Fusobacteria, followed by Firmicutes and Bacteroides, when amplifying the V3 region of 16S rRNA. Similarly, studies investigating the V1-V3 regions of 16S rRNA found very high proportions of Firmicutes

(96% OTUs) and very low proportions of other bacterial phyla. Our study investigated the V4-V6 regions of 16S bacterial rRNA, which resulted in different proportions of predominant bacterial phyla; however, the shifts in the microbiome are consistent with fiber supplementation (Middelbos et al., 2010). The supplementation of beet pulp has been found to increase the ratio of Firmicutes:Fusobacteria (Middelbos et al., 2010). A similar pattern was found in this study where increasing dietary PF concentrations elicited a quadratic increase in fecal Firmicutes and a quadratic decrease in fecal Fusobacteria.

Fermentability of fibrous substrates is dependent on the chemical composition, physical form, and amount of substrate available (Macfarlane and Macfarlane, 2003). The heat, pressure, and moisture applied in extrusion usually increases the availability of substrate for microbes to ferment and produce SCFA. Typically, the molar concentrations of SCFA in the hindgut are greatest in acetate, then propionate, and butyrate often is present in the lowest concentration. Butyrate is a major energy source for colonocytes and promotes cell apoptosis, stimulation of cell proliferation, and prevention of colon cancer (Hamer et al., 2008). Part 1 of this study found increases in all three SCFA with increasing dietary PF. Changes within the Firmicutes phylum with increasing PF was consistent with our results on fecal SCFA, and provides some insight about the physiologic relevance of this study.

Diversity within the phylum Firmicutes is indicative of a reduced risk of certain hindgut diseases, as well as concentrations of a diet containing fermentable carbohydrates (Hooda et al., 2012; Middelbos et al., 2010; Manichanh et al., 2006). Increases in butyrate and other SCFA also may be revealing of shifts in the hindgut microbiome (Wong et al., 2006). Our results do not show increased diversity within the phylum Firmicutes; however, we show an increase in abundance of key fermenters. We show an increase in *Faecalibacterium* spp. with increasing dietary PF, which

was consistent with previous studies examining fermentable fiber in dog and human diets (Hooda et al., 2012; Middelbos et al., 2010). Dogs ingesting beet pulp, a moderately fermentable fiber with a similar chemical composition to PF, were found to have more *Faecalibacterium prausnitzii* represented in fecal samples compared to dogs provided no fiber (Middelbos et al., 2010). This bacterium has received increased attention due to the human literature suggesting its protective effects in IBD (Sokol et al., 2008; Ramirez-Farias et al., 2009). In dogs, it generally has been accepted as a beneficial bacterium due to its depletion in IBD and its ability to produce butyrate; however, its physiologic relevance has not been fully elucidated (Suchodolski et al., 2012; Louis et al., 2010). Our qPCR analysis of this bacterium confirm its presence, and the analysis was highly correlated with our sequencing data. Furthermore, both the sequencing and qPCR data show similar patterns of increased concentrations of *Faecalibacterium* with increasing dietary PF.

Fecal *Blautia* and *Fusobacterium* spp. were two genera that were abundant in our sequencing data and were confirmed by qPCR. There was a significant positive correlation between qPCR and sequencing data when analyzing fecal *Blautia* spp., but no significant correlation was observed with *Fusobacterium* spp. This result was not entirely surprising, since our previous publications using these primers tended to have a bias towards *Fusobacterium* spp. (Middelbos et al., 2010; Swanson et al., 2011). The qPCR data did not show significant diet effects; however, the pattern noticed with increasing PF seemed to be similar with the sequence data. In dogs, low fecal concentrations of *Blautia* spp. have been associated with acute-hemorrhagic diarrhea and are a part of the butyrate producing superfamily, *Lachnospiraceae.* *Fusobacterium* spp. have previously been reported to have associations with liver cirrhosis and oral cavity infections in humans (Swidsinski et al., 2011; Bennett and Eley, 1993); however, the presence of this bacterial phyla is common in healthy dogs (Middelbos et al., 2010; Tolhurst et al.,

2012; Vinolo et al., 2011). An increase in fecal *Blautia* spp. and a decrease in fecal *Fusobacterium* spp. are consistent with dietary fiber intake data (Middelbos et al., 2010).

The physiologic relevance of these results was further investigated by conducting post-hoc Pearson's correlation analysis with fecal SCFA data reported previously and qPCR data (Panasevich et al., 2013). Prebiotics often are evaluated by their ability to produce butyrate (a butyrogenic effect) or to stimulate the growth of bifidobacteria (a bifidogenic effect); however, the definition of a prebiotic is not limited to this genus (De Vuyst and Leroy, 2011; Tremaroli and Backhed, 2012). We found *Bifidobacterium* spp. were positively correlated with butyrate and lactobacilli concentrations. Bifidobacteria's main carbohydrate fermentation products are acetate and lactate when substrate is available (Suchodolski et al., 2008), while lactobacilli produce lactic acid. Bifidobacteria growth has been found to accompany butyrate concentrations with inulin-type fructans (De Vuyst and Leroy, 2011). This has previously been explained by bacterial cross-feeding where primary fermenters of carbohydrate will produce secondary metabolites (acetate and lactate) or hydrolyzed substrate that can be utilized by other bacteria (De Vuyst and Leroy, 2011). Pectic oligosaccharides have been found to increase bifidobacteria *in vitro*; however, the effect is dependent on degree of methylation, and it was concluded oligo-fructose was more bifidogenic (Olano-Martin et al., 2002). An *in vitro* fermentation study utilizing human fecal inoculum found that solubilized PF that had minimal enzymatic treatment was more bifidogenic than fructooligosaccharide (Thomassen et al., 2011). The positive correlations between *Bifidobacterium* spp. with butyrate concentrations and lactobacilli growth further suggest the prebiotic potential of PF; however, the bifidogenic properties of PF need to be investigated further.

In conclusion, increasing concentrations of dietary PF were found to modulate the fecal microbiome at the phylum and genus taxonomic levels. This was confirmed by qPCR where fecal

Faecalibacterium and *Blautia* spp. showed significant positive correlations with the 454-pyrosequencing data. Furthermore, part 1 of this study found increases in fecal acetate, propionate, and butyrate with increasing dietary PF, which appears to agree with changes in the fecal microbiome. Post-hoc correlative analysis also showed significant correlations with *Bifidobacterium* spp. with butyrate concentrations and *Lactobacillus* spp. concentrations. One potential pitfall to this study that should be noted is the use of BLAST to infer taxonomy instead of an RDP classifier. Inferences made in this study were only on bacterial group's sequences confirmed by qPCR and at the phylum and genus taxonomic levels. It has been previously noted that only small differences exist between RDP and BLAST at higher taxonomic levels (Jumpstart Consortium, 2012).Overall, this research suggests that PF is beneficial to gut health and should be investigated as a novel functional ingredient in dog diets. Future research investigating other novel fiber sources effects on the microbiome could help explain how different clusters of bacterial families and genera respond to fermentable substrates.

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Table 2.1. Ingredient and chemical composition of experimental diets fed to dogs

	% potato fiber (PF)*				
Items	0	1.5	3	4.5	6
Ingredient (% as fed)					
Brewer's rice	46.55	45.90	45.25	44.60	43.95
Poultry byproduct meal-low ash	25.50	25.50	25.50	25.50	25.50
Corn, yellow, ground	12.00	12.00	12.00	12.00	12.00
Poultry fat	8.00	8.00	8.00	8.00	8.00
Potato fiber†	0.00	1.50	3.00	4.50	6.00
Cellulose§	6.00	5.15	4.30	3.45	2.60
Salt	0.70	0.70	0.70	0.70	0.70
Potassium chloride	0.56	0.56	0.56	0.56	0.56
Chromic oxide	0.20	0.20	0.20	0.20	0.20
Mineral mix|	0.18	0.18	0.18	0.18	0.18
Vitamin mix¶	0.18	0.18	0.18	0.18	0.18
Choline chloride-50%	0.13	0.13	0.13	0.13	0.13
Chemical composition (% DM)					
Dry matter	95.75	95.65	95.51	95.45	95.29
	---------- % dry matter basis ----------				
Organic matter	94.21	94.36	94.24	94.07	94.20
Crude protein	25.10	24.78	24.91	25.20	25.14
Acid-hydrolyzed fat	13.45	14.51	14.48	14.17	13.05
Total dietary fiber	10.79	11.24	11.43	11.17	11.35
Gross energy (kJ/g)	20.79	20.96	20.92	20.96	20.67

*Diet formulations contained raw PF at the specified concentrations; diets were extruded to produce the food provided to the dogs.

†Roquette Frères, Lestrem, France.

§Solka-Floc, International Fiber Corp., North Tonawanda, NY.

|Provided per kilogram of diet: Mn (as $MnSO_4$), 66.00 mg; Fe (as $FeSO_4$), 120 mg; Cu (as $CuSO_4$), 18.00 mg; Co (as $CoSO_4$), 1.20 mg; Zn (as $ZnSO_4$), 240 mg; I (as KI), 1.80 mg; Se (as Na_2SeO_3), 0.24 mg.

¶Provided per kilogram of diet: vitamin A, 5.28 mg; vitamin D_3, 0.04 mg; vitamin E, 120.00 mg; vitamin K, 0.88 mg; thiamine, 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; vitamin B_{12}, 0.11 mg.

Table 2.2. Predominant bacterial phyla and genera (expressed as a percentage of total sequences) in feces of dogs fed diets containing graded potato fiber (PF) concentrations[§]

Phylum	Family	Genus	% PF					SEM	*P*-Value
			0	1.5	3	4.5	6		
Actinobacteria			0.23[ab]	0.27[ab]	0.20[ab]	0.54[b]	0.10[a]	0.22	0.03
	Bifidobacteriaceae	*Bifidobacterium*	0.44	0.25	0.17	0.52	0.09	0.23	0.17
Bacteroidetes			1.17	0.70	1.17	1.05	0.73	0.33	0.57
	Bacteroidaceae	*Bacteroides*	0.39	0.43	0.70	0.47	0.51	0.20	0.72
	Prevotellaceae	*Prevotella*[‡]	0.78[b]	0.27[a]	0.47[ab]	0.59[ab]	0.26[a]	0.42	0.02
Firmicutes			56.66	64.33	64.52	68.20	58.17	5.99	0.17
	Clostridiaceae	*Clostridium*[†]	26.54[b]	23.00[ab]	26.96[b]	26.63[b]	14.61[a]	5.62	0.01
	Eubacteriaceae	*Eubacterium*	0.79	1.27	0.95	1.10	0.57	0.32	0.46
	Lachnospiraceae	*Blautia*[†]	11.27	12.24	12.50	13.29	10.40	2.11	0.22
		Lachnospira[‡†]	0.02[a]	0.92[ab]	1.52[b]	1.57[b]	1.51[b]	0.42	0.01
		Roseburia	0.83	0.70	0.29	0.76	0.33	0.27	0.37
	Lactobacillaceae	*Lactobacillus*	1.27	5.26	1.99	2.94	4.46	2.43	0.71
	Peptococcaceae	*Delsulfotomaculum*	1.26	1.26	0.84	0.70	0.88	0.40	0.48
	Ruminococcaceae	*Faecalibacterium*[‡*]	4.64[a]	9.38[a]	9.57[a]	10.23[ab]	15.69[b]	2.07	0.01
		Oscillospira	0.72	0.76	0.71	0.72	0.46	0.19	0.60
		Ruminococcus	5.49	5.26	4.63	4.93	4.72	1.05	0.86
	Turcibacteraceae	*Turicibacter*	0.89	0.67	0.96	1.02	0.66	0.27	0.49
	Veillonellaceae	*Megamonas*	0·62	0·71	0·80	1·03	0·96	0·38	0·93
		Phascolarctobacterium	3.30	3.57	3.65	3.45	3.49	0.85	0.99
Fusobacteria[†]			27.44	25.84	23.01	20.29	32.28	4.80	0.17
	Fusobacteriaceae	*Fusobacterium*[†]	27.44	25.84	23.01	20.29	32.28	4.80	0.17
Proteobacteria			1.53	1.77	1.80	2.72	2.78	0.83	0.62
	Succinivibrionaceae	*Succinivibrio*[‡*]	0.08	0.23	0.19	0.31	0.29	0.11	0.18
	Alicaligenaceae	*Sutterella*	1.39	1.53	1.52	2.00	2.43	0.77	0.82
Tenericutes[*]			12.74	7.09	9.29	7.20	5.20	2.52	0.18
	Erysipelotrichaceae	*Allobaculum*	10.52	4.31	6.38	5.28	3.57	2.55	0.67
		Bulleidia	0.21	0.18	0.22	0.15	0.16	0.06	0.57
		Catenibacterium	0.14	0.12	0.07	0.07	0.04	0.06	0.68
		Coprobacillus[†]	0.38	0.51	0.51	0.45	0.25	0.13	0.26
		Holdemania	0.07	0.10	0.08	0.08	0.04	0.04	0.59

Table 2.2 continued. Predominant bacterial phyla and genera (expressed as a percentage of total sequences) in feces of dogs fed diets containing graded potato fiber (PF) concentrations[§]

[§]Genera included have LSMEANs of 0.01 or higher.
[ab]Means in the same row with unlike superscript letters differ ($P < 0.05$).
[‡] Difference between 0% PF vs. all other PF diets ($P < 0.05$).
[*]Linear effect ($P < 0.05$).
[†]Quadratic effect ($P < 0.05$).

Table 2.3. Predominant bacterial species (expressed as a percentage of total sequences) in feces of dogs fed diets containing graded potato fiber (PF) concentrations[§]

	% PF						
Species	0	1.5	3	4.5	6	SEM	P-Value
Bifidobacterium pseudolongum	0.21[ab]	0.24[ab]	0.15[ab]	0.50[b]	0.09[a]	0.17	0.04
Prevotella copri[‡]	0.68[b]	0.24[a]	0.40[ab]	0.52[ab]	0.22[a]	0.36	0.02
Clostridium bifermentans	0.40	0.37	0.56	0.75	0.40	0.27	0.59
C. citroniae[‡*]	0.06	0.09	0.15	0.17	0.15	0.03	0.05
C. disporicum	0.01	0.02	0.02	0.03	0.03	0.01	0.89
C. hiranonis[*]	19.57[b]	16.64[ab]	19.64[b]	19.29[b]	10.42[a]	4.37	0.02
C. incertae sedis[†]	0.18	0.37	0.33	0.48	0.14	0.17	0.18
C. lactatifermen[†]	0.01	0.06	0.06	0.11	0.02	0.03	0.12
C. metallolevans	0.03	0.05	0.08	0.07	0.04	0.03	0.39
C. spiroforme	0.42	0.36	0.52	0.22	0.21	0.14	0.25
Allobaculum stercoricanis[#†]	2.51	3.42	3.83	2.72	2.45	0.84	0.16
Catenibacterium mitsuokai	0.13	0.11	0.06	0.06	0.03	0.05	0.64
Coprobacillus cateniformis	0.12	0.15	0.16	0.15	0.11	0.04	0.86
Turicibacter sanguinis	0.70	0.55	0.77	0.80	0.55	0.23	0.62
Eubacterium biforme	0.20	0.35	0.29	0.46	0.34	0.15	0.62
E. dolichum	0.15	0.18	0.10	0.09	0.04	0.06	0.24
Blautia producta[†]	6.33	7.81	7.61	7.56	5.98	1.34	0.31
Ruminococcus obeum	1.29[ab]	0.81[a]	1.44[ab]	2.32[b]	0.97[ab]	0.51	0.03
R. gnavus	1.04	0.94	0.83	1.06	0.60	0.38	0.38
R. torques	0.33	0.32	0.52	0.35	0.49	0.18	0.84
Lactobacillus acidophilus	0.11	0.05	0.09	0.07	0.09	0.07	0.96
L. johnsonii	0.68	3.54	0.66	2.04	3.45	1.82	0.62
L. reuteri (strain F275)	0.19	0.68	0.17	0.54	0.53	0.34	0.72
Faecalibacterium prausnitzii[‡*]	4.15[a]	8.67[a]	8.89[a]	9.63[a]	14.88[b]	2.00	0.01
Lactococcus lactis lactis	0.14	0.46	0.05	0.12	0.01	0.16	0.36
Fusobacterium varium	15.51	16.77	14.21	12.34	18.35	2.76	0.22

Table 2.3 continued. Predominant bacterial species (expressed as a percentage of total sequences) in feces of dogs fed diets containing graded potato fiber (PF) concentrations[§]

[§]Genera included have LSMEANs of 0.01 or higher.

[ab]Means in the same row with unlike superscript letters differ ($P < 0.05$).

[‡] Difference between 0% PF vs. all other PF diets ($P < 0.05$).

[*]Linear effect ($P < 0.05$).

[†]Quadratic effect ($P < 0.05$).

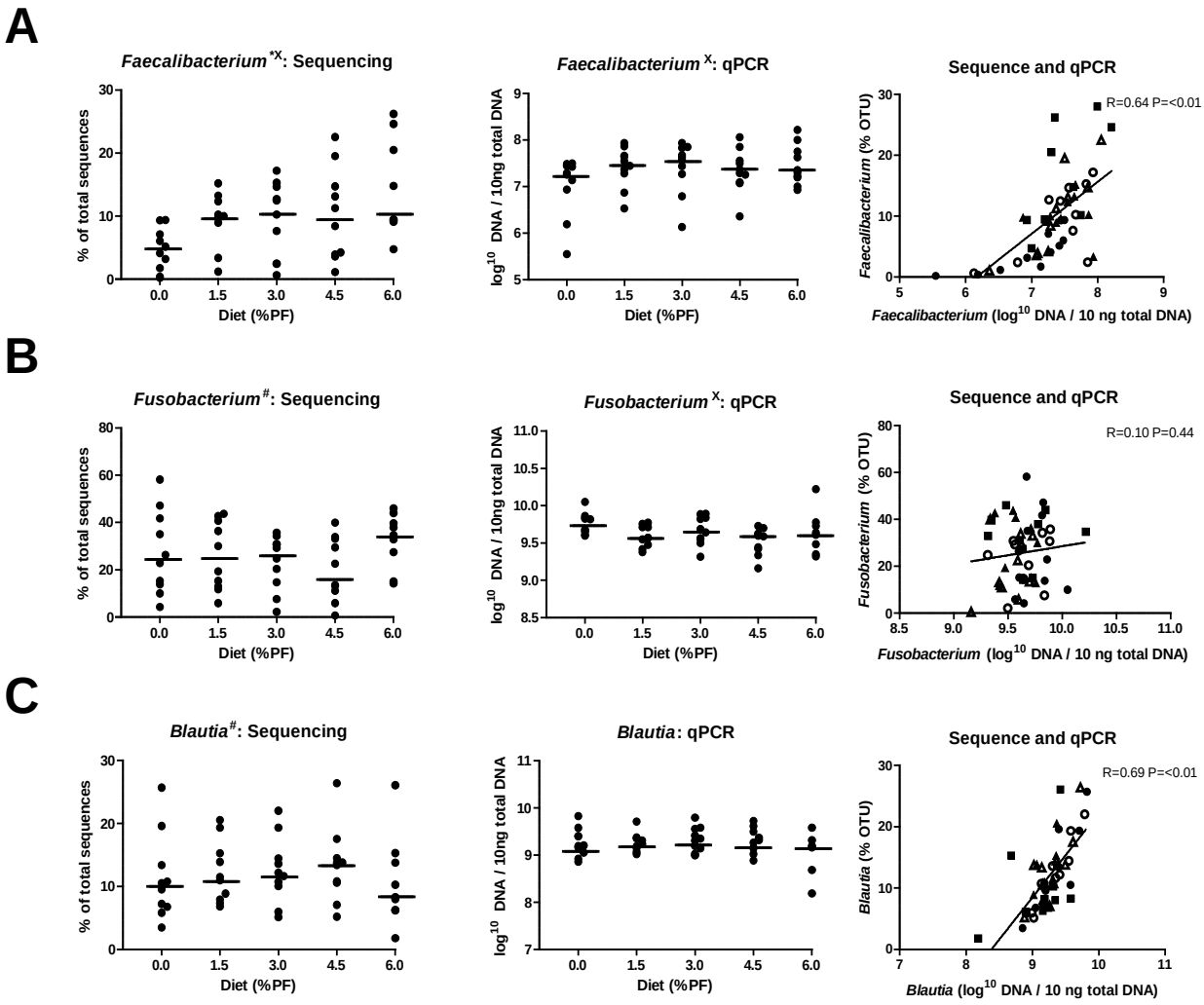

Figure 2.1. Row A: *Faecalibacterium* spp. scatter plots for the sequencing data, qPCR data, and Pearson's correlation analysis for sequence vs. qPCR data. Row B: *Fusobacterium* spp. scatter plots for sequencing data, qPCR data, and Pearson's correlation analysis for sequence vs. qPCR data. Row C: *Blautia* spp. scatter plots for the sequencing data, qPCR data, and Pearson's correlation analysis for sequence vs. qPCR data. *LSMEANs show a linear effect ($P < 0.05$); $^{\#}$LSMEANs show a quadratic effect ($P < 0.05$); ^{X}LSMEANs show a difference between 0% PF vs. all other PF diets ($P < 0.05$). Legend for Pearson's correlation plots: closed dots = 0% PF diet; closed triangle = 1.5% PF diet; open dot = 3% PF diet; open triangle = 4.5% PF diet; square = 6% PF diet.

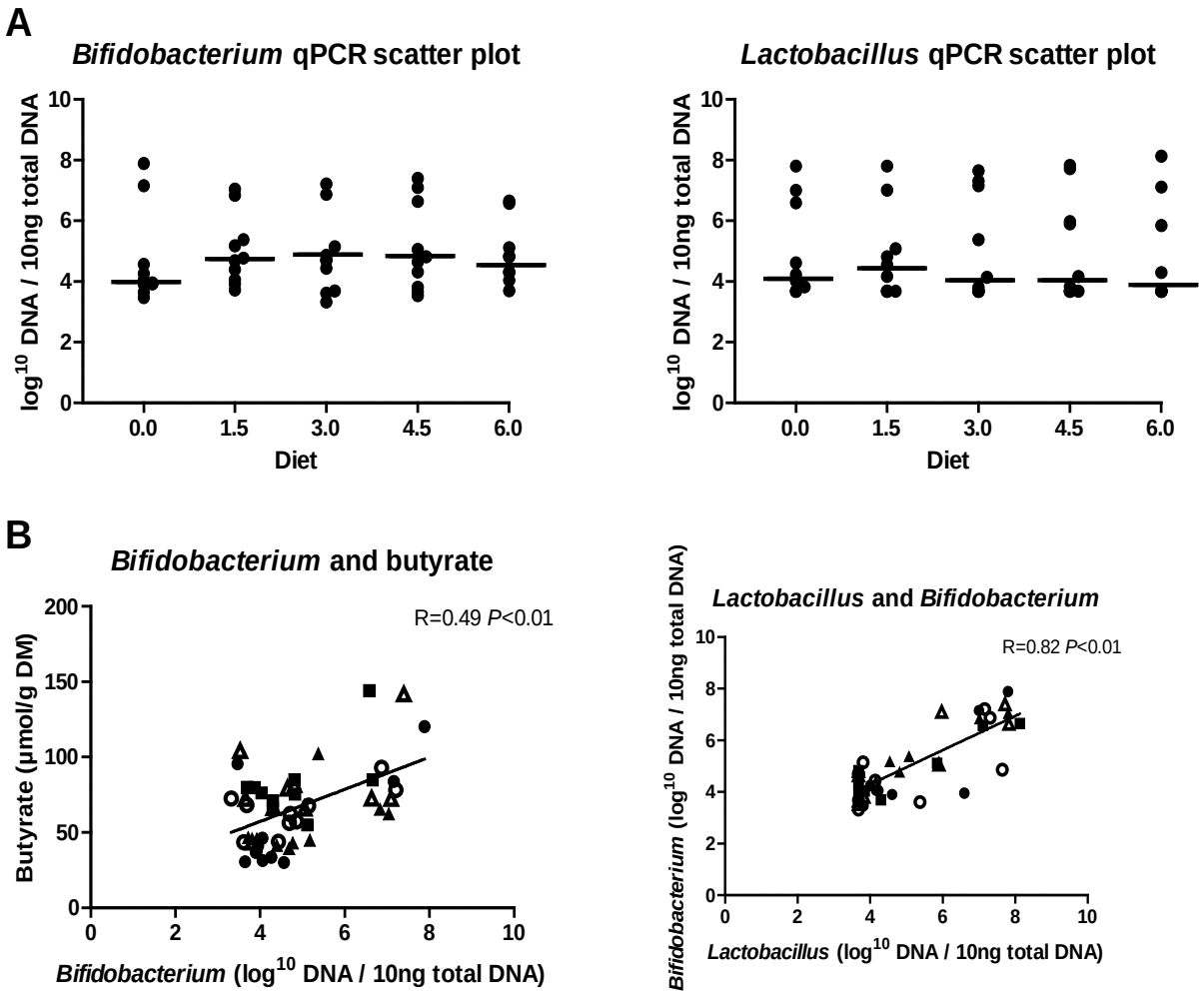

Figure 2.2. Row A: Scatter plots of qPCR data for *Bifidobacterium* and *Lactobacillus* spp. Data are represented as replicate values with a line to denote the median of each treatment group. Row B: Pearson's correlation plots for *Bifidobacterium* spp. qPCR data and butyrate and *Lactobacillus* spp. qPCR data and *Bifidobacterium* spp. Legend for row B: closed dots = 0% PF diet; closed triangle = 1.5% PF diet; open dot = 3% PF diet; open triangle = 4.5% PF diet; square = 6% PF diet.

CHAPTER 3: EVALUATION OF SOLUBLE CORN FIBER ON CHEMICAL COMPOSITION AND TMEn AND ITS EFFECTS ON IN VITRO FERMENTATION AND IN VIVO RESPONSES IN DOGS[1]

Abstract

Dietary fermentable fiber is known to benefit intestinal health of companion animals. Soluble corn fiber (SCF) was evaluated for its chemical composition, nitrogen-corrected true metabolizable energy (TMEn) content, *in vitro* digestion and fermentation characteristics, and *in vivo* effects on nutrient digestibility, fecal fermentation end-products, and modulation of the fecal microbiome of dogs. Soluble corn fiber contained 78% total dietary fiber, all present as soluble dietary fiber; 56% was low-molecular weight soluble fiber (did not precipitate in 95% EtOH). The SCF also contained 26% starch and 8% resistant starch, and had a TMEn value of 2.6 kcal/g. Soluble corn fiber was first subjected to *in vitro* hydrolytic-enzymatic digestion to determine extent of digestibility, then fermented using dog fecal inoculum, with fermentative outcomes measured at 0, 3, 6, 9, and 12 h. Hydrolytic-enzymatic digestion of SCF was only 7%. *In vitro* fermentation showed increased ($P < 0.05$) concentrations of short-chain fatty acids (SCFA) through 12 h, with acetate, propionate, and butyrate reaching peak concentrations of 1,803, 926, and 112 µmol/g DM, respectively. Fermentability of SCF was higher ($P < 0.05$) than for cellulose but lower ($P < 0.05$) than for pectin. In the *in vivo* experiment, ten female dogs (6.4 ± 0.2 yr; 22 ± 2.1 kg) received 5 diets with graded concentrations of SCF [0, 0.5, 0.75, 1.0, or 1.25% (as-is basis)] replacing cellulose in a replicated 5 x 5 Latin square design.

[1]Panasevich, M. R., K. R. Kerr, M. C. Rossoni Serao, M. R. C. de Godoy, L. Guérin-Deremaux, G. L. Lynch, D. Wils, S. E. Dowd, G. C. Fahey, Jr., K. S. Swanson, and R. N. Dilger. 2015 J. Anim. Sci. (in press).

Dogs were first acclimated to the experimental diets for 10 d followed by 4 d of total fecal collection. Fresh fecal samples were collected to measure fecal pH and fermentation end-products, and permit a microbiome analysis. For microbiome analysis, extraction of DNA was followed by amplification of the V4-V6 variable region of the 16S rRNA gene using barcoded primers. Sequences were classified into taxonomic levels using BLASTn against a curated GreenGenes database (accessed January, 2012). Few changes in nutrient digestibility or fecal fermentation end-products or stool consistency were observed, and no appreciable modulation of the fecal microbiome occurred. In conclusion, soluble corn fiber was fermentable *in vitro*, but higher dietary concentrations may be necessary to elicit potential *in vivo* responses.

Introduction

Prebiotic fibers in pet foods are becoming increasingly popular due to their favorable effects on gut function and health by increased concentrations of SCFA and changes in the intestinal microbiota (Propst et al., 2003). Most prebiotic fibers are rapidly fermentable, and if added at high concentrations to the diet, could result in negative digestive physiologic outcomes such as poor stool consistency and nutrient digestibility. Therefore, it is important to determine appropriate dietary concentrations of prebiotic fibers that modulate the microbiome and increase fermentation characteristics without affecting nutrient digestibility and (or) stool consistency.

Common prebiotic fibers often added to pet foods include inulin and oligofructose that promote SCFA concentrations and modulation of the microbiome (Propst et al., 2003). Low digestible carbohydrates are chemically-modified starches that increase SCFA concentrations and modify the microbiota in humans and animal models; however, neither the fermentation

characteristics nor the altering effects of the fecal microbiome has been studied to any extent in dogs.

Soluble corn fiber (NUTRIOSE® FM; Roquette; Lestrem, France) is a novel low digestible carbohydrate derived from hydrolysis of corn starch by heat and acid. Upon cooling, reformation of mixed β-glycosidic linkages resistant to mammalian enzymatic hydrolysis occurs. Soluble corn fiber is commonly used in the human food industry to aid in colonic health and as a low glycemic food additive (Knapp et al., 2010). Previous research has found it to be fermentable and have positive effects on changing the colonic microbiome of humans and rats; however, there is limited research on its use in dog foods. Therefore, the objectives of this research were to evaluate SCF for nutrient composition, *in vitro* digestion and fermentability, and *in vivo* responses (i.e., nutrient digestibility, fermentation end-products, and shifts in the intestinal microbiota) in dogs.

Materials and methods

Chemical analyses

Soluble corn fiber (NUTRIOSE®FM; Roquette Frères, Lestrem, France), experimental diets, and fecal samples were analyzed for dry matter (DM), organic matter, and ash according to standardized procedures (AOAC, 2006; methods 934.01 and 942.05). Crude protein was calculated from Leco (models FP2000 and TruMac; LECO Corp., St. Joseph, MI) total nitrogen values (AOAC, 2006; method 992.15). Total starch concentration of SCF was determined according to AOAC (2006; method 979.10). Total lipid content (acid-hydrolyzed fat) of each substrate was determined according to the methods of the American Association of Cereal Chemists (1983) and Budde (1952). Total dietary fiber, high-, and low-molecular weight soluble fiber of SCF were determined by AOAC (2005; method 2001.03). Briefly, high-molecular weight soluble fiber was determined as the portion that precipitated in 95% EtOH, while low-molecular weight soluble fiber

that did not precipitate in 95% EtOH was determined by HPLC. Experimental diets were analyzed for total dietary fiber, insoluble dietary fiber, and soluble dietary fiber concentrations according to Prosky et al. (1992). Free glucose and digestible starch concentrations were determined according to Muir and O'Dea (1993). Resistant starch was determined by subtracting digestible starch and free glucose from total starch concentration. 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).

Fecal SCFA and branched-chain fatty acid (BCFA) concentrations were determined by gas chromatography according to Erwin et al. (1961) using a gas chromatograph (model 5890A series II, Hewlett-Packard, Palo Alto, CA) and a glass column (180 cm x 4 mm i.d.) packed with 10% SP-1200/1% H_3PO_4 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. 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 amine concentrations were quantified using HPLC according to methods described by Flickinger et al. (2003).

In vitro hydrolytic digestion/fermentation simulation

The *in vitro* hydrolytic digestion/fermentation study was conducted according to Panasevich et al. (2013) with some modifications. Briefly, approximately 500 mg of SCF was weighed in triplicate and incubated with 12.5 mL phosphate buffer and 5 mL of a pepsin/hydrochloric acid solution at 39°C to simulate gastric digestion. After 6 h, the pH was adjusted to 6.8, and 5 mL pancreatin solution (Sigma-Aldrich Co., St. Louis, MO) was added to

each tube. Incubation continued at 39°C for 18 h to simulate small intestinal digestion (Boisen and Eggum, 1991). The set of samples prepared for enzymatic digestion then was assayed for released free sugars to correct for free glucose entering the *in vitro* fermentation.

In vitro fermentation was performed using a modification of the method of Bourquin et al. (1993). Following the *in vitro* digestion procedures described above, samples were hydrated overnight in 26 mL of anaerobic media. Fecal samples from three dogs were collected within 10 min of defecation and maintained at 39°C to prepare fresh inoculum. Prior to collection of feces, dogs had been maintained on a commercially-available food for 1 mo (Iams weight control, Procter & Gamble Pet Care, Cincinnati, OH). The fecal inoculum was prepared by blending 10 g of each fecal sample with 90 mL anaerobic diluting solution for 15 sec in a Waring blender under a stream of CO_2. The resulting solution was filtered through 4 layers of cheesecloth and sealed in 125 mL serum bottles pending the *in vitro* experiment.

Samples, blanks, and standards were inoculated with 4 mL of diluted feces. Solka floc (International Fiber Corp., North Tonawanda, NY) and high-methoxy pectin (TIC Gums Inc., Belcamp, MD) were used as negative and positive fermentation controls, respectively. Tubes were incubated at 39°C with periodic mixing. A subset of tubes was removed from the incubator at 0, 3, 6, 9, and 12 h post-inoculation and processed immediately for analyses. A 2 mL subsample of the fluid was removed and acidified for SCFA and BCFA analyses. Concentrations of SCFA and BCFA were determined by gas chromatography as described previously.

In vivo studies

Rooster study: True metabolizable energy

A nitrogen-corrected true metabolizable energy (TMEn) coefficient was determined using conventional single comb white leghorn roosters (n = 4) according to Kim el al. (2010). Briefly,

roosters were deprived of feed for 24 h and then crop-intubated with approximately 15 g of SCF and 15 g of corn with a known gross energy and nitrogen value (Sibbald et al., 1980). Roosters were crop intubated and excreta (urine plus feces) were collected for 48 h on plastic trays placed under each cage. Excreta samples were subsequently lyophilized, weighed, and ground to pass a 60-mesh screen and analyzed for gross energy content as described for samples above. Endogenous corrections for energy were made using roosters that had been food-deprived for 48 h. The TME_n values, corrected for endogenous energy losses, were calculated using the following equation:

TME_n (kcal/g) =

$$\frac{\text{Energy intake (kcal)} - \text{energy excreted by fed birds (kcal)} + \text{energy excreted by fasted birds (kcal)}}{\text{Feed intake (g)}}$$

Dog study: Animals and diets

Ten female dogs with hound bloodlines (6.4 ± 0.2 yr; 22 ± 2.1 kg) were utilized. Dogs were housed in individual kennels (2.4 x 1.2 m) in two temperature-controlled rooms with a 16 h light:8 h dark cycle. A replicated 5 x 5 Latin square design experiment was conducted with 5 diets and 10 dogs in two different rooms for five, 14-d periods. The first 10 d of each period served as an adaptation phase, followed by 4 d of total fecal collection. Five diets containing SCF were formulated to contain approximately 32% CP and 18% crude fat (DM basis; Table 4.1). Each diet contained graded concentrations of SCF [0, 0.5, 0.75, 1.0, or 1.25% (as-is basis)] that replaced cellulose (Solka floc; International Fiber Corp., North Tonawanda, NY) in the diet. Low-ash poultry by-product meal, poultry fat, brewer's rice, ground corn, and vitamin and mineral premixes constituted the remainder of the dry, extruded, kibble diets. All diets were formulated to exceed

NRC (2006) recommended allowances for an adult large breed dog. Diets were mixed and extruded at the Kansas State University Bioprocessing and Industrial Value-Added Program facility (Manhattan, KS) under the supervision of Pet Food and Ingredient Technology, Inc. (Topeka, KS). Dogs were offered 155 g of diet twice daily (0800 and 1700 h) to meet the required energy needs based on the estimated ME content of the diet. Food refusals were recorded daily, and fresh water was provided to the dogs *ad libitum*. Chromic oxide was added as a digestion marker but was not needed because of excellent stool quality and ease of fecal collection from the pen floor.

Sample handling and processing

Total feces excreted during the collection phase of each period were taken from the pen floor, weighed, and frozen at -20°C until analysis. All fecal samples during the collection period were subjected to a consistency score according to the following scale: 1 = hard, dry pellets, small hard mass; 2 = hard, formed, dry stool; remains firm and soft; 3 = soft, formed, and moist stool, retains shape; 4 = soft, unformed stool, assumes shape of container; and 5 = watery, liquid that can be poured.

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 used for 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 approximately 2 mL of 2 N hydrochloric

acid for ammonia, SCFA, and BCFA analyses. Additional aliquots were used for pH measurement and fresh fecal DM determination.

Microbiome analysis

Fecal DNA Extraction and 454 Pyrosequencing

Bacterial DNA was extracted according to McInnes and Cutting (2010) using the PowerSoil™ Kit (MO BIO Laboratories, Carlsbad, CA). Extracted DNA concentrations were quantified using a Qubit® 2.0 Fluorometer (Life Technologies, Carlsbad, CA) and diluted to 5 ng/mL. Quality of DNA was assessed by electrophoresis using precast agarose gels (E-Gel® EX Gel 1%, Invitrogen, Grand Island, NY). Amplification of a 600-bp sequence of the V4–V6 variable region of the 16S rRNA gene was done using barcoded primers (Cephas et al., 2011). Amplicons from PCR then were purified further utilizing AMPure XP beads (Beckman-Coulter Inc., Indianapolis, IN). Amplicons were combined in equimolar ratios to create a DNA pool that was used for pyrosequencing. Quality of DNA from amplicon pools was assessed before pyrosequencing using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Pyrosequencing was performed at the Roy J. Carver Biotechnology Center at the University of Illinois utilizing a 454 Genome Sequencer and FLX titanium reagents (Roche Applied Science, Indianapolis, IN).

Bioinformatics

High quality (quality value > 25) sequence data derived from the sequencing process was processed using a proprietary analysis pipeline (www.mrdnalab.com) and as described previously (Dowd et al., 2008a,b; Edgar, 2010; Capone et al., 2011; Dowd et al., 2011; Eren et al., 2011; Swanson et al., 2011) . Briefly, sequences were depleted of barcodes and primers, short sequences (< 200 bp), sequences with ambiguous base calls, and sequences with homopolymer runs

exceeding 6 bp. Sequences then were denoised, and chimeras were removed. Operational taxonomic units (OTU) were defined after removal of singleton sequences and clustering at 3% divergence (97% similarity). Then, OTUs were taxonomically classified using BLASTn against a curated GreenGenes database (accessed January 2012) (DeSantis et al., 2006) and compiled into each taxonomic level into both "counts" and "percentage" files. Only genera and species that represented greater than 0.01% of the total sequences were reported.

Statistical analysis

Data were analyzed as a completely randomized design using the Mixed procedure of SAS (Version 9.2; SAS Institute, Inc., Cary, NC). The UNIVARIATE procedure was used to assure equal variance, normal distribution, and to identify outliers. Any observation that was more than three standard deviations away from the mean was considered an outlier. Data were transformed by log or square root if the normality assumption was not met. The *in vitro* experimental data were analyzed using mean separation with a Tukey's adjustment to determine differences among substrates. For the *in vivo* dog experiment, diet was considered a fixed effect, whereas random effects included animal and period. Linear and quadratic effects were tested, using orthogonal polynomial contrasts. Differences among dietary treatments were determined using the least significant difference method. A probability of $P < 0.05$ was accepted as being statistically significant. Additionally, sequence percentages were compared using single degree of freedom orthogonal contrasts to test linear and quadratic effects of providing graded concentrations of dietary SCF, and all SCF treatments (0.5 to 1.25%) were compared with the 0% SCF control using a single degree of freedom contrast. Principal component analysis was used to assess shifts in variability between diets and Chao 1 and rarefaction curves were used to assess microbial diversity and species richness.

Results

Substrate chemical analysis

Soluble corn fiber was devoid of crude protein and ash and had very low concentrations of acid-hydrolyzed fat (Table 3.1). It contained a high amount of total dietary fiber (78.3%) that was completely soluble. Starch concentration was 25.5% with a notable proportion of resistant starch 7.8%. These values sum to 103.8% because a portion of the resistant starch was included in the TDF value. More low-molecular weight soluble fiber (55.5%) was present in SCF, as compared with high molecular weight soluble fiber (22.8%). The concentration of total free sugars was very low (28.5 mg/g DM), with glucose serving as the predominant free sugar (24.9 mg/g DM).

In vitro hydrolytic digestion/fermentation

Concentrations of SCFA produced over time from cellulose, SCF, and pectin are shown in Figure 3.1. During the *in vitro* hydrolytic-enzymatic digestion, SCF was only 7% digestible (data not shown), leaving 93% as indigestible material for subsequent *in vitro* fermentation. Once hydrolytic-enzymatic digestion was complete, the fermentation experiment was corrected for the release of free sugars.

Over the 12 h *in vitro* fermentation, a numerical decrease in pH due to concomitant increases ($P < 0.05$) in acetate, propionate, and butyrate concentrations with SCF was noted. Concentrations of acetate, propionate, and total SCFA were greater ($P < 0.05$) for SCF at each time-point compared with cellulose. Soluble corn fiber elicited higher ($P < 0.05$) butyrate concentrations at 6, 9, and 12 h compared with cellulose. In comparison with pectin, SCF produced lower ($P < 0.05$) acetate, propionate, butyrate, and total SCFA concentrations throughout the 12 h fermentation, which translated into less ($P < 0.05$) of a decrease in pH over time.

In vivo experiments

Rooster study: TMEn

The TMEn value was determined to be 2.6 kcal/g (Table 3.1).

Dog study

Table 3.2 presents the ingredient composition of the basal diet fed to dogs, and Table 3.3 presents the analyzed chemical composition of all experimental diets. All diets had similar DM, OM, CP, acid-hydrolyzed fat, TDF, and GE concentrations. During the feeding study, food intake was similar among treatments at 310 g/d as-is (288 g DM/d) and dogs consumed all of their food allotment (data not shown). Fecal output, apparent total tract nutrient digestibility, and fecal consistency scores are all presented in a supplementary table (Table 3.6). Briefly, fecal output and nutrient digestibility were not affected by increasing concentrations of dietary SCF. Fecal consistency was excellent across all diets, and no differences due to dietary treatment were observed.

Table 3.4 presents fecal SCFA, BCFA, and ammonia concentrations, as well as fecal pH values, for dogs. Fecal concentrations of acetate, propionate, and total SCFA were lowest ($P < 0.05$) when dogs were fed the 0.75% SCF diet. When compared with the 0.75% SCF diet, dogs fed 1.25% SCF had higher ($P < 0.05$) fecal acetate, propionate, and total SCFA concentrations. Fecal butyrate concentrations were not affected by treatment. Fecal BCFA and ammonia concentrations were low and showed no significant differences due to treatment. Other markers of protein fermentation, including phenols, indoles, and biogenic amines, were measured; however, these compounds were present at low concentrations and were not affected by dietary SCF concentration (data not shown).

Pyrosequencing of 16S rRNA gene-barcoded amplicons resulted in a total of 769,200 sequences, with an average of 15,384 reads (range: 8,776 to 32,349) per sample. Samples had an

average read length of 506 base-pairs. According to Chao 1 values and rarefaction curves (data not shown), microbial diversity and species richness were similar among dietary treatments. Principal component analysis revealed no separation among dietary treatments (data not shown).

Regardless of dietary treatment, Firmicutes (24.78 to 92.69% of all sequences) was the predominant bacterial phylum in dog feces, followed by Fusobacteria (0.11 to 52.21% of all sequences) and Tenericutes (2.58 to 54.04% of all sequences; Table 3.5). Actinobacteria (0 to 9.68% of all sequences), Bacteroidetes (0 to 3.22% of all sequences), and Proteobacteria (0 to 8.41% of all sequences) were also present. No statistically significant changes were noted among treatments, but there was a numeric increase in the proportions of Firmicutes and a numeric decrease in Fusobacteria with SCF supplementation.

Fusobacterium (0.11 to 52.21% of all sequences), *Clostridium* (7.31 to 53.29% of all sequences), *Blautia* (4.38 to 34.04% of all sequences), and *Allobaculum* (0.27 to 53.58% of all sequences) were the predominant genera in dog feces (Table 3.5).Fecal *Lachnospira* increased ($P < 0.05$) with increasing concentrations of dietary SCF. The proportions of *Roseburia* and *Ruminococcus* decreased ($P < 0.05$) linearly with increasing concentrations of SCF. Within the Tenericutes phylum, the proportion of *Catenibacterium* increased linearly ($P < 0.05$) with increasing SCF concentrations. Fecal *Coprobacillus* exhibited a linear decrease ($P < 0.05$) and, overall, dogs fed diets containing SCF had lower ($P < 0.05$) *Coprobacillus* compared with dogs fed the 0% SCF diet. Bacterial populations at the species level are presented as supplementary material (Table 3.7).

Discussion

Functional food ingredients that are becoming increasingly popular include low digestible carbohydrates that induce prebiotic effects. Prebiotics are defined as non-digestible food

ingredients that, when consumed in sufficient amounts, selectively stimulate the growth, activity or both of one or a limited number of microbial genera or species in the gut microbiota that ultimately benefits health of the host (Tremaroli and Backhed, 2012). Common prebiotic fibers present in companion animal and human foods include fructooligosaccharides, inulin, and resistant starch (Tomasik and Tomasik, 2003). Low digestible carbohydrates often are low-molecular weight and resist mammalian hydrolytic/enzymatic digestion, and will enter the large bowel to be fermented by microbes to produce SCFA and lower digesta pH (Mussato and Manchilha, 2007). They are similar to dietary fiber in that they have the ability to provide health-promoting effects on the host (Knapp et al., 2010).

Soluble corn fiber contained 78% total dietary fiber, all of which was soluble fiber, with 55% in a low molecular weight form. Soluble corn fiber is a purified fiber source having no or very low concentrations of ash, CP, AHF, and free sugars, and a moderate amount of both digestible and type-4 resistant starch. The SCF used in this study was a soluble fiber dextrin derived from corn starch that is considered a low digestible carbohydrate due to its high proportion of low-molecular weight soluble fiber. Normally, corn starch is made up of α-1,4 and α-1,6 glycosidic bonds that are easily degraded by mammalian pancreatic amylase. The dextrinization process uses heat and acid to hydrolyze the α-glycosidic bonds. Upon cooling, the reformation of both digestible glycosidic bonds (α-1,4 and α-1,6), as well as non-digestible glycosidic bonds (β-1,4, 1,6, 1,3, and 1,2), make up the short-chain oligosaccharides that then can enter the large bowel for fermentation by the resident microbiota (Knapp et al., 2010). Previous studies have shown that SCF is a good candidate for low energy and low glycemic dog diets (de Godoy et al., 2013), but no information was available regarding prebiotic potential of SCF fed to dogs.

The *in vitro* hydrolytic-enzymatic digestion experiment suggested that SCF was only 7% digestible, leaving 93% of the substrate available for fermentation. The SCF was highly fermentable throughout the entire 12 h fermentation, exhibiting increases in SCFA concentrations at each time point. Wheat dextrin soluble fiber (NUTRIOSE®FB06) was tested for *in vitro* fermentation properties using human fecal inoculum (Hobden et al., 2013). In that study, acetate, propionate, and butyrate were reported to increase in the distal portion of the gut model compared with the proximal portion, indicating that the wheat dextrin soluble fiber substrate was potentially fermentable throughout the gastrointestinal tract. Furthermore, wheat dextrin soluble fiber modulated the microbiota in the model, with increases in butyrate-producing taxa (Hobden et al., 2013). Knapp et al. (2010) determined that a variety of soluble fiber dextrins, including those derived from corn starch, may be potential substrates for hindgut fermentation due to their ability to resist *in vitro* hydrolytic-enzymatic digestion. This was supported further by low glycemic responses in dogs (Knapp et al., 2010).

Previous studies that have determined TMEn values of various SCF substrates that were obtained by different methods of starch hydrolysis reported values as low as 1.7 kcal (Knapp et al., 2010) and as high as 3.0 kcal (de Godoy et al., 2014). Our TMEn value of 2.6 kcal/g is accurate since the SCF used in this study was treated with hydrochloric acid and was consistent with the TMEn value obtained previously using the same processing method (2.4 kcal/g) (de Godoy et al., 2014). The variation in TMEn values of different SCF substrates has been attributed to differences in processing methods and molecular structures of the carbohydrates (de Godoy et al., 2014).

Nutrient digestibility was not affected by SCF inclusion in this study, and there were no significant changes in fecal SCFA concentrations, fecal pH, or fecal consistency relative to the 0% SCF diet. Similarly, we observed no changes in markers of protein fermentation as evidenced by

a lack of change in fecal BCFA, phenolic and indolic compounds, and biogenic amines with increasing SCF supplementation. Higher dietary concentrations of SCF than those used here may be necessary to affect these outcomes. Dogs may also have a lower ability to ferment the SCF substrate compared to humans, perhaps due to differences in abundance of select microbial taxa (i.e. Firmicutes, Fusobacteria, Bacteroidetes, Proteobacteria and Actinobacteria), volume of the large bowel, and anatomical/physiological differences, such as sacculations and transit time.

Previous *in vivo* studies in rodent and human models investigating dietary SCF determined this substrate to be highly fermentable (defined by SCFA concentration), modulators of the microbiome, and overall favorable to indices of gut health. The objectives of these studies, however, were to determine the efficacy of dietary SCF as a fermentable fiber source. Therefore, the concentrations of SCF added to the diet were high to elicit these responses. Normally, prebiotic fibers are added at low concentrations in the diet to increase SCFA concentrations and stimulate the growth of potentially beneficial bacteria. The objective of this current study was to assess the prebiotic potential of SCF, which entails adding graded concentrations of SCF of only up to 1.25% of the diet. The previous *in vivo* studies in humans and rodents have shown the potential health benefits of SCF as a fiber source at higher dietary concentrations. Dietary SCF concentrations of 5% or higher in rats has been found to improve cecal and colonic fermentation characteristics, as well as indices of gut health (i.e., increased crypt depth, goblet cell numbers, and acidic mucins) (Guerin-Deremaux et al., 2010; Knapp et al., 2013). Similarly, in humans, consumption of SCF at 20 g/d resulted in a decrease in colonic pH, suggesting increased fermentative activity (Lefranc-Millot et al., 2012).

In recent years, the development of novel high-throughput sequencing techniques has led to a more comprehensive understanding of the microbial populations present in the colon.

Specifically, the effect of diet on the microbial populations, as well as their functional capacity to metabolize nutrients, can be measured using these techniques. Very limited research using these techniques has been conducted with dietary SCF. In humans, consumption of 21 g/d of SCF elicited increases in select butyrate-producing taxa (i.e., *Faecalibacterium* spp. and *Facaelibacterium prausnitzii*), as well as increases in lactobacilli (Hooda et al., 2012).

In our study, there were no significant differences in the diversity of gut bacteria among diets, nor was there a clear clustering by diet as indicated by principal component analysis. The predominant bacterial phyla present in feces of dogs fed all diets in this study were Firmicutes and Fusobacteria. Previously published data showed that a normal dog fecal microbiome was variable, with studies showing 14 to 48% Firmicutes and 7 to 40% Fusobacteria (Middelbos et al., 2010; Suchodolski et al., 2008; Swanson et al., 2011). At the microbial genus level, there was only slight modulation of the fecal microbiome with increasing dietary SCF. Dogs fed the 1% SCF diet showed increases in *Lachnospira*, which is a part of the butyrate-producing superfamily Lachnospiraceae (Marounek and Duskova 2002). However, this did not translate into increases in fecal butyrate concentrations.

Previous studies in humans have suggested that SCF and other fibers similar in chemical composition (e.g. wheat dextrin soluble fiber) are fermentable *in vitro* and *in vivo* and can beneficially modulate the microbiome, but these effects were observed only at dietary concentrations well above the highest concentration used in the present study (Pasman et al., 2006; Lefranc-Millot et al., 2012; Hobden et al., 2013). The integration of both *in vitro* fermentation and *in vivo* dog data suggests that SCF elicits some modulation of the microbiome; however, the doses provided were insufficient to induce a robust response.

Soluble corn fiber added at concentrations similar to proven prebiotics did not elicit the same effects on SCFA concentration, pH decline, or shifts in the microbial populations in dogs. Prebiotic fibers such as fructooligosaccharides and galactooligosaccharides are effective at all of the dietary concentrations tested in this experiment, putting this particular novel fiber at a disadvantage because of the higher concentrations that ostensibly would be required to elicit an effect. Overall, establishing an effective dose of SCF to elicit effects of increased SCFA concentrations, modulation of the microbiome, and other indices of gut health is needed.

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Table 3.1. Chemical composition of soluble corn fiber

Item	Concentration
Dry matter, %	96.5
---------- dry matter basis ----------	
Organic matter, %	100.0
Crude protein, %	0.0
Acid-hydrolyzed fat, %	0.5
Total dietary fiber, %	78.3
Insoluble dietary fiber	0.0
Soluble dietary fiber	78.3
HMWSF[1]	22.8
LMWSF[2]	55.5
Starch, %	
Digestible	17.7
Resistant	7.8
Total	25.5
Free sugars, mg/g	
Arabinose	0.7
Galactose	0.3
Glucose	24.9
Sucrose	0.7
Mannose	0.1
Fructose	1.8
Total	28.5
Gross energy, kcal/g	4.1
TME_n[3], kcal/g	2.6

[1]High molecular weight soluble fiber defined as the portion that precipitated in 95% ethanol.
[2]Low molecular weight soluble fiber defined as the portion that did not precipitate in 95% ethanol.
[3]Nitrogen corrected true ME.

Table 3.2. Ingredient composition of the basal diet fed to dogs[1]

Ingredient	% as-fed
Brewer's rice	46.55
Poultry byproduct meal-low ash	25.50
Corn, yellow, ground	12.00
Poultry fat	8.00
Soluble corn fiber[2]	Variable
Cellulose[3]	6.00
Salt	0.70
Potassium chloride	0.56
Chromic oxide	0.20
Mineral mix[4]	0.18
Vitamin mix[5]	0.18
Choline chloride-50%	0.13

[1]Soluble corn fiber was added at 0, 0.5, 0.75, 1.0, or 1.25% of the diet at the expense of cellulose and brewer's rice to main isofibrous and isonitrogenous diets.

[2] NUTRIOSE®FM: Roquette;. Lestrem, France.

[3]Cellulose: Solka-Floc, International Fiber Corp., North Tonawanda, NY.

[4]Provided per kilogram of diet: Mn (as $MnSO_4$), 66.00 mg; Fe (as $FeSO_4$), 120 mg; Cu (as $CuSO_4$), 18 mg; Co (as $CoSO_4$), 1.20 mg; Zn (as $ZnSO_4$), 240 mg; I (as KI), 1.8 mg; Se (as Na_2SeO_3), 0.24 mg.

[4]Provided per kilogram of diet: vitamin A, 5.28 mg; vitamin D_3, 0.04 mg; vitamin E, 120 mg; vitamin K, 0.88 mg; thiamine, 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; vitamin B_{12}, 0.11 mg.

Table 3.3. Chemical composition of experimental diets fed to dogs.

	% Soluble corn fiber				
Item	0	0.5	0.75	1.0	1.25
Dry matter, %	92.9	92.7	93.4	92.9	93.2
	---------- % dry matter basis ----------				
Organic matter	94.5	94.6	94.5	94.2	94.1
Crude protein	23.4	23.8	24.2	24.3	24.9
Acid-hydrolyzed fat	14.1	13.8	13.7	14.0	14.0
Total dietary fiber	7.34	7.38	7.39	7.43	7.44
GE, kcal · g^{-1}	4.99	4.98	4.98	5.00	4.99

Table 3.4. Fecal short-chain fatty acid (SCFA), branched-chain fatty acid (BCFA), and ammonia concentrations, and pH values for dogs fed diets containing graded soluble corn fiber concentrations

	% Soluble corn fiber							*P* -value	
Item	0	0.5	0.75	1	1.25	SEM	*P*-value	Linear	Quadratic
pH	6.67	6.45	6.47	6.62	6.23	0.13	0.11	0.09	0.73
SCFA, µmol/g DM									
Acetate	268.7^{ab}	313.6^{ab}	254.7^{a}	292.2^{ab}	317.2^{b}	17.1	0.02	0.15	0.58
Propionate	96.7^{ab}	116.5^{b}	91.8^{a}	111.9^{ab}	118.9^{b}	7.5	0.01	0.09	0.64
Butyrate	53.8	57.8	44.8	48.9	53.8	6.1	0.19	0.98	0.79
Total	419.2^{ab}	487.9^{b}	391.3^{a}	453.1^{ab}	490.0^{b}	27.6	0.02	0.21	0.57
BCFA, µmol/g DM									
Isobutyrate	8.34	9.00	7.72	8.23	8.14	0.71	0.51	0.66	0.86
Isovalerate	12.93	14.63	12.44	13.27	13.16	1.23	0.10	0.22	0.77
Valerate	0.87	0.94	0.84	0.82	1.04	0.15	0.63	0.65	0.62
Total	22.13	24.96	21.00	22.32	22.34	2.01	0.53	0.85	0.79
Ammonia, µmol/g DM	176.1	194.9	162.4	178.8	177.9	13.94	0.22	0.80	0.91

^{ab}Mean values within a row with unlike superscript letters differ ($P < 0.05$).

Table 3.5. Predominant bacterial phyla and genera expressed as a percentage of total sequences in feces of dogs fed diets containing graded soluble corn fiber concentrations[1]

			% Soluble corn fiber						
Phylum	Family	Genus	0	0.5	0.75	1	1.25	SEM	*P*-value
Actinobacteria			0.26	0.34	0.33	0.17	0.36	0.21	0.74
	Bifidobacteriaceae	*Bifidobacterium*	0.24	0.32	0.31	0.15	0.34	0.21	0.73
Bacteroidetes			1.00	0.56	0.91	0.68	0.71	0.21	0.44
	Bacteroidaceae	*Bacteroides*	0.49	0.28	0.31	0.47	0.38	0.08	0.08
	Prevotellaceae	*Prevotella*	0.34	0.26	0.46	0.41	0.31	0.19	0.91
Firmicutes			56.94	56.44	59.06	59.26	64.89	5.30	0.40
	Acidaminococcaceae	*Acidaminococcus*	0.22	0.22	0.27	0.32	0.18	0.05	0.35
	Clostridiaceae	*Clostridium*	24.73	21.81	25.17	22.34	26.54	3.87	0.54
	Eubacteriaceae	*Eubacterium*	1.41	2.07	2.08	1.63	3.08	0.83	0.21
	Lachnospiraceae	*Blautia*	10.97	12.80	14.09	12.71	15.15	2.23	0.34
		Dorea	0.24	0.08	0.08	0.25	0.04	0.08	0.08
		Lachnospira	0.02^{a}	0.92^{ab}	1.52^{b}	1.57^{b}	1.51^{b}	0.42	0.01
		Roseburia[*]	0.69	0.56	0.54	0.50	0.35	0.14	0.23
	Lactobacillaceae	*Lactobacillus*	3.38	4.92	2.75	4.20	5.88	2.86	0.49
	Paenibacillaceae	*Paenibacillus*	0.19	0.25	0.37	0.18	0.39	0.14	0.47
	Peptococcaceae	*Delsulfotomaculum*	0.75	1.00	1.68	0.75	1.42	0.59	0.39
	Ruminococcaceae	*Faecalibacterium*	2.79	3.64	2.43	3.15	5.50	0.97	0.20
		Oscillospira	0.79	0.82	0.62	1.11	0.66	0.30	0.35
		Ruminococcus[*]	5.64	5.06	3.96	5.00	4.12	1.04	0.13
	Turcibacteraceae	*Turicibacter*	0.54	0.59	1.23	0.98	1.39	0.48	0.55
	Veillonellaceae	*Megamonas*[†]	1.27	0.69	1.13	0.90	1.80	0.32	0.06
		Phascolarctobacterium	4.90	4.38	4.86	6.97	4.05	1.05	0.15
Fusobacteria			28.37	27.05	23.69	28.69	17.70	4.52	0.20
	Fusobacteriaceae	*Fusobacterium*	28.37	27.05	23.69	28.69	17.70	4.52	0.20
Proteobacteria			2.67	1.92	1.86	2.72	2.61	0.68	0.13
	Succinivibrionaceae	*Succinivibrio*	0.16	0.09	0.12	0.08	0.25	0.08	0.20
		Anaerobiospirillum	0.22	0.38	0.14	0.57	0.17	0.18	0.22
	Alicaligenaceae	*Sutterella*	1.99	1.31	1.45	1.76	1.12	0.47	0.28
Tenericutes			10.75	12.75	14.14	10.78	14.06	4.10	0.32
	Erysipelotrichaceae	*Allobaculum*	7.71	9.20	10.32	4.74	8.13	3.96	0.12
		Bulleidia	0.32	0.22	0.35	0.18	0.26	0.11	0.62
		Catenibacterium[*]	0.14	0.26	0.16	0.24	1.33	0.39	0.08
		Coprobacillus[‡*]	0.33^{b}	0.17^{a}	0.19^{ab}	0.25^{ab}	0.14^{a}	0.05	0.01

Table 3.5 continued. Predominant bacterial phyla and genera expressed as a percentage of total sequences in feces of dogs fed diets containing graded soluble corn fiber concentrations[1]

[1]Genera included have least squares means of 0.01 or higher.
[ab]Mean values in the same row with unlike superscript letters differ ($P < 0.05$).
[‡] Difference between 0% SCF vs. all other SCF diets ($P < 0.05$).
[*]Linear effect ($P < 0.05$).
[†]Quadratic effect ($P < 0.05$).

Table 3.6. Nutrient digestibility and fecal scores from dogs fed diets containing graded soluble corn fiber concentrations[1]

	% Soluble corn fiber						*P*-value	
Item	0	0.5	0.75	1	1.25	SEM	*P*-value	Linear	Quadratic
Feed intake, g/d DM	287.9	287.4	289.4	288.0	288.8				
Fecal output, g									
As-is	597.4	609.1	573.4	571.2	592.5	23.4	0.75	0.53	0.81
Dry matter basis	223.1	220.6	212.0	210.9	211.9	7.41	0.67	0.17	0.84
Fecal DM, %	37.5	36.2	37.1	37.0	36.0	0.70	0.05	0.27	0.98
Apparent total tract digestibility, %									
Dry matter	80.6	80.8	81.7	81.7	81.7	0.64	0.61	0.15	0.84
Organic matter	83.3	83.5	84.3	84.5	84.2	0.57	0.49	0.12	0.80
Crude protein	81.7	81.3	80.7	80.2	80.2	0.84	0.31	0.22	0.76
Acid hydrolyzed fat	94.2	94.2	94.7	94.4	94.7	0.31	0.36	0.17	0.87
Energy	84.4	84.3	85.2	85.3	85.0	0.58	0.66	0.24	0.86
Digestible energy intake, kcal·d^{-1}	1213	1206	1228	1230	1224	8.39	0.23	0.12	0.95
Fecal score	2.6	2.6	2.4	2.6	2.7	0.12	0.17	0.79	0.37

[1]Values expressed on a dry matter basis.

Table 3.7. Predominant bacterial species expressed as a percentage of total sequences in feces of dogs fed diets containing graded soluble corn fiber concentrations[1]

	% Soluble corn fiber						
Species	0	0.5	0.75	1	1.25	SEM	*P*-Value
Acidaminococcus intestini	0.22	0.22	0.27	0.32	0.18	0.05	0.35
Allobaculum sp id4	4.64	4.31	4.41	3.84	3.86	3.87	0.63
A.stercoricanis	3.07	4.89	5.91	3.20	4.27	1.08	0.28
Anaerobiospirillum succiniciproducens	0.22	0.38	0.14	0.57	0.17	0.18	0.28
Anaerotruncus colihominis	0.06	0.06	0.05	0.07	0.04	0.02	0.72
Bacteroides cellulosilyticus[‡*]	0.09	0.04	0.06	0.05	0.04	0.02	0.11
B. coprocola[*]	0.03[a]	0.03[a]	0.05[ab]	0.07[ab]	0.10[b]	0.02	0.02
B. eggerthii	0.11	0.09	0.08	0.15	0.09	0.02	0.13
B. plebeius	0.14	0.06	0.12	0.08	0.08	0.03	0.20
Bifidobacterium pseudolongum	0.23	0.85	0.28	0.12	0.95	0.48	0.44
B. hydrogenotrophica	0.12	0.14	0.25	0.08	0.25	0.10	0.28
Blautia producta[‡*]	9.85	12.00	13.06	11.92	13.95	2.05	0.23
B. ruminococcus obeum[†]	1.01	0.65	0.79	0.71	0.95	0.17	0.32
Catenibacterium mitsuokai	0.14	0.26	0.16	0.24	0.20	0.10	0.29
Clostridium bartlettii	0.30	0.25	0.38	0.38	0.51	0.12	0.53
C. bifermentans	0.17	0.19	0.22	0.15	0.08	0.08	0.55
C. bolteae	0.23	0.28	0.20	0.32	0.27	0.07	0.55
C. cocleatum	0.17	0.11	0.08	0.12	0.12	0.07	0.82
C. hathewayi	0.37	0.33	0.20	0.32	0.36	0.07	0.15
C. hiranonis	21.42	18.74	22.14	18.74	22.95	3.62	0.54
C. methoxybenzovorans	0.05	0.08	0.04	0.04	0.05	0.01	0.09
C. perfringens	0.17	0.11	0.01	0.03	0.11	0.08	0.43
C. ramosum	0.13	0.34	0.27	0.27	0.26	0.15	0.80
C. spiroforme	0.91	0.63	0.72	1.09	0.99	0.32	0.43
C. thermosuccinogenes	0.09	0.14	0.11	0.15	0.19	0.06	0.60
C. xylanolyticum	0.08	0.11	0.05	0.07	0.06	0.02	0.13
Coprobacillus cateniformis[‡*]	0.33[b]	0.17[ab]	0.19[a]	0.25[ab]	0.14[a]	0.05	0.01
Coprococcus catus	0.04[a]	0.08[b]	0.02[a]	0.05[ab]	0.03[a]	0.01	0.01
Desulfotomaculum halophilum	0.74	0.98	1.65	0.74	1.38	0.58	0.55
Dorea formicigenerans[‡*]	0.20[b]	0.08[ab]	0.06[a]	0.11[ab]	0.04[a]	0.05	0.01
Eubacterium dolichum	0.09	0.08	0.07	0.09	0.05	0.04	0.42
E. biforme[‡*]	0.58[a]	1.60[a]	1.48[a]	1.12[a]	2.44[b]	0.77	0.08
E. sulci	0.44	0.31	0.42	0.37	0.34	0.15	0.84
Faecalibacterium prausnitzii	2.79	3.64	2.43	3.15	5.50	0.97	0.20
Fusobacterium mortiferum[‡]	7.68	3.76	2.97	4.89	2.70	1.57	0.13
F. varium	20.52	23.21	20.64	23.68	14.92	3.76	0.25
Lactobacillus acidophilus	0.00	0.01	0.05	0.27	0.42	0.20	0.48
L. apodemi	0.03	0.03	0.05	0.05	0.17	0.05	0.32
L. johnsonii	2.56	3.05	1.45	2.45	0.36	1.70	0.50
L. lactococcus lactis	0.31	0.47	0.34	1.00	1.84	0.67	0.47
L. reuteri	0.05	0.12	0.10	0.04	0.03	0.06	0.64
L. reuteri (strain F275)	0.41	0.31	0.72	0.39	0.32	0.32	0.72
Megamonas hypermegale[†]	1.27[ab]	0.69[a]	1.13[ab]	0.90[ab]	1.80[b]	0.32	0.06
Paenibacillus borealis	0.17	0.24	0.35	0.17	0.20	0.10	0.39
Prevotella copri	0.34	0.26	0.46	0.41	0.31	0.19	0.95
Roseburia eubacterium rectale	0.67	0.53	0.49	0.73	0.32	0.17	0.18
Ruminococcus flavefaciens	0.48	0.58	0.44	0.58	0.46	0.12	0.77
R. gnavus[‡]	3.40	2.14	2.22	2.94	2.16	0.88	0.15
R. torques	1.76[ab]	2.33[b]	1.30[a]	1.46[ab]	1.49[ab]	0.35	0.02
Sutterella sanguinus	0.46	0.29	0.50	0.47	0.44	0.17	0.73
S. stercoricanis	0.15	0.08	0.13	0.15	0.10	0.04	0.44

Table 3.7 continued. Predominant bacterial species expressed as a percentage of total sequences in feces of dogs fed diets containing graded soluble corn fiber concentrations[1]

[ab]Mean values in the same row with unlike superscript letters differ ($P < 0.05$).
[‡] Difference between 0% NU vs. other diets ($P < 0.05$).
[*]Linear effect ($P < 0.05$).
[†]Quadratic effect ($P < 0.05$).

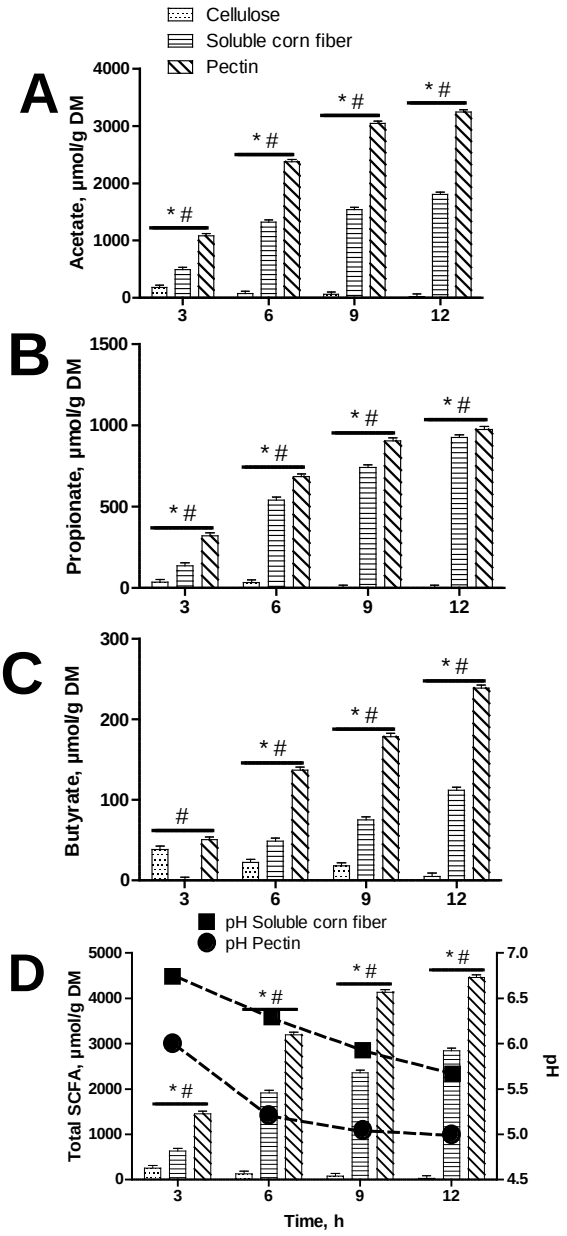

Figure 3.1. *In vitro* experiment: concentrations of acetate (A), propionate (B), butyrate (C), and total short-chain fatty acids (SCFA) (D), and pH values during a 12 h *in vitro* fermentation. Standard error bars are presented for each mean value.* denotes a significant ($P < 0.05$) time by treatment interaction. # denotes a significant difference ($P < 0.05$) between pectin and SCF within each time point.

CHAPTER 4: MODERATELY FERMENTABLE POTATO FIBER ATTENUATES BODY WEIGHT LOSS AND IS ANTI-INFLAMMATORY DURING EXPERIMENTAL COLITIS

Abstract

Potato fiber (PF), a co-product of potato starch isolation, is a moderately fermentable (30-70% fermentable) dietary fiber that was evaluated for its efficacy in attenuating the acute inflammatory response in a dextran sodium sulfate (DSS)-induced colitis mouse model. We hypothesized that dietary PF would attenuate the inflammatory response more effectively than non-fermentable cellulose (Cell) due, in part, to the influence of fermentation end-products (i.e., short-chain fatty acids; SCFA). Male C57Bl/6J mice (n=67) were randomized based on initial body weight to diets containing either 8% Cell (n=34) or 14.5% PF (n=33) for a 22-d feeding study. Starting on study d 14, mice were provided either distilled water or 2% (wt/vol) DSS in drinking water for 5 d, after which all mice received distilled water until study termination. Daily weights of mice, food, and water were collected from d 14 through d 22. Distal colon tissue was analyzed for histological outcomes and changes in gene expression, and cecal contents were analyzed for SCFA concentrations. All data were analyzed by ANOVA with repeated measures analyses applied where necessary. Mice provided the PF/DSS treatment exhibited a delayed ($P < 0.05$) loss in body weight compared with mice provided the Cell/DSS treatment following DSS administration. Furthermore, by study end (d 8 post-DSS administration), mice on the PF/DSS treatment exhibited earlier recovery from body weight loss ($P < 0.05$) compared with Cell/DSS mice. A diet by DSS treatment interaction ($P < 0.05$) was noted for cecal SCFA concentrations, which were higher in the PF/DSS treatment compared with the Cell/DSS treatment. This evidence was corroborated by positive changes in histology scores, with PF/DSS-treated mice exhibiting

lower ($P < 0.05$) infiltration of leukocytes into the lamina propria compared with Cell/DSS-treated mice. The PF/DSS treatment also decreased ($P < 0.05$) mRNA expression of distal colon inflammatory cytokines TNF-α, IL1-β, IL-6, and IL-17A, as well as the potent neutrophil chemoattractant chemokine, CXCL1, compared with the Cell/DSS treatment. These results suggest that ingestion of PF during chemically-induced experimental colitis may attenuate the inflammatory response through microbial production of SCFA.

Introduction

Ulcerative colitis and Crohn's disease are both inflammatory bowel diseases (IBD) that are marked by intermittent bouts of inflammation throughout the gastrointestinal tract (Goto et al., 2010). The etiology of IBD is not fully understood; however, there are known genetic, immunologic, and environmental factors (Pillai et al., 2013). The human intestine is enumerated with 10^{13} to 10^{14} microorganisms that have the ability to metabolize non-digestible carbohydrates (i.e., dietary fiber) and produce fermentative end-products, mainly the short-chain fatty acids (SCFA), acetate, propionate, and butyrate (Gill et al., 2006). Microbes residing in the human colon and increases their fermentative end-products (acetate, propionate, and butyrate), have been associated with diseases such as diabetes mellitus, obesity, IBD, and others (Turnbaugh et al., 2009; Larson et al., 2010; Kranich et al., 2011). Interest in using dietary fiber to improve overall health has increased with new insights into the beneficial effects of the microbiome and, in particular, their ability to produce SCFA.

Potato fiber (PF) is a co-product of potato starch isolation that has a chemical composition of fiber components (e.g., hemicelluloses, cellulose, and pectins) that are favorable to colonic fermentation (Mayer, 1998). Previous research in our lab using dogs suggested that PF is moderately fermentable, exhibiting dose-dependent linear increases in fecal acetate, propionate,

and butyrate concentrations (Panasevich et al., 2013). Furthermore, with increasing concentrations of PF, we found shifts in the fecal microbiome of key SCFA-producing bacteria, and, in particular, butyrate producers (Panasevich et al., 2014). To this end, *Faecalibacterium*, and, more specifically, *Faecalibacterium prausnitzii*, a well-characterized butyrate-producing species, was increased by feeding graded dietary levels of PF to dogs. This bacterial species often is found in low concentrations in humans and dogs exhibiting IBD. These previous results led us to investigate whether PF, with its *in vivo* fermentation characteristics and ability to modulate the microbiome, would be efficacious in attenuating the inflammatory response in an animal model of ulcerative colitis.

Our main objective in this study was to utilize a model of acute colitis, induced by oral dextran sodium sulfate (DSS), to test the efficacy of moderately fermentable PF in attenuating the inflammatory response. Dextran sodium sulfate dissolved in drinking water and provided ad libitum to mice has been used extensively as an animal model for IBD (Okayasu et al., 1990). Our study investigated diets containing identical concentrations of dietary fiber from two different sources; PF, which is moderately fermentable, and cellulose (Cell), which is an inert, non-fermentable dietary fiber. We hypothesized that ingestion of PF would (1) ameliorate symptoms associated with DSS-colitis more effectively than Cell, and (2) SCFA concentrations would explain the anti-inflammatory effects during DSS colitis.

Materials and Methods

Animals and diets

Male C57Bl/6J mice (8-10 weeks of age) were purchased from Jackson Laboratories (Bar Harbor, ME) and singly housed in a temperature-controlled room. Animals were provided ad libitum access to food and water throughout the study and housed under a reversed light cycle

(lights on at 2100, lights off at 0900). Mice were acclimated to the facility and provided a standard AIN93G diet (5% fiber) (LabDiet/TestDiet; St. Louis, MO) for 14 d prior to starting the experimental diets. Following acclimation, mice were provided a modified AIN93G (8% total dietary fiber (TDF)) containing either a Cell (n=34) or PF (n=33) in the diet for a 22 d experimental feeding period. Each diet was formulated to be isofibrous (8% TDF), isonitrogenous, and isocaloric on the basis of the guaranteed analysis provided by the manufacturer, thereby applying experimental focus strictly on fermentability of the dietary fiber source. Potato fiber was added at 14.5% of the diet to achieve an 8% TDF at the expense of corn starch to achieve equal concentrations of carbohydrate between diets.

After receiving the experimental diets for 14 d, mice within each diet group were randomly assigned to receive either distilled water (Cell, n=17; PF, n=16) or DSS (Cell, n=17; PF, n=16) dissolved in drinking water for 5 d, after which time all mice then received distilled water until study end. Mice were euthanized 8 d after initiating water treatments, at which point tissues were collected. All corncob bedding and cages were sterilized before use and were changed weekly. All experiments were approved by the University of Illinois at Urbana-Champaign Institutional Animal Care and Use Committee.

DSS treatment

Dextran sodium sulfate was provided at a concentration and duration that would induce an acute inflammatory response (Perše and Cerar, 2012; Cook et al., 2013). Briefly, regular drinking water was replaced by 2% (wt/vol) DSS (MP Biochemicals; Santa Ana, CA; 36,000–50,000 MW) in sterile water for a 5 d period. The DSS solution was monitored daily, refilled appropriately, and replaced on d 3 after initial administration. On d 5 post-water treatment induction, all mice were provided distilled water until the study conclusion.

Morbidity and tissue collection

Mouse body weights were recorded upon arrival and once weekly until DSS was administered. Daily measurements of body weight and food and water intake were recorded during and after DSS administration. Mouse feces were evaluated in an observer-blinded manner for consistency/diarrhea (e.g., formed pellet vs. semi-formed/soft). Home-cage physical activity was assessed using a 4-point scale (1 = normal, 2 = slight reduction, 3 = limited, 4 = immobile), and response to capture using a 3-point scale (1 = normal evasion, 2 = some evasion, 3 = no evasion), as indicators of sickness behavior.

Eight days after initiating water treatments, mice were euthanized by CO_2 asphyxiation, and blood was collected into a heparin-coated tube by cardiac puncture and stored on ice. Plasma was separated and stored at −80°C. Whole ceca were excised and weighed with contents. The colon was removed (distal to the cecum extending to the anus) and colon length was assessed using digital calipers (Tresna©; Guilin, China) to the nearest mm. Mesentery tissue was removed from the colon, which was subsequently flushed with PBS and separated into proximal and distal sections. A 2-cm segment was cut from the top portion of the distal section, fixed in 10% buffered formalin for 24 h, and switched to 70% ethanol until sectioned for histological analysis. Additional samples of distal colon tissue were collected and stored at −80°C until analysis.

Short-chain fatty acid analysis

Cecal SCFA concentrations were determined according to Panasevich et al. (2013). Briefly, SCFA 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 × 4 mm i.d.), packed with 10% SP-1200/1% H_3PO_4 on 80/100 + mesh

Chromosorb WAW (Supelco Inc., Bellefonte, PA). Nitrogen was the carrier gas with a flow rate of 75 mL/min. Oven, detector, and injector temperatures were 125, 175, and 180°C, respectively.

Histology

Formalin-fixed distal colon samples were paraffin embedded, and 3 μm sections were stained with hematoxylin and eosin. Colon damage (i.e., necrosis) and immune cell infiltration were scored by a blinded observer based on a published scoring system that considers architectural derangements, goblet cell depletion, edema/ulceration, and degree of inflammatory cell infiltration (Wirtz et al., 2007). Each sample was ranked twice, once for necrosis and once for inflammation. Scoring for inflammatory cell infiltration was as follows: 0 = normal, 1 = mild, 2 = moderate, 3 = severe. Necrosis was scored as: 0 = normal, 1 = mild (∼1–25% necrotic), 2 = moderate (∼26–75% necrotic), 3 = severe (>75% necrotic).

Distal colon gene expression

Quantitative real-time polymerase chain reaction (qRT-PCR) of colon tissue was used to assess changes in gene expression. Approximately 30 mg of distal colon tissue was homogenized, subjected to RNA extraction using a modified RNeasy mini kit protocol (QIAGEN, Valencia, CA) with the addition of DNase, and stored at −80°C. Complementary DNA was synthesized using the reverse transcription reaction utilizing commercial first strand kits (Life Technologies Co. [Applied Biosystems], Carlsbad, CA) and random hexamer primers, as described in the manufacturer's protocol. The RNA sample concentrations and purities were analyzed by a spectrophotometer, and RNA integrity was assessed by agarose gel electrophoresis (Labnet Gel XL Ultra V-2 Mini-Gel System; Labnet International, Inc. Edison, NJ). Expression of both pro-inflammatory cytokines (IL-1β, TNF-α, IL-6, IL-17A) and the anti-inflammatory cytokine, IL-10, were quantified as markers of an inflammatory response (primers: IL-1β, Mm00434228; TNF-α,

Mm00443258; IL-6, Mm00446190; Mm00518984; IL-17A, Mm00439618; IL-10, Mm00439616). Additionally, expression of the chemokines CCL6 and CXCL1 (primers: CCL6, Mm01302419; CXCL1, Mm04207460) were quantified as markers of immune cell trafficking. Expression of free-fatty acid receptor 2 (FFAR2) (primer: Mm02620654) was evaluated as a potential mechanism of associating SCFA concentrations and leukocyte recruitment. A qRT-PCR instrument (7900HT Fast Real-Time PCR system and SDS Enterprise Database; Life Technologies; Carlsbad, CA) was used to assess gene expression, and Taqman® gene expression q-PCR master mixes were used (Applied Biosystems, Carlsbad, CA) to perform the q-PCR reaction. Expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control (Su et al., 2007). Target genes were expressed relative to GAPDH using the ΔΔ-Ct method as fold change, with the Cell/distilled treatment combination serving as the calibrator group (Livak and Schmittgen, 2001).

Plasma serum amyloid A

Concentrations of the acute phase protein, serum amyloid A (SAA) were measured in plasma by ELISA (Alpco Diagnostics, Salem, NH) as an indicator of systemic inflammation.

Statistical analysis

Differences from baseline in daily body weight, as well as fluid and water intake, were analyzed by 3-way ANOVA, with time as the repeated measure, to determine the combined effects of diet and water treatment combinations. Significant differences between diet and water treatments were set at $P < 0.05$ within each day. A significant interactive effect was set at $P < 0.10$ for colon and cecal content measures. Gene expression, plasma SAA, and histology data were subjected to 2-way ANOVA at a single time-point (i.e., study conclusion). All data were analyzed using the MIXED procedure of SAS (SAS Inst. Inc., Cary, NC), and the UNIVARIATE procedure

was used to identify outliers and ensure equal variance and normal distribution within the complete dataset. Data were transformed by log or square root if the assumption of equal variances was not met. Any observations more than 3 standard deviations from the mean were considered as outliers and removed.

Results

Body weight loss, as well as food and water intake, were measured from the beginning of DSS induction to study conclusion (Figure 4.1A-C). There were no significant differences in body weight between dietary treatment groups before beginning water treatments (24.47 ± 0.38 g and 24.36 ± 0.38 g for Cell and PF, respectively). Throughout the 8-d water treatment period, consumption of DSS decrease ($P < 0.05$) BW of mice compared with the distilled water treatment. Mice on the Cell/DSS treatment showed an earlier ($P < 0.05$) decrease in BW starting at d 4 post-DSS, whereas PF/DSS treatment started at d 5 post-DSS. A diet by DSS interaction ($P < 0.05$) was observed both on d 5 and d 8 post-DSS induction. At study end, PF-fed mice had lost less ($P < 0.05$) BW compared with Cell-fed mice. Assessments of behavioral changes and fecal pellet consistency in response to DSS were recorded, but no significant changes were noted (data not shown).

Compared with mice provided distilled water, the Cell/DSS treatment combination resulted in decreased ($P < 0.05$) water intake on d 2, and d 4-8 post-DSS induction. Decreases ($P < 0.05$) in water intake did not begin until d 5 post-DSS in mice provided the PF/DSS treatment. Similarly, the Cell/DSS treatment elicited decreased ($P < 0.05$) food intake compared with distilled water mice on days 4-8 post DSS, whereas PF/DSS showed a decrease ($P < 0.05$) in food intake of even greater magnitude on d 5 and 6 post-DSS compared with healthy mice. Both the PF/DSS and Cell/DSS treatment groups, however, did regain similar food and water intakes by study end.

Colon length and cecal weight (including contents) were measured to assess magnitude of DSS-induced sickness symptoms and extent of fermentation, respectively (Figures 4.2A-B). Colon length and cecal weights were lower ($P < 0.05$) in DSS-treated mice compared with mice receiving distilled water. Moreover, cecal weight was increased ($P < 0.05$) in PF-fed mice compared with Cell-fed mice. No interactive effects were noted for these outcomes. Concentrations of SAA were measured in plasma as an indication of systemic inflammation (Figure 4.3). Mice provided DSS had higher ($P < 0.05$) concentrations of SAA compared to mice provided distilled water, and no interactive effect was noted.

Cecal contents were analyzed for SCFA concentrations to assess the magnitude and profile of fermentation end-product formation (Figure 4.4A-D). Concentrations of SCFA exhibited interactive effects ($P < 0.10$) and, in particular, cecal acetate, butyrate, and total SCFA. Mice fed the PF/DSS treatment had increased ($P < 0.05$) cecal acetate, butyrate, and total SCFA ($P < 0.10$) compared with mice on the Cell/DSS treatment. There was a negative effect on cecal fermentation with the DSS treatment, with PF-fed mice receiving distilled water having higher ($P < 0.05$) concentrations of acetate, butyrate, and total SCFA in cecal contents compared with PF-fed mice receiving DSS, but no differences between water treatments for mice fed Cell. Propionate concentrations in cecal contents did not show an interactive effect ($P = 0.41$); however, there was a significant main effect ($P < 0.05$) of dietary treatment, where mice fed PF had higher ($P < 0.05$) propionate concentrations in cecal contents compared with Cell-fed mice.

Histology of distal colon was scored by independent, blinded observer to assess symptoms of DSS colitis (Figures 4.5A-C). Specifically, percent necrosis and inflammatory cell infiltration of innate immune cells were assessed. Both cellular infiltration and necrosis scores resulted in a main effect ($P < 0.05$) of water treatment, with mice receiving DSS having higher ($P < 0.05$)

values compared with mice receiving distilled water. A diet by DSS interaction ($P < 0.05$) resulted in the assessment of infiltration of leukocytes into the lamina propria, with mice on the PF/DSS treatment having lower ($P < 0.05$) scores for infiltration of leukocytes compared with the Cell/DSS mice. Infiltration of leukocytes were not statistically different between dietary treatments in mice provided distilled water. Necrosis scores were higher ($P < 0.05$) in DSS-treated vs. control mice, with no interactive effects noted.

Colon gene expression of pro-and anti-inflammatory cytokines (Figures 4.6A-E) and chemokines (Figures 4.7A-B) were analyzed as indications of inflammation and leukocyte recruitment, respectively. In general, DSS resulted in greater ($P < 0.05$) expression of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6, and IL-17A) compared with distilled water. Dietary PF blunted the inflammatory response induced by DSS, thereby producing lower ($P < 0.05$) colonic mRNA expression of TNF-α, IL-1β, IL-6, and IL-17A compared with Cell. A significant ($P < 0.05$) diet by DSS treatment interaction was observed with CXCL1 expression. Specifically, mice in the PF/DSS group having lower ($P < 0.05$) expression of CXCL1 compared with the Cell/DSS group. These changes were observed between diets in CXCL1 expression in mice provided distilled water. Expression of CCL6 showed no significant differences between dietary treatments ($P = 0.27$); however, an effect of DSS approached significance ($P = 0.06$) in the expression of CCL6 compared with the distilled water treatment. Free-fatty acid receptor 2 (FFAR2) mRNA expression was also quantified as a potential mechanism linking leukocyte recruitment and SCFA concentrations (Figure 4.8). A main effect of water treatment was observed, with FFAR2 expression in DSS-treatment mice exhibiting higher ($P < 0.01$) expression compared with mice receiving distilled water. However, no diet by DSS treatment interaction was observed for FFAR2 expression ($P = 0.36$).

Discussion

Our main objective in this experiment was to determine if a moderately fermentable dietary fiber could attenuate the acute-inflammatory response compared with a non-fermentable fiber source (i.e., cellulose). Therefore, we formulated these diets to be isofibrous to ensure that experimental focus is strictly on fermentability. In terms inducing an acute-inflammatory response, it has previously been determined that DSS administered at 2-5% for a duration of 4-9 days is the best model for acute inflammation (Perše and Cerar, 2012). Observations of C57bl mice provided a 5% DSS solution however, have become too diseased (Melgar et al., 2005). Cook et al. (2013), found that a concentration of 2% for a duration of 5 days induced a peak acute inflammatory response at day 8 post DSS induction with low amounts of mortality. Therefore, we used this concentration for the same duration to induce a peak acute-inflammatory response at day 8 post induction.

Previous chemical analysis of PF used in this study confirmed that it contains 55% TDF with 33% insoluble fiber and 22% soluble fiber. It also contains 30% starch, with 4.6% of this fraction as resistant starch, and has low concentrations of fat (2%) and crude protein (4.8%) (Panasevich et al., 2013). The Cell used in this study is a highly purified wood cellulose that is 100% TDF and non-fermentable. Our diets were formulated to contain 8% TDF, due to previous results in dogs showing increases in fecal SCFA concentrations up to 6% addition into the diet (Panasevich et al., 2013). Therefore, PF was added at 14.5% of the diet, while Cell was added at 8% to achieve equal fiber concentrations between diets.

Dietary PF modulated the dog fecal microbiome in such a manner to suggest anti-inflammatory effects as related to findings in IBD patients (Panasevich et al., 2015). In particular, ingestion of PF increased a known butyrate producer, *Faecalibacterium* (*Faecalibacterium*

prausnitzii), which is a bacterial species often negatively associated with IBD in both dogs and humans (Suchodolski et al., 2012; Sokol et al., 2008; Ramirez-Farias et al., 2009). These results, however, were in healthy adult dogs and remained mere associations. Therefore, we sought to test the efficacy of PF in attenuating the inflammatory response in an experimentally-induced colitis model. We hypothesized that ingestion of PF would attenuate the symptoms of DSS-colitis by modulating the inflammatory response through the concentrations of SCFA (acetate, propionate, and butyrate) by fermentation of PF in the cecum of mice.

Dietary fermentable fibers have previously been tested for their efficacy in attenuating the inflammatory response in DSS-colitis. Many of these studies, however, tested these fiber sources using diets that were unequal in dietary fiber, or where cellulose was supplemented to achieve equal dietary fiber concentrations (Moreau et al., 2003; Komiyama et al., 2011; Zarepor et al., 2014). Our study aimed to use the DSS-mouse model to specifically study fermentable vs. non-fermentable dietary fiber when diets were iso-fibrous. In humans, inflammatory bowel disease encompasses both ulcerative colitis and Crohn's disease, which together affect more than 3.6 million people in the United States and Europe (Kostic et al., 2014; Loftus, 2004). These diseases display symptoms of intermittent bouts of colonic and systemic inflammation (i.e., increases in pro-inflammatory cytokines and chemokines), diarrhea, abdominal pain, and increased rectal bleeding. Pharmaceutical agents such as antibiotics and immunosuppressants often are used in symptomatic treatment of IBD, but these remedies can be expensive and often have severe side-effects. The efficacy of dietary fibers in suppressing an inflammatory response is dependent on the fermentation profile of the fiber (Rose et al., 2007). Therefore, an extensive evaluation of dietary fiber sources is needed in order to determine their efficacy in controlling such inflammatory diseases.

Our indices of morbidity included food and water intakes, body weight loss, colon length, and cecum weight with contents. Fermentable fibers such as FOS and partially hydrolyzed guar gum have been found to decrease body weight loss in response to DSS (Goto et al., 2010; Naito et al., 2006). Compared with resistant starch, FOS was less beneficial in delaying body weight loss during DSS (Moreau et al., 2003). The GI tract and, in particular, the colon, has been found to become more fibrotic and will ultimately decreases in length in response to DSS (Diaz-Granados et al., 2000). Colon length has previously been found to be an accurate indicator of disease activity (Diaz-Granados et al., 2000). Furthermore, cecum weight with contents provides an idea of DSS symptoms, as well as an indirect measure of the extent of fermentability. Our results suggest that fermentable fiber intake did not have an effect on DSS-induced reduction in colon length; however, fermentation of PF in the cecum occurred during DSS-colitis. In general, PF-fed mice exhibited higher cecal weights, which translates to more fermentation in both control and DSS-treated mice, and lessening of symptoms in the PF/DSS treatment group.

Previous studies investigating the effects of dietary fiber on attenuating the symptoms of chemically-induced acute colitis in animal models have provided contradictory findings. This could be explained by the differences in physicochemical properties of the fiber and, ultimately, their fermentability. For instance, highly fermentable fibers such as fructooligosaccharides (FOS) have been found to be protective against DSS-colitis when orally gavaged (Winkler et al., 2007; Goto et al., 2010); however, supplementation in a purified diet (i.e., cellulose as the only fiber source) showed no beneficial effects (Goto et al., 2010). Consumption of resistant starch, which is a more butyrogenic and moderately fermentable than FOS, with red meat was found to exert beneficial effects compared with consumption of red meat alone in an acute-colitis model (Le Leu et al., 2013). Furthermore, resistant starch, but not FOS, reduced macroscopic colonic cecal

damage in a DSS model (Moreau et al., 2003). Other non-digestible carbohydrates such as goat milk oligosaccharides, lactulose, and lactulose-derived synthetic and natural milk saccharides also have been shown to reduce macroscopic damage in DSS-colitis animal models (Lara-Villoslada et al., 2006; Boehm and Stahl, 2007). Moreover, plant-derived oligosaccharides, such as hydrolyzed guar gum, enzymatically-treated rice fiber, cellulose, and germinated barley foodstuff, have been found to be beneficial in the DSS-colitis model by either ameliorating the symptoms (body weight or colon tissue integrity) or eliciting lower expression of inflammatory markers (Naito et al., 2006; Komiyama et al., 2011; Nishimura et al., 2010; Kanauchi et al., 2001). Overall, we believe our results are consistent with those testing other moderately fermentable fibers in an acute DSS-colitis model.

The duration and concentration of DSS administration used in this study were intended to initiate an acute-inflammatory response. The predominant innate immune cell types recruited in this type of colitis include neutrophils, monocytes, eosinophils, and dendritic cells. Chemokines are highly expressed in the colon of patients with IBD, and are known to direct immune cell trafficking, and exert anti-microbial effects (Kotarsky et al., 2010; Coelho et al., 2007). Here we measured both CCL6, a chemotactic agent for innate immune effector cells such as dendritic cells and macrophages, and CXCL1, a neutrophil chemoattractant (Coelho et al., 2007; Puleston et al., 2005). Although expression of CCL6 was not affected by dietary treatment, there was a strong trend for a water main effect. This suggests that DSS caused detrimental colonic epithelial barrier function, thereby potentially allowing bacterial translocation from the intestinal lumen (Kotarsky et al., 2010). What was particularly interesting were the changes in CXCL1 distal colon gene expression. Mice treated with DSS and provided with dietary Cell, a non-fermentable fiber, had a 7-fold increase in CXCL1 expression compared with DSS-treated mice receiving PF. In this acute-

colitis model, neutrophils would be the predominant innate immune cell type at the site of inflammation.

We hypothesized that a moderately fermentable fiber would attenuate the acute-inflammatory response in DSS-colitis more effectively than cellulose, an inert non-fermentable fiber. Therefore, we analyzed cecal SCFA concentrations as an indication of fermentation, thereby providing quantitative evidence of the protective effects PF may provide against DSS-colitis. Even with induction of DSS-colitis, PF-fed mice had higher concentrations of acetate, butyrate, and total SCFA in cecal contents. In terms of fermentability and prebiotic potential, these results support our previous experiments investigating PF *in vitro* and in the dog (Panasevich et al., 2013; Panasevich et al., 2014). Previous studies testing prebiotic fibers in DSS-colitis rodent models have typically elicited anti-inflammatory effects. Ingestion of dietary fibers such as rice bran, resistant starch, and hydrolyzed plant polysaccharide were found to reduce mucosal damage and had anti-inflammatory properties (Komiyama et al., 2011; Moreau et al., 2003; Naito et al., 2006). Conversely, dietary flaxseed, which contains anti-inflammatory bioactive components such as fermentable fiber, phenolic compounds, and the polyunsaturated fatty acid, α-linolenic acid, was found to exacerbate colonic damage and inflammatory cytokines, even with increases in cecal SCFA concentrations (Zarepor et al., 2014). Consumption of highly fermentable FOS also produced mixed results in reducing DSS-induced colonic damage (Winkler et al., 2007; Goto et al., 2010). A study investigating fiber-enriched vs. low-fiber germinated barley in DSS-colitis suggested the fiber component was anti-inflammatory (Kanauchi et al., 2001). Collectively, these studies suggest that moderately fermentable and butyrogenic dietary fibers are the most effective at attenuating DSS-colitis.

Our histopathological analysis and distal colon gene expression of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6, and IL-17a) support our hypothesis of SCFA-induced anti-inflammation during DSS. Dietary PF exhibited decreases in leukocyte infiltration scores in distal colon compared to Cell. Furthermore, mice on the PF/DSS treatment reduced distal colon inflammation by exhibiting decreases in TNF-α, IL-1β, IL-6, and IL-17a compared to the Cell/DSS group. Taken together, the cecal SCFA concentrations, infiltration of leukocyte pathology scores, and pro-inflammatory cytokine gene expression, indicate an association of SCFA affecting leukocyte recruitment during DSS-induced colitis.

The low viability of leukocytes and, in particular, neutrophils, make them very difficult to study them in tissues. We measured degree of leukocyte infiltration using a previously validated scoring system to look at the degree of total leukocyte recruitment (Wirtz et al., 2007). One proposed mechanism for SCFA to affect the innate immune response is through taste-receptors present on the cell surface of leukocytes and, more specifically, neutrophils (Vinolo et al., 2009; Maslowski et al., 2009). Indeed, butyrate is considered to be the most beneficial SCFA in terms of gut health, as it is known to down-regulate nuclear factor-beta (NF-κB), a master regulatory switch closely involved in inflammatory processes, and increase peroxisome proliferator-activated receptor gamma, to ultimately reduce paracellular permeability of colonic epithelial cells (Segain et al., 2000; Russo et al., 2012; Kinoshita et al., 2002). Butyrate also is considered the primary energy source for colonic epithelial cells (Topping and Clifton, 2001). More recently, however, there has been interest in the role of SCFA (in particular, acetate) in controlling leukocyte recruitment (mainly neutrophils) to the site of inflammation via the taste receptor free fatty-acid receptors (FFAR) 2 and 3.

Prevailing hypotheses suggest SCFA produced by the colonic microbiota could control leukocyte recruitment by two main mechanisms: (1) activation of FFAR 2 and 3, and (2) inhibition of histone deacetylase (Vinolo et al., 2009). Neutrophil recruitment to the site of inflammation has been of recent interest in terms of controlling the inflammatory response. Short-chain fatty acids have been found to bind members of the FFAR2 family, which are highly expressed on neutrophils, monocytes, adipocytes, and on enterendocrine cells in the GI tract (Vinolo et al., 2009; Nøhr et al., 2013). Since SCFA are binding agonists for FFAR2, they serve to activate several intracellular pathways such as protein kinase C, mitogen-activated protein kinases, and other transcriptional factors involved with the chemotactic movement of neutrophils (Vinolo et al., 2009). Although SCFA are a chemoattractant for neutrophils *in vitro,* there are limited studies that investigate the implications of this *in vivo* where there are other FFAR2 binding agonists (e.g., formyl-methionine-leucine-phenylalanine, chemokines, and leukotriene B4) (Vinolo et al., 2009).

Recent evidence from a DSS-induced colitis model involving FFAR2-knockout mice suggested that ingestion of solubilized acetate in the presence of colonic microbiota ameliorated the acute inflammatory response (Maslowski et al., 2009). Furthermore, the ingestion of SCFA also had a systemic effect on inflammation by reducing inflammation in mice with asthma (Maslowski et al., 2009). The *in vitro* and *in vivo* studies clearly show an effect of SCFA on mounting a neutrophil chemotactic response; however, these results are contingent on ingestion of SCFA, not on intake of dietary fermentable fiber. We measured distal colon gene expression of FFAR2 as a possible involvement of SCFA in controlling leukocyte recruitment. While there was no interactive effect of diet and water treatments, mice induced with DSS-colitis exhibited higher expression of FFAR2 in distal colon, which supports the notion that FFAR2 has a role in the inflammatory response initiated by chemically-induced colitis.

An alternative explanation for the immune response seen here is the bridge between the adaptive and innate immune systems through changes in IL-17a expression. During DSS-induced colitis, mice ingesting PF exhibited IL-17a expression levels comparable to mice given distilled water. Ingestion of non-fermentable Cell during DSS exhibited a 40-fold increase in expression of IL-17a relative to mice fed PF. Specialized T-cells, called T-helper 17 (Th17) cells, are considered a major source of IL-17a, which attracts neutrophils (Jin and Dong, 2013). Recently, there has been interest in a T-cell subtype (i.e., innate-Th17 cells) that reside in the lamina propria of the intestine. Enteric bacteria such as *Citrobacter rodentium* and *Salmonella typhimurium* have been shown to activate resident Th17 cells and, ultimately, produce IL-17 in cecal tissue (Jin and Dong, 2013). Other innate immune cell types produce IL-17 as well, including lymphoid tissue induced cells, natural killer and natural killer T cells, macrophages, and Paneth cells (Reynolds et al. 2010; Cua et al., 2010). Furthermore, IL-17a signal transduction mediates NF-κB and MAPK pathways that ultimately lead to the concentrations of pro-inflammatory cytokines, chemokines, and eventually myeloid cell recruitment to the site of inflammation (Shalom-Barak et al. 1998; Awane et al. 1999). Recently, SCFA have been found to induce both effector and regulatory T cells by suppression of histone deacetylases in a manner independent of both FFAR2 and 3 (Park et al., 2015). Inhibition of histone deacetylases by SCFA and, in particular, acetate, has been shown to regulate the mTOR pathway required for the generation of Th17 cells (Park et al., 2015).

Inflammatory bowel diseases such as Crohn's disease and ulcerative colitis are autoimmune disorders marked by flares of uncontrolled inflammation, and non-pharmaceutical remedies to control this inflammation are desperately needed. Overall, our present study, taken together with previous investigations of dietary PF *in vitro* and in the dog, strongly suggest that PF is a prebiotic fiber that has anti-inflammatory properties in an acute-colitis model. Results of

this study confirm that ingestion of moderately fermentable PF is protective against weight loss and pro-inflammatory cytokines induced by DSS-colitis compared with non-fermentable cellulose. Furthermore, ingestion of PF elicited decreases in leukocyte recruitment pathological scores in the distal colon of DSS-treated mice. Our data associate concentrations of SCFA in leukocyte recruitment, as indicated by changes in chemokine expression (CXCL1). This response, however, may be due to decreasing induction of effector and regulatory T-cells and, in particular, innate Th17 cells independent of FFAR2 as indicated by changes in expression of IL-17a.

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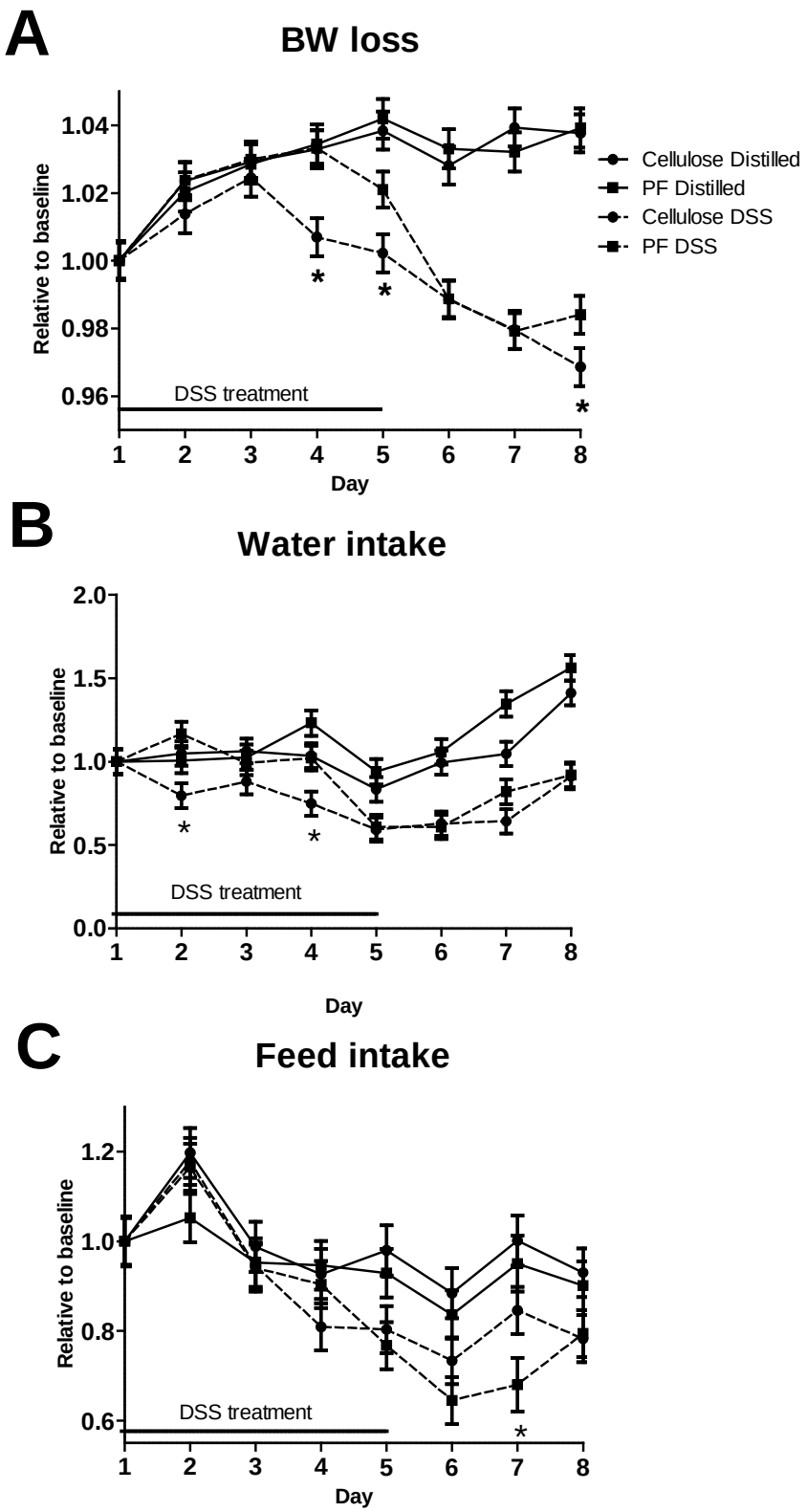

Figure 4.1. Effects of DSS on body weight (A), water intake (B), and food intake (C). PF; potato fiber; DSS; dextran sodium sulfate. All values are expressed relative to baseline, which was the percentage of the body weight, food and water intakes on the day of DSS induction. *Signifies a difference ($P < 0.05$) within day between dietary treatments for mice receiving DSS. Cellulose/distilled (n=17); potato fiber/ distilled (n=16); cellulose/DSS (n=17); potato fiber/DSS (n=17).

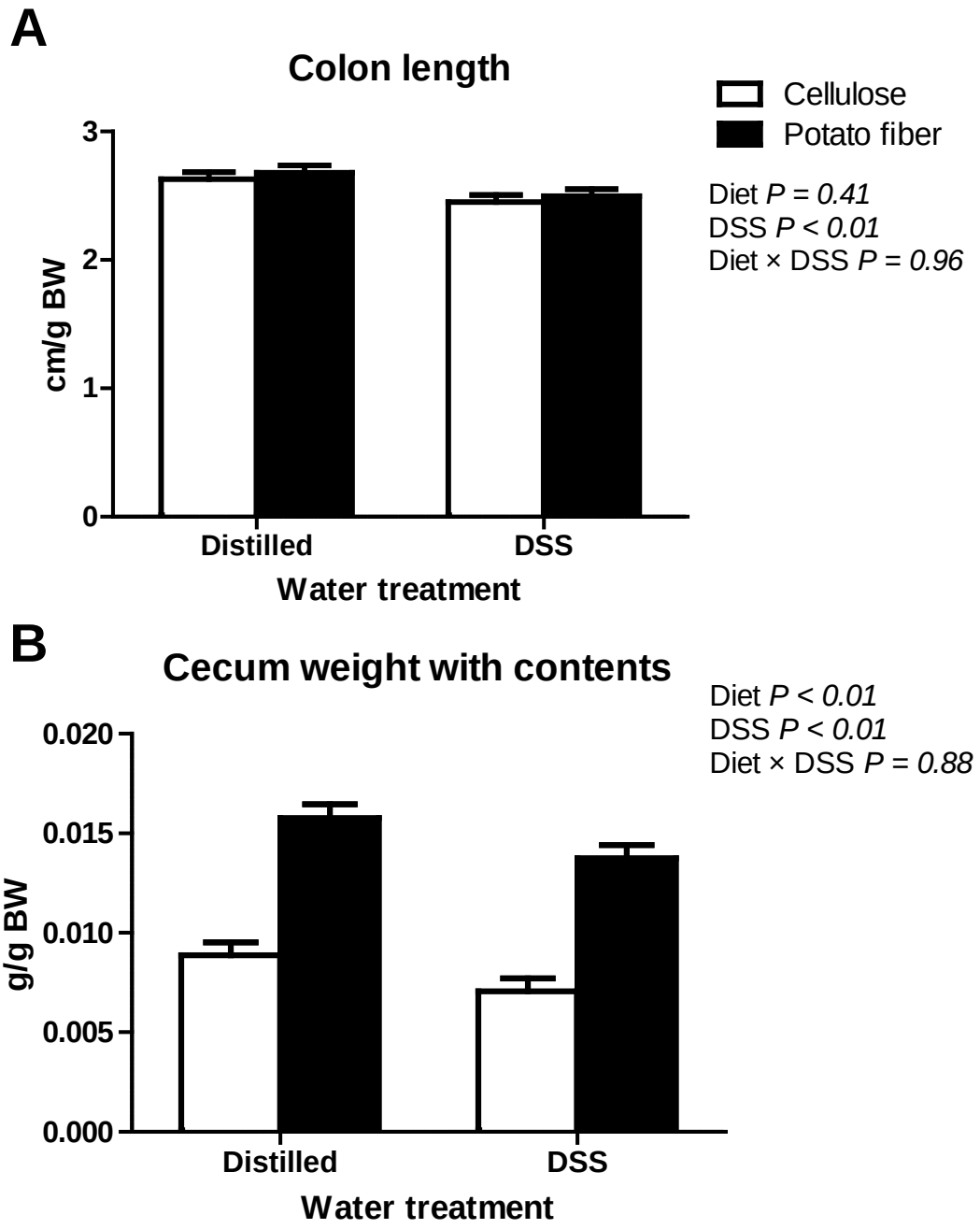

Figure 4.2. Effects of DSS on colon length (**A**) and cecum weight with contents (**B**). Both colon length and cecum weights with contents are represented relative to total BW on d 8 post-DSS (i.e., study conclusion). Cellulose/distilled (n=17); potato fiber/distilled (n=15); cellulose/DSS (n=17); potato fiber/DSS (n=17).

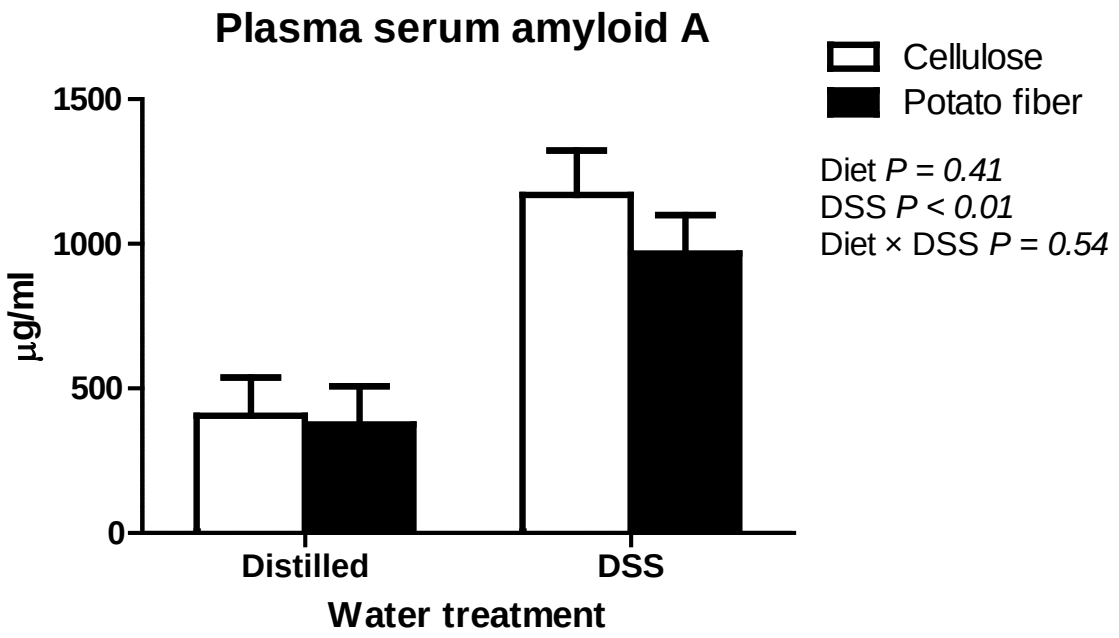

Figure 4.3. Effects of DSS on plasma concentrations of serum amyloid A. Cellulose/distilled (n=8); potato fiber/ distilled (n=8); cellulose/DSS (n=6); potato fiber/DSS (n=8).

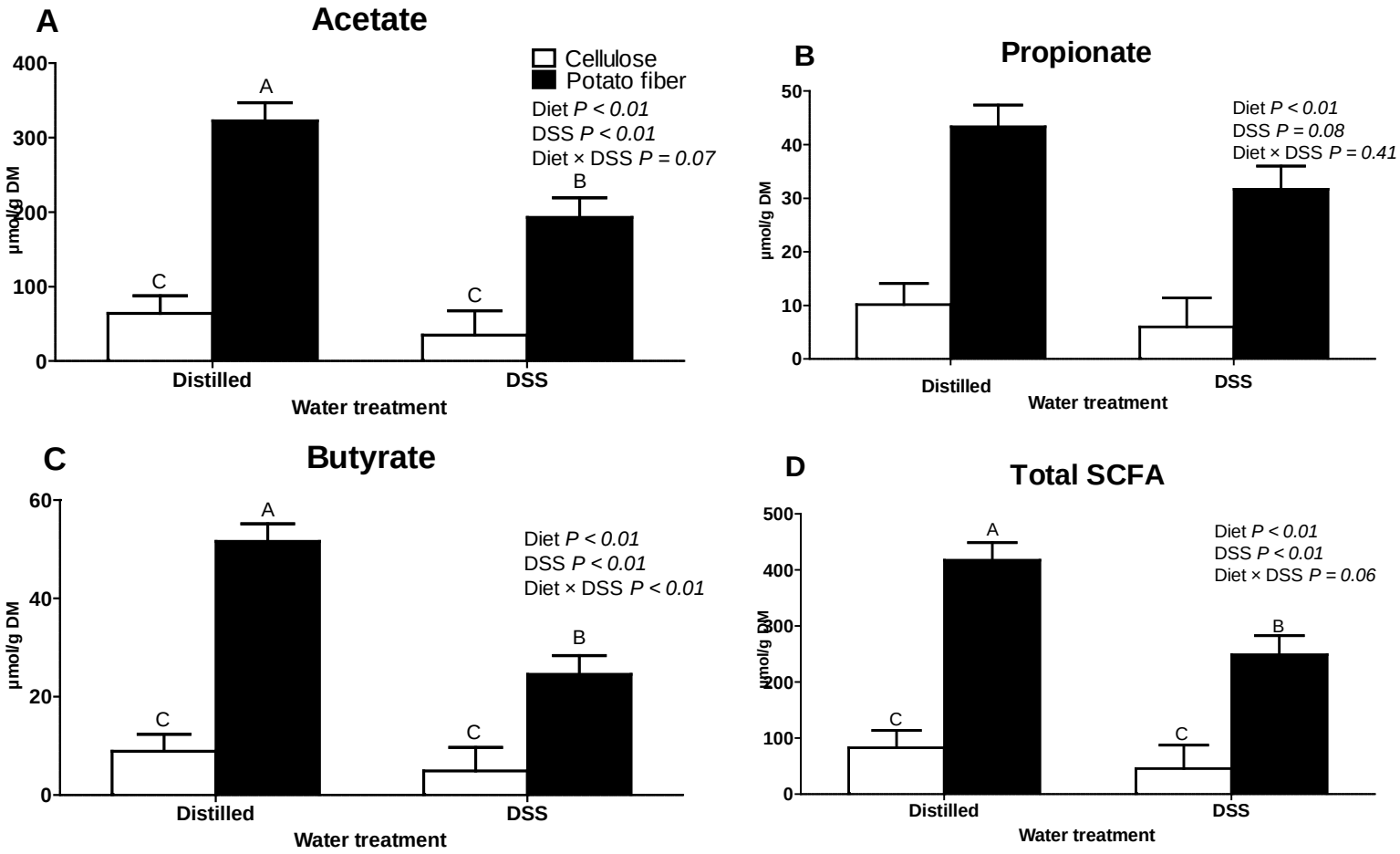

Figure 4.4. Effects of DSS on cecal concentrations of acetate (A), propionate, (B), butyrate (C), and total short-chain fatty acids (SCFA) (D) when mice were fed a cellulose or potato fiber diet. Different letters denote differences ($P < 0.05$) between treatment groups (cellulose/distilled (n=17); potato fiber/distilled (n=16); cellulose/DSS (n=9); potato fiber/DSS (n=14)). All SCFA concentrations are represented as micromoles per gram of cecal content on a dry matter basis.

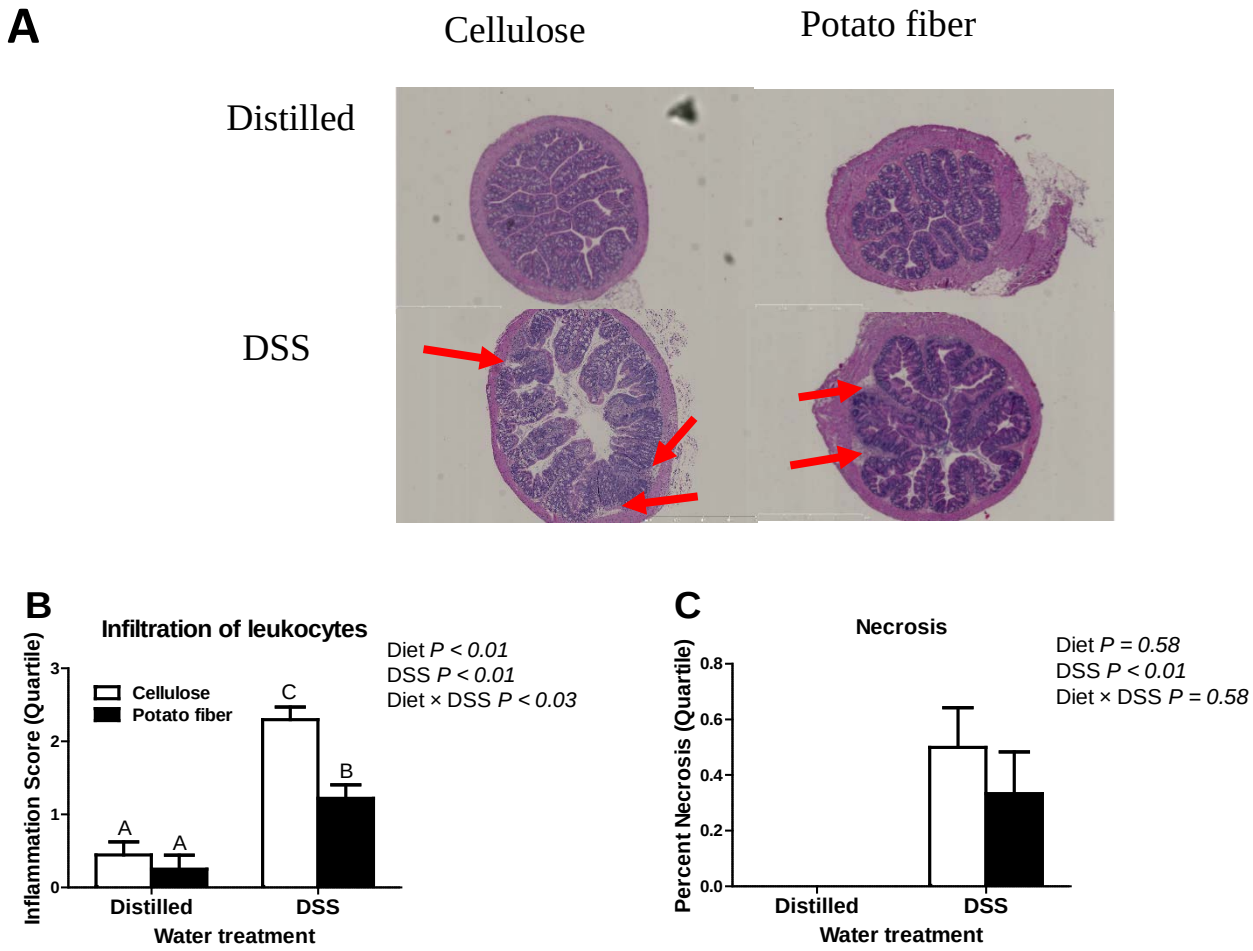

Figure 4.5. Effects of PF on visual differences in leukocyte infiltration and edema (**A**). Red arrows represent areas of leukocyte (mainly neutrophils) accumulation in the lamina propria. Histology scores were based of a previously validated scoring system (Wirtz et al., 2007) for infiltration of leukocytes (**B**) and percent necrosis (**C**) are presented for all treatments. Cellulose/distilled (n=9); potato fiber/ distilled (n=8); cellulose/DSS (n=10); potato fiber/DSS (n=9). Different letters represent differences ($P < 0.05$) between treatment groups.

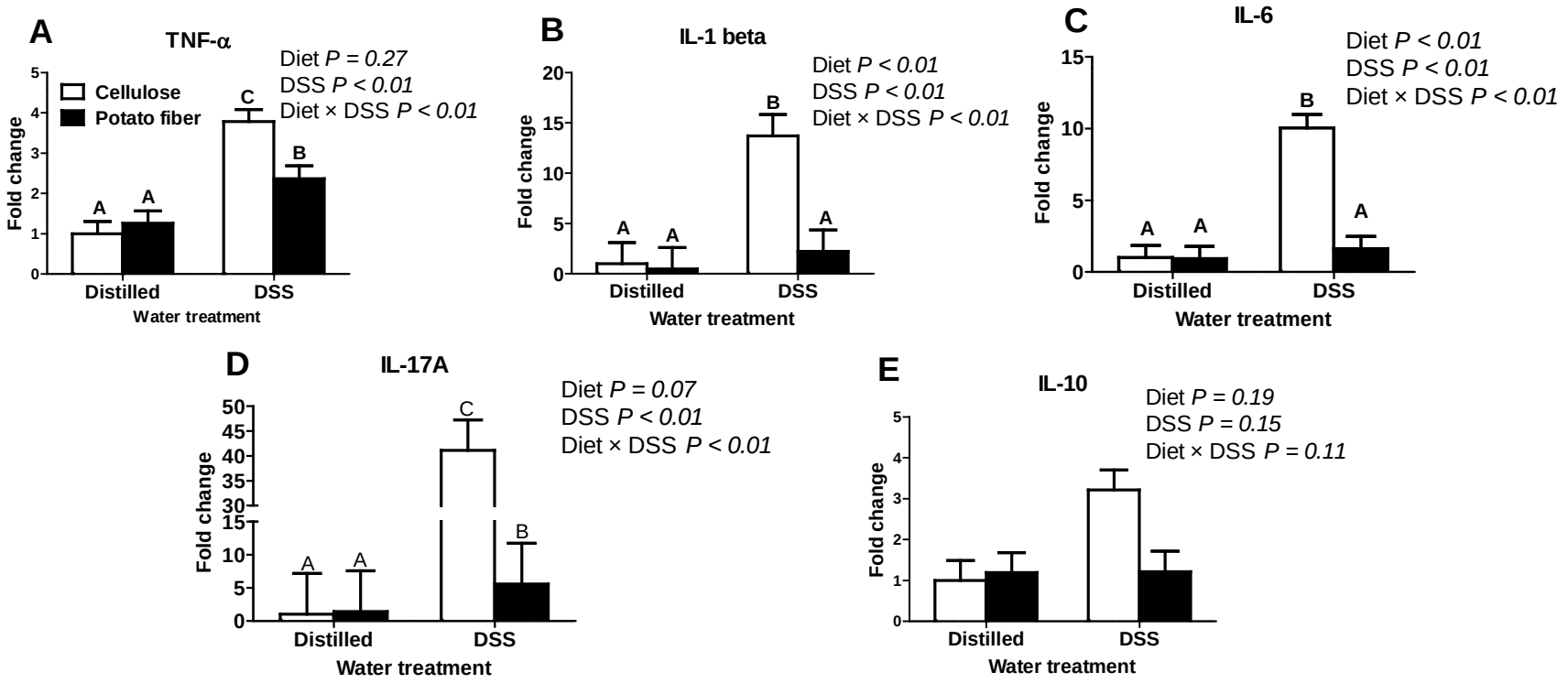

Figure 4.6. Effects of DSS on distal colon gene expression of pro-inflammatory cytokines (n=10-12 per group) (TNF-α, IL-1β, IL-6, and IL-17A) (**A-D**, respectively) and anti-inflammatory cytokine IL-10 (**E**) in both cellulose (Cell)-and potato fiber (PF)-containing diets. All gene expression is represented as fold change in ΔΔ-Ct values relative to the cellulose/distilled treatment group. Different letters represent differences ($P < 0.05$) between treatment groups.

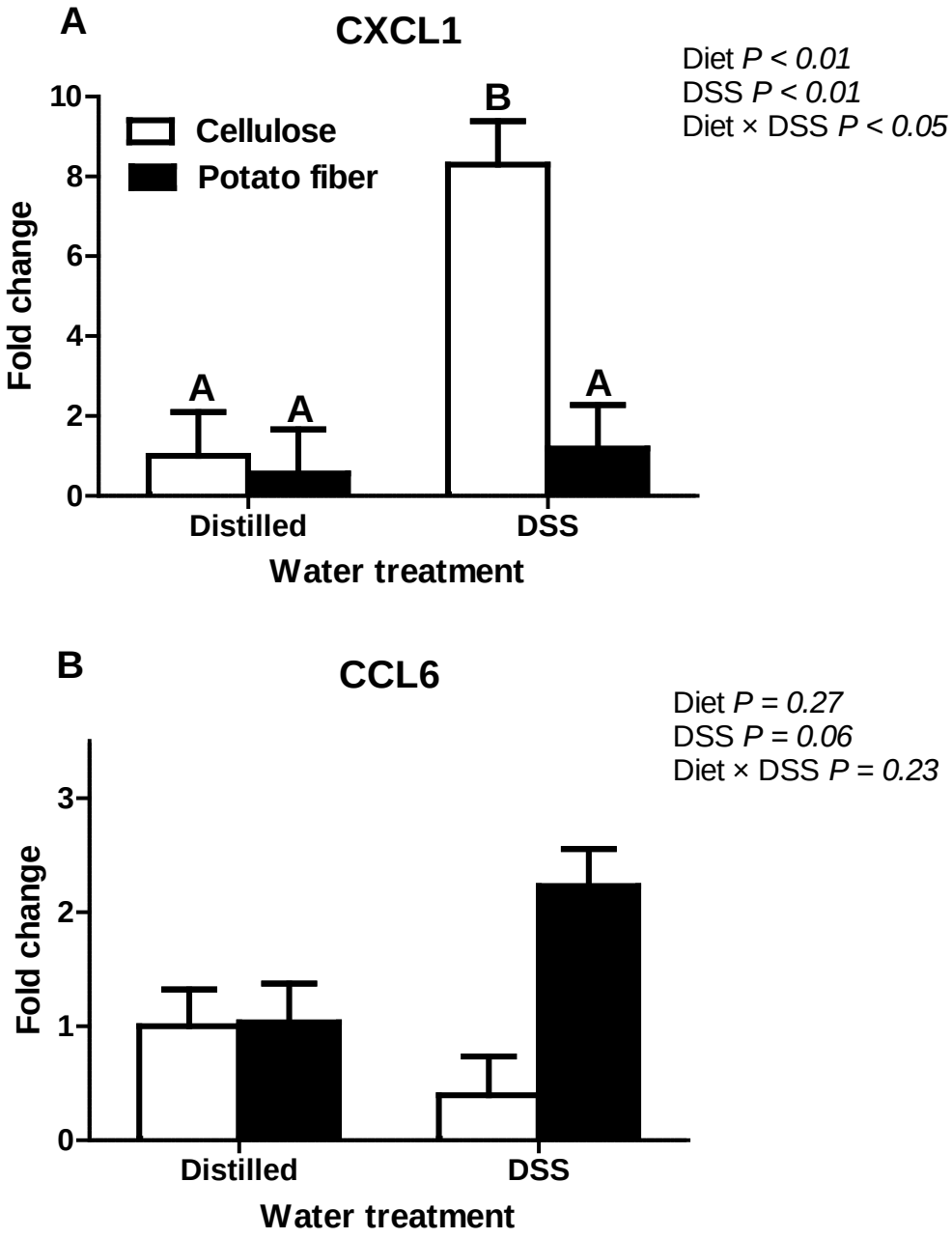

Figure 4.7. Effects of DSS on distal colon gene expression of the chemokines CXCL1 (n=12 per group) (**A**) and CCL6 (n=11-12 per group) (**B**) when mice were fed either cellulose or potato fiber diets. All gene expression is represented as fold change in ΔΔ-Ct values from the cellulose/distilled treatment group. Different letters represent differences ($P < 0.05$) between treatment groups.

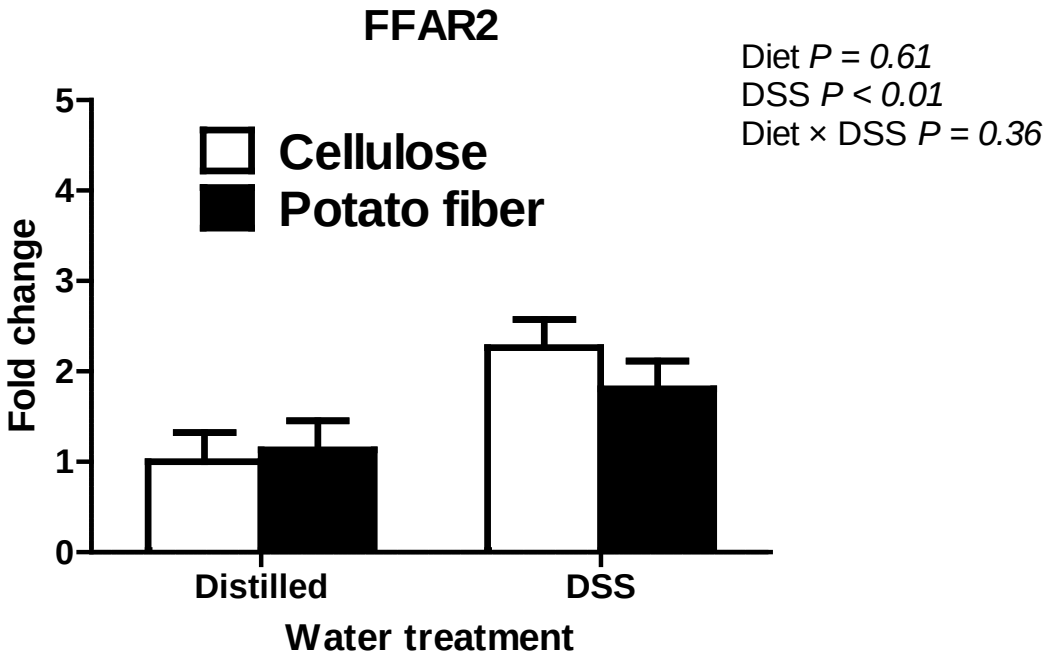

Figure 4.8. Effects of DSS on distal colon gene expression of free fatty acid receptor 2 (FFAR2) when mice were fed either cellulose or potato fiber diets. All gene expression is represented as fold change in ΔΔ-Ct values from the cellulose/distilled treatment group. Cellulose/distilled (n=11); potato fiber/ distilled (n=11); cellulose/DSS (n=12); potato fiber/DSS (n=12)

CHAPTER 5: FUTURE DIRECTIONS

The collection of studies presented in this thesis display how changes in the fecal microbiome through ingestion of dietary fiber in healthy dogs were associated with health and disease. In particular, PF ingestion modulated the fecal microbiome in dogs that was suggestive of anti-inflammatory effects. Therefore, we designed a study to evaluate the efficacy of PF in attenuating the inflammatory response in an acute-colitis mouse model. This study revealed that a PF-containing diet was more anti-inflammatory than the Cell diet in DSS colitis. We investigated this further by showing associations with SCFA concentrations and leukocyte recruitment. Specifically, we made an association with DSS and FFAR2, which is found on enteroendocrine cells and leukocytes in the lamina propria of colon (Nøhr et al., 2013). This association was also made with innate Th17 cells that reside in the lamina propria. Therefore, future studies are needed to investigate the mechanism of how SCFA production influences immune cell recruitment and thereby alters inflammatory processes.

Utilizing a genetic knockout mouse would be most effective in studying the role FFAR2 in relation to dietary fiber intake and inflammation. Specifically, an FFAR2 knockout mouse provided either a PF or Cell diet in response to DSS colitis would help to elucidate a mechanism for how SCFA may control leukocyte recruitment in the distal colon. Previous *in vivo* studies have investigated how ingestion of SCFA could affect the inflammatory response in an FFAR2-dependent manner; however, this has not been related back to the ingestion of specific dietary fibers (Maslowski et al., 2011; Vinolo et al., 2011). It is advisable to investigate how a fermentable vs. non-fermentable diet would impact leukocyte recruitment and, in particular, neutrophil recruitment in a FFAR2 knockout given DSS colitis. Our previous study utilized a hematoxylin and eosin stain on distal colon tissue to assess leukocyte recruitment by an independent observer.

In order to specifically link SCFA concentrations and neutrophil recruitment, one could utilize immunohistochemistry (IHC) of the distal colon tissue incubated with the primary antibodies Ly6G and p38. It has been previously shown that phosphorylation of p38 MAPK lessens the chemotactic response of neutrophils (Sina et al., 2009). Assessment of distal colon by IHC with these primary antibodies would assess: (1) a link between SCFA concentrations and neutrophil recruitment, and (2) changes in the chemotactic response of neutrophils in response to fermentable fiber intake. We hypothesize that dietary PF would decrease neutrophil recruitment and increase phosphorylation of p38 MAPK intracellular pathway in colon-derived neutrophils.

One other future direction of this work would be to investigate different immune cell phenotypes in response to fermentable fiber intake. More specifically, looking at immune cells involved in the adaptive immune response. The DSS model is effective at investigating the role of the innate immune response; however, the major limitation of this model is that it does not require a T or B cell response (Chassing et al., 2014). Therefore, the more appropriate model to investigate the adaptive immune response would be utilizing the IL-10 knockout model. We would use the same diets in order to evaluate fermentable fiber's influence on attenuating the inflammatory response in this model. Immunofluorescence (IF), IHC, or flow cytometry would be appropriate in quantifying different immune cell types. We observed a robust response in mRNA expression of IL17a in distal colon tissue in Chapter 4 of this thesis, and we associated this effect with innate Th17 cells. Therefore, interest in evaluating these cells in distal colon by IF using primary antibodies for IL17 and CD4 may be useful in evaluating their role in this type of colitis (Zhang et al., 2013).

Overall, our combination of studies revealed associations between fermentable fiber intake and leukocyte recruitment. In order to evaluate the exact mechanism that shows a direct link

between SCFA and leukocyte recruitment and activation, more studies utilizing genetic knockout mice and different techniques, such as IF, IHC, and flow cytometry, are needed. These current studies are a pivotal step in showing the immunomodulatory effects of dietary fiber on colonic inflammation. In conclusion, more studies are needed to investigate the mechanisms of how fermentation of dietary fiber impacts different immune cell types to be recruited to the site of inflammation.

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