EFFECTS OF PROBIOTICS, PREBIOTICS AND DIETARY FIBERS ON THE GASTROINTESTINAL HEALTH AND IMMUNE INDICES OF ADULT DOGS AND CATS

BY

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DISSERTATION

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ABSTRACT

Functional ingredients are those that provide health benefits beyond that of basic nutrients, and their inclusion in pet foods has become a popular practice in the industry. This is largely due to the increased owner awareness pertaining to pet health and well-being. A variety of functional ingredients, including fibers, prebiotics, probiotics, postbiotics, and milk oligosaccharides have been considered and added to pet foods to support gastrointestinal and immune health. While many of these ingredients have been tested individually, others have yet to be tested. Moreover, commercial foods often include ingredient blends that also require testing. Therefore, the goal of this dissertation was to investigate the effects of individual or blends of functional ingredients on the gastrointestinal health and immune responses of healthy adult dogs and cats.

The aim of the first study was to evaluate the effects of diets containing blends of fibers, 'biotics', and/or spray-dried plasma on apparent total tract digestibility (ATTD), stool quality, fecal microbiota and metabolites, and immune health outcomes of adult dogs. Twelve healthy adult intact English pointer dogs (6 M; 6 F; age = 6.4 ± 2.0 yr; BW = 25.8 ± 2.6 kg) were used in a replicated 3x3 Latin square design to test diets formulated to: 1) contain a low concentration of fermentative substances (control diet; CT); 2) be enriched with a fiber-prebiotic-probiotic blend (FPPB); and 3) be enriched with a fiber-prebiotic-probiotic blend + immune-modulating ingredients (iFFPB). In each 28-d period, 22 d of diet adaptation was followed by a 5-d fecal collection phase and 1 d for blood sample collection. Dogs fed FPPB or iFPPB had decreased ($P<0.01$) dry matter (DM), organic matter, fat, fiber, and energy ATTD, improved ($P<0.01$) fecal scores and higher ($P<0.01$) fecal DM% than CT. Serum triglyceride and cholesterol concentrations were lower ($P<0.01$) in dogs fed FPPB or iFPPB than those fed CT. Fecal protein

catabolite (isobutyrate, isovalerate, indole, ammonia) and butyrate concentrations were lower ($P<0.05$), while fecal immunoglobulin A (IgA) concentration was higher ($P<0.01$) in dogs fed FPPB and iFPPB than those fed CT. Fecal microbial alpha diversity was lower ($P<0.05$) in dogs fed iFPPB and the relative abundance of 20 bacterial genera were altered in dogs fed FPPB or iFPPB compared to those fed CT. The circulating helper T cell:cytotoxic T cell ratio was higher ($P<0.05$) in dogs fed iFPPB than those fed CT. Circulating B cell numbers were lower ($P<0.05$) in dogs fed FPPB than those fed iFPPB, and lower ($P<0.05$) in dogs fed iFPPB than those fed CT.

The aim of the second study was to evaluate the ATTD, stool quality, fecal microbiota and metabolites, and immune health outcomes of dogs fed a novel milk oligosaccharide (MO) biosimilar (GNU 100). 32 adult dogs were used in a completely randomized design study. Following a 2-wk baseline, dogs were assigned to one of four treatments and fed for 26 wk: 0%, 0.5%, 1% and 1.5% GNU100. On wk 2, 4 and 26, fresh fecal samples were collected to measure stool quality, IgA concentration, and calprotectin concentration, and blood samples were collected to measure serum chemistry and inflammatory markers and hematology. On wk 2 and 4, fresh fecal samples were collected to measure metabolites and microbiota. On wk 4, total feces were collected to assess ATTD. All dogs were healthy and had no signs of GI intolerance or illness. All diets were well-accepted and food intake, fecal characteristics and metabolite concentrations and macronutrient digestibilities were not altered. GNU100 modulated fecal microbiota, increasing evenness and *Catenibacterium*, *Megamonas*, and *Prevotella* (SCFA producers) and reducing *Collinsella*. Overall, results suggest that GNU100 is palatable and well-tolerated, causes no genotoxicity or adverse effects on health, and beneficially shifts the fecal microbiota, supporting the safety of GNU100 for inclusion in canine diets.

The aim of the third study was to evaluate the effects of diets enriched in resistant starch or fibers on the fecal characteristics, microbiome, and metabolite profiles of cats. Twelve healthy adult domestic shorthair cats (age = 9.6±4.0 yr; body weight = 3.9±1.0 kg) were used in a replicated 3x3 Latin square design to test diets that were enriched with: 1) resistant starch (ERS), 2) a fiber-prebiotic-probiotic blend (FPPB), or 3) a fiber-prebiotic-probiotic blend + immune-modulating ingredients (iFPPB). In each 28-d period, 22d of diet adaptation was followed by fecal and blood sample collection. Fecal microbiota was assessed with shotgun and 16S sequencing. In addition, fecal and blood metabolite measurements and white blood cell stimulation was performed to assess immune function. A total of 1,690 bacterial species were identified via shotgun sequencing, with 259 species differing between fiber-rich and ERS treatments. Fiber-rich treatments increased diversity and promoted Firmicutes and Bacteroidetes populations, while resistant starch reduced ($P<0.01$) microbial diversity and fecal pH, led to a bloom in Actinobacteria, and modified KO terms (KEGG Orthology; KEGG: Kyoto Encyclopedia of Genes and Genomes) pertaining to starch and sucrose metabolism, fatty acid biosynthesis and metabolism, epithelial cell signaling, among others. Resistant starch increased ($P<0.01$) butyrate concentrations; decreased ($P<0.01$) propionate and protein catabolite (branched-chain fatty acids; phenols and indoles; ammonia) concentrations and reduced ($P<0.05$) blood cholesterol, which correlated strongly with microbial taxa and KO terms, and allowed for a high predictive efficiency of diet groups by random forest analysis.

Our results indicate that supplementation of a fiber-prebiotic-probiotic blend may have positive impacts on stool quality, fecal metabolites, fecal microbiota and immune function in dogs. Different effects were observed in cats fed a fiber-prebiotic-probiotic blend, however, likely being impacted by the ERS diet they were compared with. Our results indicate that the

feline microbiome and metabolite profiles were highly responsive to resistant starch intake. In addition, the MO biosimilar tested did not produce a beneficial health effect, though no adverse effects were observed. The utilization of these fiber-prebiotic-probiotic + immune modulating ingredient blends and MO on geriatric or obese dog and cat populations that are more susceptible to inflammation and gut microbial dysbiosis may also be of interest in future studies.

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CHAPTER 1: INTRODUCTION

According to an American Pet Products Association 2021-2022 survey, it is estimated that 70% of U.S. households, or 90.5 million homes, own a pet (APPA, 2021). Those numbers translate to approximately 69.0 million U.S. households owning dogs and 45.3 million U.S. households owning cats. The estimated dollar amount spent on pet foods and treats was 42.0 billion dollars in the U.S. in 2021. The pet food market is expected to maintain its steady growth in the upcoming years. This market growth has not only been fueled by the increasing number of pets, but also because of pet humanization and payment for higher priced products. Although it was not always the case, pets are now considered to be family members by most owners, which are highly concerned about the overall health, longevity, and quality of life of their pets.

Factors such as age, disease status, environmental exposures, and diet can impact the overall health and quality of life of an animal. Diet is a factor that can be easily altered to beneficially affect gastrointestinal and immune health. The gut microbiota harbors trillions of microbes that carry out metabolic activities essential to maintaining the gastrointestinal and immune health (Suchodolski, 2011; Pilla and Suchodolski, 2021). This complex interplay comes from microbial fermentation of non-digestible substrates in the gut, producing a variety of metabolites that have different functions. For instance, microbial fermentation of non-digestible carbohydrates promotes the production lf short-chain fatty acids (SCFA), primarily acetate, propionate and butyrate (Valdes et al., 2018). These SCFA not only lower the pH of the colon and inhibit pathogen binding to the mucosa, but the butyrate produced serves as a primary source of energy to colonocytes. Additionally, different signaling pathways carry out the cross-talk between the microbes and host immune system (Kau et al., 2011; Nicholson et al., 2012; Hooper et al., 2012). Such changes in the gut microbiota can be achieved through dietary modification

(Pilla and Suchodolski, 2021). This has led to an increased demand for premium pet foods that contain functional ingredients that can provide health benefits beyond basic nutritional needs. Some of these ingredients include probiotics, prebiotics, yeast products, spray-dried animal plasma and milk oligosaccharides. While many of these functional ingredients have been evaluated for application in dog and cat food, the understanding of these ingredients on GI and immune health outcomes are often limited. Therefore, the overall objective of this dissertation was to investigate the effects of different functional ingredients on the gastrointestinal health and immune responses of healthy adult dogs and cats.

The objective of the first study was to evaluate the effects of diets containing blends of fibers, 'biotics', and/or spray-dried plasma on apparent total tract digestibility (ATTD), stool quality, fecal microbiota and metabolites, and immune health outcomes of adult dogs. A control diet was formulated to be a premium diet that provided a low level of substrate for microbial fermentation. The second diet contained a blend of fibrous ingredients (i.e., oat groats; beet pulp; pea fiber), a probiotic, and a prebiotic (i.e., inulin), which was expected to impact GI and immune health primarily through fermentation and SCFA production. The third diet contained the fiber-probiotic-prebiotic blend plus ingredients thought to support immune function [i.e., spray-dried animal plasma (SDAP); yeast fermentation product] via different mechanisms.

The second study evaluated the safety and GI tolerance of GNU100 and explore its effects on ATTD, stool quality, fecal microbiota and metabolites, and immune health outcomes by conducting a 26-wk study in healthy adult dogs. The objective of the third study was to explore the fecal microbial community profiles and their functions in domestic cats fed diets enriched in either RS or a combination of dietary fibers, prebiotics, and probiotics utilizing

shotgun sequencing. Secondary objectives were to identify significant fecal bacterial taxa-bacterial gene-metabolite changes in response to the different dietary treatments.

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CHAPTER 2: LITERATURE REVIEW

Canine and feline gastrointestinal health and microbiota

The mammalian body is densely populated with microorganisms, which includes bacteria, archaea, fungi, protozoa, and viruses, collectively known as microbiota. Microbes are present throughout the mammalian body, with habitats on the skin, in the oral cavity, in the urogenital tract, and in the GI tract. Among the different body habitats, the GI tract harbors the most dense population of microbes, with the GI tract being estimated to contain as many bacterial cells as there are host cells in the body (Sender et al., 2016). In healthy adult dogs and cats, the predominant GI bacterial phyla are the Firmicutes, Proteobacteria, Fusobacteria, Bacteroidetes and Actinobacteria, although the relative abundances vary by GI region, the host, laboratory methods used for analyses, age, sex, health status, among many other factors (Ritchie et al., 2008; Suchodolski et al., 2008; Middelbos et al., 2010; Deng and Swanson, 2015). Within Firmicutes, *Enterococcus* and *Lactobacillus* are predominant in cats, while *Streptococcus* and *Lactobacillus* are predominant in dogs (Handl et al., 2011). Moreover, *Prevotella* and *Bacteroides* within the Bacteroidetes phyla are typically the most abundant bacterial genera in canine and feline species (Alessandri et al., 2019 a-c, 2020). In addition, *Fusobacterium* within the Fusobacteria phylum has been identified as a primary member of canine GI microbiota, though it is present at a lower abundance in cats (Suchodolski et al., 2008; Alessandri et al., 2019 a-c, 2020).

Diet and GI microbiota

There are many factors that may influence the composition of the GI microbiota, as well as its long-term stability and resilience. Some of these factors include host species, host age, diet, environmental factors, disease status, and medical interventions. Of these factors, dietary

intervention plays an important role in defining the composition and activity of the microbiota community and one that has been proven to quickly respond to dietary changes and alter the GI microbiota (David et al., 2014; Maukonen and Saarela, 2015).

Dietary substrates that are not digested by the host enter the colon, where microbial fermentation may take place. Different bacterial species ferment and utilize different substrates, primarily carbohydrates and protein. The fermentation of these substrates yields secondary metabolites such as short-chain fatty acid (SCFA), which are generally the result of carbohydrate fermentation. These organic acids are considered beneficial, as they provide energy to colonocytes, lower GI pH, and prevent pathogen colonization. On the other hand, substrates of protein catabolism have often been associated with negative health effects, as they lead to the production of branched-chain amino acids (BCFA), ammonia, phenol, P-cresol, or biogenic amines that can cause intestinal epithelial cell damage (Aguirre et al., 2016). Therefore, by manipulating diet, the gut microbiota may be modulated in ways that produce beneficial health effects on the host.

In dogs and cats, studies have shown that the macronutrient profile, ingredient composition and even different diet processing methods can affect the gut microbiota (Sandri et al., 2017; Algya et al., 2018; Do et al., 2021). Recent studies have demonstrated that diets manufactured using different processing methods (processing temperatures) or containing different ingredients (raw meat, etc.) may impact the gut microbiota differently (Sandri et al., 2017; Algya et al., 2018; Do et al., 2021).

Dietary ingredients such as fibers, prebiotics, and probiotics have been studied most frequently, as they induce specific changes in the microbiome. These ingredients often enrich Firmicutes because many of the members of that phylum are fiber degraders and SCFA

producers. Moreover, the evaluation of GI microbiomes fed these ingredients often reports increased abundance of beneficial microbes such as *Bifidobacterium, Lactobacillus, and Faecalibacterium*, which ferment carbohydrates to produce SCFA. Details of how some of these ingredients modulate the GI microbiota will be discussed in the following sections.

Canine and feline immunity

Innate immunity

Immunity is the state of protection from different infectious diseases, with the body employing strategies that are either innate or adaptive (Hoebe et al., 2004). The innate immune system is composed of anatomic barriers and serum proteins that are part of the complement system and innate immune cells. They act as the first line of defense by quickly recognizing and licensing effector function to kill pathogens in an unspecific manner. Cells of the innate immune system include macrophages, monocytes, dendritic cells, neutrophils, eosinophils, basophils, mast cells and natural killer (NK) cells. Innate immune cells have pattern recognition receptors (PRRs) that recognize and bind pathogen-associated molecular patterns (PAMPs), leading to cytokine/chemokine production to initiate inflammation and phagocytosis to destroy pathogenic cells. Toll-like receptors (TLR) make up a family of PRRs. They are transmembrane glycoprotein receptors located either on the cell surface or intracellularly. Their main responsibility is to recognize pathogens such as bacteria, fungi, and viruses. When a PAMP binds to its corresponding TLR, signal protein complexes are formed, and a signal transduction cascade is initiated so that a pro-inflammatory cellular response can occur. There are about 12 different functional TLR present in mammals (**Table 2.1**). Excluding TLR3, all other TLR utilize myeloid differentiation primary response (MyD) 88, which is a protein that activates transcription factors including mitogen activated protein (MAP) kinase, interferon regulatory

factor 3 (IRF-3), and nuclear factor kB (NF-kB). These transcription factors activate the genes for inflammatory cytokines, interleukin (IL)-1, IL-6, tumor necrosis factor-α (TNF-α), and others.

NK cells also mediate inflammatory processes and act differently than other cells by recognizing tumor or virus-infected cells and killing them by cell lysis (Medzhitov and Janeway, 2000). Macrophages and dendritic cells are known as antigen-presenting cells (APC) that bind and present pathogens to B cells via major histocompatibility complex (MHC) class II. This initiates the adaptive immune process.

Adaptive immunity

The adaptive immune system is an acquired process that develops after immune cells have been exposed to a specific pathogen. The primary cells in an adaptive response are B cells and T cells. T cells can recognize a pathogen, differentiate, and proliferate to carry out effector function to kill the pathogen and stimulate B cells to proliferate and produce antibodies against it. Antibodies, also known as immunoglobulins (Ig), are B cells of the adaptive immunity and are activated by antigens. Their mechanism of action is through bacterial neutralization, opsonization, and complement activation (Tizard, 2018). In dogs and cats, there are 4 classes of Ig, namely IgA, IgE, IgG, and IgM. The Ig characteristics are listed in **Table 2.2**. There are two types of T cells, T cytotoxic (T_C) and T helper (T_H) cells. When T_C cells are activated by APC and T_H cells, they are able to kill the infected cells by recognizing the antigenic peptides presented by MHC class I.

Dietary fiber

Dietary fibers are carbohydrate polymers that escape enzymatic digestion and absorption in the small intestine, with partial to complete fermentation in the large intestine. According to the Codex Alimentarius Comission (2009), dietary fibers are carbohydrate polymers made up of more than 10 monomeric units. This is a broad category that contains many different types and sources of ingredients, including non-starch polysaccharides, non-digestible oligosaccharides, synthesized carbohydrates, resistant starches, and non-digestible carbohydrates of animal origin. In 2016, FDA proposed the definition of dietary fiber to be "non-digestible soluble and insoluble carbohydrates (with 3 or more monomeric units) and lignin that are intrinsic and intact in plants; isolated or synthetic non-digestible carbohydrates (with 3 or more monomeric units) determined by FDA to have physiological effects beneficial to human health" (FDA, 2016). The physiological effects that FDA refers to includes lowering blood glucose, cholesterol, and blood pressure, improved laxation, increased mineral absorption in the intestinal tract, and reduced energy intake.

Inclusion of dietary fibers as a functional ingredient in pet foods has gained interest in the pet food industry because they possess many potential health benefits. In addition to the beneficial health effects proposed by FDA, inclusion of dietary fibers in companion animal diets have led to improved GI health by modulating gut microbiota and improving fecal quality, improving immune function, increasing production of SCFA, aiding in weight loss, and aiding in hairball excretion in cats (Banta et al., 1979; Fahey et al., 1992; Massimino et al., 1998; Swanson et al., 2002; Phungviwatnikul et al., 2022).

Dietary fibers and prebiotics have been of much interest in the pet food industry over the years due its many beneficial health effects. Common sources of dietary fiber present in pet

foods are beet pulp, cellulose, wheat bran, soybean hulls, pectin, with many of the above-mentioned beneficial effects being reported in the literature (Fahey et al., 1990; Elliott et al., 2006; Middlebos et al., 2007; Loureiro et al., 2017; Detweiler et al., 2019; Donadelli and Aldrich, 2019). Many plant-based ingredients that are byproducts of human food processing may provide an excellent yet sustainable source of dietary fiber. Although many fibers already exist, research may continue to test the potential benefits of novel dietary fiber sources.

Prebiotics

Prebiotics were initially defined as a "non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health" (Gibson and Roberfroid, 1995). Because much has changed in recent years, the definition has been updated to "a substrate that is selectively utilized by host microorganisms conferring a health benefit" and includes three important criteria. A prebiotic must be: 1) resistant to mammalian enzyme breakdown; 2) fermented by intestinal microbiota, and 3) selectively utilized by host microorganism to confer a health benefit (Gibson et al., 2017). Prebiotics are often recognized as functional food ingredients and most of them can be classified as dietary fibers, although few fibers are able to meet the criteria of a prebiotic. Prebiotics may be fermented to SCFA that provide energy to maintain epithelial homeostasis in the large intestine, lower colonic pH that limits pathogen growth, and stimulate gut peptide synthesis, which has many other consequent effects on the GI tract and host. Prebiotics have been shown to protect the gut epithelium by increasing epithelial cell numbers and cell turnover, increasing the mucus layer, preventing adherence of pathogens to the epithelial cells, and improving microbial balance (Roberfroid et al., 2010).

Current prebiotics include inulin, galactooligosaccharides (GOS) and fructooligosaccharides (FOS). Although the optimal prebiotic supplementation level depends on the source and purity, high doses (>20% DM) of fructans may have negative effects such as flatulence and loose stools (Flickinger et al., 2003a). A meta-analysis of 15 studies testing 65 different treatments in various breeds of dog showed a linear increase in fecal SCFA with increasing prebiotic (FOS, inulin, MOS, arabinogalactan and yeast cell wall) dose (Patra, 2011). Although dietary inclusions 1-1.5% of prebiotic have been shown to be effective, the cost of using these ingredients can be expensive on a commercial scale. Therefore, more research is needed exploring novel ingredients with prebiotic potential, as well as searching for other functional ingredients that may be used as a blend by adding prebiotic at a lower inclusion level.

Resistant starch

Starch molecules are polysaccharides composed of glucose units linked by α-1,4 and α-1,6 glycosidic bonds. Amylose and amylopectin are the two major forms of starch. Amylose is a linear polymer composed of α-1,4 linkages with a degree of polymerization up to 1,500 to 6,000, whereas amylopectin is a much larger, branched polymer composed of α-1,4 and α-1,6 linkages with a degree of polymerization of 300,000 to 3 million (Buleon et al., 1998; Perez et al., 2009). Based on their rate and extent of digestion, starches are classified into three different categories: rapidly digested starch (RDS), slowly digestibly starch (SDS), and resistant starch (RS) (Englyst et al., 1992; Englyst and Englyst, 2005; Lehmann and Robin 2007). Both RDS and SDS are completely digested in the GI tract, but at different rates. RDS has been associated with elevated glucose and insulin peaks and has been linked with diabetes and coronary heart disease in humans (Coulston et al., 1983; Soral-Smietana and Gallant, 2009).

RS is the fraction of starch that is not digested in the small intestine and escapes to the large intestine for partial or complete fermentation. RS may serve as a substrate for colonic microbial fermentation, providing health benefits through gut microbiome-mediated metabolism. There are currently 5 different categories of RS (RS1-RS5), with RS1 referring to starches that cannot be hydrolyzed due to the barrier effect of cell walls, and which are usually present in beans and grains. RS2 refers to naturally resistant starch granules such as unripe banana and uncooked potato starch. RS3 is difficult to hydrolyze by amylase due to crystal formation during cooling and storage after starch gelatinization, a process called retrogradation (Englyst et al., 1992). RS4 is cross-linked starch that is resistant to hydrolysis due to the change in molecular structure and RS5 is characterized by complexes of amylose and lipids that form a helical structure that is difficult to digest (Jiang et al., 2020). Common sources of RS in pet foods include raw potato starch, legumes, high amylose cornstarch, and chemically modified starches usually produced through processing (Spears and Fahey, 2004).

Many studies have reported increased GI SCFA concentrations, in particular butyrate, with RS consumption. Elevated SCFA production typically lowers colonic pH, which in turn can aid in preventing bacterial overgrowth (Roy et al., 2006). Additionally, butyrate provides energy to colonocytes, prevents, and inhibits colon carcinogenesis, protects the colon against oxidative stress, and exhibits anti-inflammatory properties (Hamer et al., 2008). Due to its GI health implications in humans, RS has received increased attention from pet food manufacturers. There are still few studies that have investigated the effects of RS in dogs and cats, however. A study conducted in beagle dogs of different ages (n=8, 4.0 yr old; n=8, 11.5 yr old) tested diets containing corn that had been extruded to contain low RS (2.2 g/kg DM; 99.9% starch gelatinization) or high RS (15.3 g/kg DM; 62.6% starch gelatinization) (Ribeiro et al., 2019).

After 28 d of diet adaptation, results indicated that high RS-fed dogs in both age groups had increased ($P < 0.01$) fecal total SCFA, acetate, propionate, and butyrate concentrations. In addition, an increase in fecal lactate and a reduction in fecal ammonia was observed (Ribeiro et al., 2019).

Jackson et al. (2020) investigated the physiological effects of consuming dry pet food processed under different extrusion conditions generating either lower or higher concentrations of RS on the gut microbiome of cats. A total of 36 healthy cats were fed identically formulated diets either processed under high (HRS; n = 17) or low (LRS; n = 19) extrusion conditions for 6 weeks. Results showed that cats fed HRS had 43% higher butyrate concentrations at week 3, and 29% higher butyrate concentrations at week 6 of study compared to cats in the LRS group. At week 6, fecal IgA concentrations were 56% higher in the HRS group compared with the LRS group. Though the changes observed in the microbiome were fewer than expected, there were increases ($P < 0.05$) in *Lactobacillus*, a lactate producer, and *Megasphera*, a lactate utilizer in cats fed HRS compared with those fed LRS. There were also decreased ($P < 0.05$) abundances of proteolytic bacteria, namely *Peptostreptococcus* and *Tepidibacter*, in HRS fed cats. Though interest is growing on the use or RS in pet food, research studies are still limited. More studies using RS are needed in dogs and cats to understand their effects on host metabolism and immune function.

Probiotics

Probiotics are “live microorganisms that, when administered in adequate amounts, confer a health benefit on the host (Hill et al., 2014). There are several mechanisms by which probiotics may improve host health, such as increasing gut barrier function (Collado et al., 2007a), maintaining epithelial tight junction integrity, enhancing immune response (Pagnini et al., 2010),

increasing secretory IgA production, preventing and displacing pathogenic bacteria growth (Lee et al., 2003), and increasing production of SCFA (Sakata et al., 2003; Oelschlaeger, 2010; Thomas and Versalovic, 2010). Because these beneficial mechanisms are strain-specific, with some strains increasing the adherence of pathogens to intestinal mucus (Collado et al., 2007b), each potential probiotic must be tested to confirm efficacy.

Despite the growing interest in probiotics, research in companion animals has been limited. In a shelter-based study, cats (n=182) administered with a *Enterococcus faecium* SF68 ($2.1x10^9$ CFU/g) probiotic had lower incidence of diarrhea lasting more than 2 d (7.4%) when compared with the placebo group (20.7%) (Bybee et al., 2011). Studies have also shown improved immune function following probiotic supplementation. For example, in a study in puppies (n=7; 8 wk old), SF68 supplementation ($5x10^8$ CFU/d) for 44 wk increased ($P < 0.05$) serum and fecal IgA concentrations (Benyacoub et al., 2003). In another study, 60 d of supplementation of a *Lactobacillus casei* Zhang, *Lactobacillus plantarum* P-8, and *Bifdobacterium animalis* subsp. *lactis* V9 blend ($2x10^9$ cfu/g) increased ($P < 0.01$) serum IgG and IFN-γ, increased fecal IgA, and reduced TNF-α in dogs when compared with the placebo group (Xu et al., 2019). Probiotics also seem to improve non-specific dietary sensitivity in dogs (n=6), with supplementation of *L. acidophilus* ($6x10^6$ CFU/g) for 12 wk showing improvements in fecal consistency, dry matter content, and defecation frequency, although no significant changes in fecal lactobacilli and bifidobacteria were observed (Pascher et al., 2008).

Although these studies show the potential beneficial effects of probiotic supplementation, the physiological improvements are typically only observed during supplementation. A study testing a synbiotic that contained a mixture of seven different microbial strains (*Enterococcus faecium, Streptococcus salivarus ssp., Thermophilus, Bifidobacterium longum, Lactobacillus*

acidophilus, Lactobacillus casei ssp. rhamnosus, Lactobacillus plantarum, Lactobacillus delbrueckii ssp. bulgaricus ; $5x10^9$ CFU) and a blend of FOS and arabinogalactans was administered to healthy cats (n=12) and dogs (n=12) for 21 d (Garcia-Mazcorro et al., 2011). Although the supplemented probiotic species were detected in 11/12 cats and 10/12 dogs during probiotic administration, these populations returned to baseline once the supplementation ceased. While interest in probiotics continues to grow, more research evaluating different probiotic strains and their mechanisms of action is needed in the future.

Spray-dried animal plasma

Spray-dried animal plasma (SDAP) is a protein concentrate usually of porcine or bovine origin primarily composed of albumin, globulin and fibrinogen type proteins. It is obtained by separating plasma and the cellular fraction of blood through centrifugation, followed by spray drying into a heated chamber at 207 °C to convert it into powder form (Gatnau, 1990). Many animal research studies have shown health benefits of SDAP, with most being used in the livestock industry. Several researchers have evaluated the effect of SDAP on growth performance in weanling pigs. A review of 13 studies evaluating the dose-responsiveness of SDAP during the first 2 wk after weaning reported a 26.8% increase in average daily gain (ADG), 24.5% increase in average daily feed intake (ADFI), and a 3.2% decrease in feed conversion ratio (FCR) compared to controls (Van Dijk et al., 2001). The response to ADG and ADFI was consistent at up to 6% inclusion of SDAP, while FCR response was only consistent at a level below 6%.

Spray-dried animal plasma may also improve fecal scores and decrease incidence of post-weaning diarrhea in piglets (weaned at 17-19 d old; 5.49 ± 0.1 kg). In piglets fed 5% SDAP, lower and improved ($P < 0.05$) fecal scores were observed compared to pigs fed 2.5% SDAP or

control (0%) at 7 and 14 d after weaning (Peace et al., 2011). In that study, evaluation of gut barrier function and intestinal inflammation after SDAP supplementation was reported to reduce paracellular permeability of C-inulin in the colon, reduce ileal C-inulin, and reduce H-mannitol permeability. The level of TNF-α, a pro inflammatory cytokine, was lower in pigs supplemented with SDAP (Peace et al., 2011). A beneficial effect on gut barrier function has also been observed in Wistar Lewis rats challenged with *Staphylococcus aureus* enterotoxin B (SEB), whereby 34 or 35 d of SDAP (80 g/kg) supplementation reduced dextran and HRP paracellular flux, reduced permeability of tight junctions, reduced the passage of microbes, and lowered local inflammation (Pérez-Bosque et al., 2006). Other studies have shown SDAP to modulate GI microbiota. Tran et al. (2018), for instance, evaluated the effects of SDAP on fecal microbiota of nursery pigs (20 ± 1.2 d old) for 28 d. Pigs were fed 5% SDAP during 14 d, followed by another 14 d of diet containing 2.5% SDAP. Results showed that there was an increase in beneficial bacteria *Lactobacillus*, but a reduction in *Clostridium difficile* in pigs supplemented with SDAP compared to control (0% inclusion).

In dogs, 3 different SDAP trials were conducted using a total of 22 beagles. Across all trials, dogs fed diets containing 2% SDAP had an increase ($P < 0.04$) in dry matter and organic matter digestibility, while fecal output (DM basis) was decreased by an average of 15% (Quigley et al., 2004). In cats, SDAP was compared to gluten as a binder in wet food. In a crossover digestibility study, a total of 11 cats (11.9 ± 0.9 yr old ; 4.08 ± 0.20 kg BW) were acclimated to wet food containing 30 g/kg SDAP or 30 g/kg wheat gluten for 7 d, followed by 5 d of fecal collection (Rodriguez et al., 2016). Similar to the dog study, SDAP consumption increased ($P < 0.05$) digestibility of DM, crude fiber, ash, phosphorus and calcium compared to those consuming wheat gluten. Those results indicate that SDAP may have beneficial effects in GI

health of dogs and cats. However, there are no studies evaluating other physiological outcomes of SDAP supplementation so more research in dogs and cats is needed.

Postbiotics

The term postbiotics has been utilized for some time, but there was no formal consensus on the definition until recently. In 2021, the International Scientific Association for Probiotics and Prebiotics (ISAPP) defined a postbiotic as "a preparation of inanimate microorganisms and/or their components that confers a health benefit on the host" (Salminen et al., 2021). This means that a postbiotic must contain inactivated microbial cells or its components with or without fermentative metabolites and contribute to observable health benefits. The benefits of using a postbiotic in a diet as opposed to a probiotic is the stability of the product during industrial processes. This is especially important in pet foods that undergo extrusion, a process that requires high heat treatment, and that may be stored for 12-18 months prior to consumption, both reducing the viability of the live microorganisms in a probiotic. Some of the proposed mechanisms on how postbiotics may confer health benefits include modulation of resident gut microbiota, systemic metabolic responses, local and systemic immune responses, enhanced epithelial barrier, and systemic signaling through the nervous system (Salminen et al., 2021). Some of the postbiotics that have been evaluated in dogs and cats include heat-killed probiotics, lyophilized probiotics, and yeast fermentation products (Osumi et al., 2019; Santoro et al., 2021). Most studies testing heat-killed or lyophilized probiotics have been focused on improving clinical signs of atopic dermatitis in dogs so there is still lack of literature evaluating the effect of postbiotics on GI and immune health. There are many more studies in yeast fermentation products focusing on its effects on dogs and cats immunity and GI health. This will be discussed in more detail in the following section.

Yeast fermentation products

Ingredients that are yeast derivatives or that contain yeast cell walls, usually from different strains of *Saccharomyces cerevisiae,* are known as yeast products. The Association of American Feed Control Officials (AAFCO) has defined various forms of yeast products allowed in animal feeds, but broadly, they can be classified as: 1) whole yeast, 2) yeast cell walls, 3) yeast solubles, and 4) yeast fermentation products. These different forms of yeast products have been shown to improve livestock performance and with potential health benefits to pets.

Yeast cell walls are composed of β-glucans, mannanoligosaccharides, chitin, proteins, lipids, and B vitamins (Kollár et al., 1997; Lipke and Ovalle, 1998). One mechanism by which yeast products may function is by inhibiting pathogen binding through mannan-binding type 1 fimbriae, which are present in some pathogenic bacteria like *E. Coli* and *Salmonella,* thus preventing their binding to enterocytes (Ofek et al., 1977; Neeser et al., 1986; Tiago et al., 2012). Another mechanism is through the binding of β-glucans to dectin-1, a C-type lectin. Dectin-1, which is expressed on the surface of dendritic cells, monocytes, macrophages and a subset of B and T cells, can initiate a downstream immune activation upon binding and recognition of β-glucans (Xiao et al., 2020).

Yeast products have been shown to improve the intestinal health of animals by modulating gut microbial composition and maintaining gut epithelial barrier integrity. In dogs, yeast products have shown to decrease *E. coli* counts in feces. In one study, 0.65% of dietary yeast cell wall supplementation to dogs for 10 d decreased 10% of the *E. coli* counts present in feces (Stercova et al., 2016). Similarly, a 6-wk supplementation of 1g/kg BW of live *S. cerevisiae* in dogs decreased fecal *E. coli* by 22% (Middelbos et al., 2007). In addition, yeast-derived mannanoligosaccharides (MOS) have been shown to modulate gut microbiota by

increasing beneficial bacteria such as *Bifidobacterium* and *Lactobacillus* (Swanson et al., 2002; Grieshop et al., 2004). In cats, similar effects have been observed. Cats supplemented with 0.6% of yeast cell walls for 34 d had increased fecal *Bifidobacterium* and *Lactobacillus* and lower fecal *C. perfringens* when compared to a non-supplemented control group (Santos et al., 2018). In addition, that study also reported an elevated concentration of fecal butyrate and acetate when cat were supplemented 0.6% of yeast cell walls compared to controls. Not all studies have produced positive results, however, with some dogs not having differences in fecal SCFA concentrations with yeast product supplementation (Swanson et al., 2002; Stercova et al., 2016).

In addition to modulation of the GI microbiota, yeast products have been shown to have immunomodulatory effects. There are few studies in dogs, however, and results have been contradictory. While two studies reported a decrease in serum lymphocyte number in healthy dogs, including those supplemented with 1% MOS for 28 d and those supplemented with *S. cerevisiae* fermentation product (SCFP) for 21 d (Grieshop et al., 2004; Lin et al., 2019), another study reported an increase in serum lymphocytes in dogs supplemented with 0.25% MOS for 10 d (Swanson et al., 2002). In addition, Lin et al. (2019) reported that SCFP-supplemented dogs had a linear increase in IFN-γ secreting helper cells, cytotoxic T cell populations, and serum IgE. Another study reported an increase in $CD4^+$ lymphocyte population in dogs treated with 1.5% MOS for 60 d (Pawar et al., 2017). Because the type and dosage of yeast products have differed among studies, that may be the reason for contradictory results. From a digestibility perspective, some have reported decreased nutrient digestibility following yeast cell wall supplementation (Zentek et al., 2002), while others have reported the opposite (Middelbos et al., 2007). In the study by Middelbos et al. (2007), a dose-response (0, 0.05, 0.25, 0.45, and 0.65% of diet) of spray-dried yeast cell walls tended to increase ($P < 0.10$) ileal digestibility of dry matter, organic

matter, crude protein, acid-hydrolyzed fat, and gross energy, with greatest response observed at the 0.25% inclusion level. In that study, a quadratic response was observed in ileal IgA concentrations, with the highest concentrations measured with the 0.25% inclusion level. That research seemed to indicate that a 0.25% inclusion level of pure yeast might be ideal in dogs, but research would be needed to test for similar effects in cats.

Milk oligosaccharides

Mammalian milk contains high concentrations of structurally diverse, complex carbohydrates in free or conjugated form known as milk oligosaccharides (MO) that act as bioactive compounds. In addition to providing essential nutrients to neonates, there is evidence that MO confer health benefits by serving as an anti-adhesive, an anti-microbial, and helping to modulate immunity, epithelial cell responses, and enhance brain and cognitive function of neonates (Kunz et al., 2000; Ruiz-Palacios 2003; Eiwegger et al., 2004; Newburg et al., 2005; Newburg, 2009 ; Bode, 2012; Jantscher-Krenn, 2012; Hester et al., 2013; Kavanaugh et al., 2013; Manthey 2014; Comstock et al., 2017; Plaza-Diaz 2018; Kong et al., 2019; Bode, 2019). In addition, MO are known for their prebiotic effect, with *in vitro* fermentation studies showing fermentation of MO by beneficial microbes that produce SCFA (Ward et al., 2006; LoCascio et al., 2010; Asakuma et al., 2011).

There are hundreds of milk oligosaccharides (MO) that have been identified in mammals, being composed of various monomers, including D-glucose, D-galactose, N-acetylglucosamine, L-fucose and sialic acid (N-acetylneuraminic acid) (Kunz et al., 2000; Kobata, 2010; Bode, 2019). Over 20 MO structures have been identified from dogs through liquid chromatography coupled with mass spectrometry (Macias Rostami et al., 2014; Hughes et al., 2020; Wrigglesworth et al., 2020). Though it has been of great interest to find substitutes of MO that

can provide the same health benefits, success has been limited due to the lack of structural diversity and complexity of traditional oligosaccharides (FOS and GOS) as well as a difficulty in large-scale supply (Bode et al., 2016; Thomson, 2018).

Functionally, both mucin glycans and MO have been shown to enrich similar taxa of gut resident microbes, help mitigate perturbations in the gut microbiota and provide host health benefits in mice (Pruss et al., 2021). In addition, mucin glycans have been shown to provide mucosal immune homeostasis through SCFA production in a rat model (Hino et al., 2020). Results from a previous *in vitro* fermentation studies have shown that when MO or MO mimics were inoculated with feces from dogs, cats, and piglets, a beneficial shift in microbial composition (increased *Bifidobacterium* and *Lactobacillus*; reduction in *Escherichia*/*Shigella* and *Salmonella*) and an increased production of SCFA occurred (Li et al., 2012; Oba et al., 2020). Although they have been tested using *in vitro* fermentation assays, MO have not been studied in companion animals.

Research aims

Many studies conducted in companion animals, humans, and other animal species have reported the beneficial effects of dietary fibers, prebiotics, probiotics, yeast products, SDAP and MO. These ingredients may affect health by modulation of GI microbiota, protection of the GI tract, improvement of gut integrity, and modulation of the immune system. Many studies have been conducted to test the benefits of fibers and traditional prebiotics (e.g., FOS) in companion animals, but most other ingredients listed above have limited research on how they may affect canine and feline health. In addition, few studies have examined these ingredients in the form of blends, which is becoming a popular practice in the pet food industry. More research, therefore, is needed to increase our understanding of how these ingredients may affect dogs and cats when

used as blends. Hence, the proposed research aims are: 1) to determine the effects of diets enriched in dietary fiber, prebiotics and immune modulators on nutrient digestibility, fecal characteristics and microbiota, and immune function of adult dogs; 2) to study the effects of a novel animal milk oligosaccharide biosimilar on macronutrient digestibility and gastrointestinal tolerance, fecal metabolites, and fecal microbiota of adult dogs; 3) to evaluate the effects of diets enriched in dietary fiber, prebiotics and immune modulators on nutrient digestibility, immune cell responsiveness, and fecal characteristics and metabolites of adult cats; and 4) to evaluate the effects of diets enriched in dietary fiber, prebiotics and immune modulators on the fecal metagenome of adult cats.

Tables

Table 2.1. Pathogen-associated molecular patterns recognized by mammalian toll-like receptors

TLR	Location	Ligand	Source
TLR1	Cell surface	Triacylated lipoprotein	Bacteria
TLR2	Cell surface	Lipoproteins	Bacteria, viruses, parasites
TLR3	Intracellular	Double strand RNA	Viruses
TLR4	Cell surface	Lipopolysaccharide	Bacteria, viruses
TLR5	Cell surface	Flagellin	Bacteria
TLR6	Cell surface	Diacylated lipoprotein	Bacteria, viruses
TLR7	Intracellular	ssRNA	Bacteria, viruses
TLR8	Intracellular	ssRNA	Bacteria, viruses
TLR9	Intracellular	Cytosine-guanosine DNA, Double strand DNA	Bacteria, viruses, protozoa
TLR10	Intracellular	Unknown	Unknown
TLR11	Cell surface	Toxoplasma profilin-like molecule	Protozoa
TLR12/13	Found in mice, not humans		Unknown

Table 2.2. Immunoglobulin types, characteristics and concentrations in dogs and cats

Immunoglobulin	Characteristics	Serum Ig concentrations (mg/dL)	
		Dogs	Cats
IgG	- Synthesized in spleen and lymph nodes - Smallest Ig, can easily pass from blood vessel to tissues, important in inflammation - Activates complement pathway and opsonization	1000 - 2000	400 - 2000
IgM	- Synthesized in spleen and lymph nodes - Primary Ig produced in an immune response - Efficient at activating complement pathway and opsonization - Remains in the blood vessel due to large size	70 - 270	30 - 150
IgA	- Synthesized in intestine, urinary system, skin, mammary gland, and respiratory tract - Primarily available as secretory IgA - Acts by agglutination and neutralization of viruses	50 - 150	30 - 150
IgE	- Synthesized in intestine and respiratory tract - Binds eosinophil, basophil, and mast cells to activate and target against parasitic worms	2.3 - 4.2	

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CHAPTER 3: EFFECTS OF DIETS ENRICHED IN DIETARY FIBER, PREBIOTICS, AND IMMUNE MODULATORS ON NUTRIENT DIGESTIBILITY, FECAL CHARACTERISTICS AND MICROBIOTA, AND IMMUNE FUNCTION OF ADULT DOGS

*Lee, A. H., C.-Y. Lin, S. Do, P. M. Oba, S. E. Belchik, A. J. Steelman, A. Schauwecker, and K. S. Swanson. Dietary supplementation with fiber, 'biotics', and spray dried plasma affects apparent total tract macronutrient digestibility and the fecal characteristics, fecal microbiota, and immune function of adult dogs. J. Anim. Sci. (in press). doi: 10.1093/jas/skac048.

Abstract

A variety of functional ingredients, including fibers, prebiotics, probiotics, and postbiotics may be added to pet foods to support gastrointestinal and immune health. While many of these ingredients have been tested individually, commercial foods often include blends that also require testing. This study was conducted to evaluate the effects of diets containing blends of fibers, 'biotics', and/or spray-dried plasma on apparent total tract digestibility (ATTD), stool quality, fecal microbiota and metabolites, and immune health outcomes of adult dogs. Twelve healthy adult intact English pointer dogs (6 M; 6 F; age = 6.4 ± 2.0 yr; BW = 25.8 ± 2.6 kg) were used in a replicated 3x3 Latin square design to test diets formulated to: 1) contain a low concentration of fermentative substances (control diet; CT); 2) be enriched with a fiber-prebiotic-probiotic blend (FPPB); and 3) be enriched with a fiber-prebiotic-probiotic blend + immune-modulating ingredients (iFFPB). In each 28-d period, 22 d of diet adaptation was followed by a 5-d fecal collection phase and 1 d for blood sample collection. All data were analyzed using SAS

9.4, with significance being P<0.05 and trends being P<0.10. FPPB and iFPPB diets led to shifts in numerous outcome measures. Dry matter (DM), organic matter, fat, fiber, and energy ATTD were lower (P<0.01), fecal scores were lower (P<0.01; firmer stools), and fecal DM% was higher (P<0.0001) in dogs fed FPPB or iFPPB than those fed CT. Serum triglycerides and cholesterol were lower (P<0.01) in dogs fed FPPB or iFPPB than those fed CT. Fecal protein catabolites (isobutyrate, isovalerate, indole, ammonia) and butyrate were lower (P<0.05), while fecal immunoglobulin A (IgA) was higher (P<0.01) in dogs fed FPPB and iFPPB than those fed CT. Fecal microbiota populations were affected by diet, with alpha diversity being lower (P<0.05) in dogs fed iFPPB and the relative abundance of 20 bacterial genera being altered in dogs fed FPPB or iFPPB compared to CT. The circulating helper T cell:cytotoxic T cell ratio was higher (P<0.05) in dogs fed iFPPB than those fed CT. Circulating B cells were lower (P<0.05) in dogs fed FPPB than those fed iFPPB, and lower (P<0.05) in dogs fed iFPPB than those fed CT. Our results demonstrate that feeding a fiber-prebiotic-probiotic blend may provide many benefits to canine health, including improved stool quality, beneficial shifts to fecal microbiota and metabolite profiles, reduced blood lipids, and increased fecal IgA.

Introduction

Dogs are considered to be family members by most pet owners today and addressing their health and longevity has become a priority. The inclusion of functional ingredients such as dietary fibers, prebiotics, probiotics and postbiotics in commercial pet foods has become a widespread practice. The addition of these ingredients aims to provide functional benefits beyond the basic nutritional needs of the animal and to support gastrointestinal (GI) and immune health. The GI microbiota and metabolite profiles are a common target, with many experiments

in recent years demonstrating the impacts of diet on canine fecal microbiota populations (Barko et al., 2018; Mondo et al., 2019; Alessandri et al., 2020; Pilla and Suchodolski, 2020; Wernimont et al., 2020). Bacteria may utilize non-digestible nutrients that reach the colon, converting carbohydrate-based substrates into compounds such as short-chain fatty acids (SCFA) that serve as an important energy source for colonocytes and provide many other benefits to host health (Schmitz and Suchodolski, 2016). Undigested proteins that reach the colon may be fermented by bacteria to yield compounds such as branched-chain fatty acids (BCFA), phenols, indoles, and ammonia that are typically thought to have a negative impact on intestinal health and contribute to fecal odor.

Many of the functional ingredients used to support GI health in pet foods are designed to manipulate the activity and composition of the resident microbiota populations, leading to increased SCFA, modulation of GI immune cell response, pathogen inhibition, or other responses leading to health benefits. Providing health benefits may be done by providing ingredients that serve as substrates for beneficial GI microbiota (i.e., fibers or prebiotics; Gibson et al., 2017) or those that contain live microbes (i.e., probiotics; Hill et al., 2014), a mixture of live microbes and substrates (i.e., synbiotics; Swanson et al., 2020), or a mixture of inanimate microorganisms and/or their components (i.e., postbiotics; Salminen et al., 2021). While much of the focus is on SCFA production, the modulation of the commensal bacteria may also prevent gut colonization of pathogens via other mechanisms, including the diversification of B cells and increasing immunoglobulin A (IgA) production (Kamada et al., 2013; Butler et al., 2016; Tizard and Jones, 2018).

In the past, studies have tested many different types of functional ingredients and described the beneficial health effects in dogs as well as livestock species. Dietary

supplementation of fibers and/or prebiotics may aid in glucose control and homeostasis, improve stool quality, increase production of SCFA, lower caloric density of food to aid in weight loss, and improve GI immune function (Banta et al., 1979; Fahey et al., 1992; Massimino et al., 1998; Swanson et al., 2002a). Common sources of dietary fiber in pet foods include beet pulp, cellulose, wheat bran, soybean hulls, Miscanthus grass, pectin, and others (Elliott et al., 2006; Middelbos et al., 2007; Detweiler et al., 2019; Donadelli and Aldrich, 2019). The most common prebiotics in pet foods are fructan-based ingredients that are present as short-chain fructooligosaccharides (scFOS; Swanson et al., 2002a; 2002b), inulin (Alexander et al., 2018), or natural sources such as chicory (Grieshop et al., 2004). Probiotics may contain a variety of bacterial taxa alone or in mixtures, but most tend to be lactic acid-producing bacteria. Probiotics provide health benefits and function via several mechanisms, including increased gut barrier function (Collado et al., 2007a), maintenance of epithelial tight junction integrity, enhanced immune response (Pagnini et al., 2010), increased secretory IgA production, prevention and displacement of pathogenic bacterial growth (Lee et al., 2003), and increased production of SCFA (Sakata et al., 2003; Oelschlaeger, 2010; Thomas and Versalovic, 2010). Dietary fibers, prebiotics, and probiotics may impact immune function, but other ingredients have this capability as well. Yeast-based products, including yeast cell wall extracts and yeast fermentation products, have been studied and shown to modulate GI immune cell response (Swanson et al., 2002a), blood cell populations and activity (Grieshop et al., 2004; Lin et al., 2019), and fecal microbiota populations (Lin et al., 2019). Spray-dried animal plasma (SDAP) has been shown to promote immune health in pigs and rodent models (Pérez-Bosque et al., 2006; Peace et al., 2011; Miró et al., 2020). It has potential application to pet health, but has only been tested for its impact on diet characteristics in pet foods thus far (Rodríguez et al., 2016).

Although studies have demonstrated efficacy and identified effective dosages for many of the ingredients listed above, most have been tested individually and prior to the advent of high-throughput molecular tools to measure GI microbiota and canine-specific assays to measure immune responses. Moreover, because many commercial pet foods contain blends of these functional ingredients, research is necessary to determine whether they provide the same benefits. Therefore, the objective of this study was to evaluate the effects of diets containing blends of fibers, 'biotics', and/or spray-dried plasma on apparent total tract digestibility (ATTD), stool quality, fecal microbiota and metabolites, and immune health outcomes of adult dogs. The control diet was formulated to be a premium diet that provided a low level of substrate for microbial fermentation. The second diet contained a blend of fibrous ingredients (i.e., oat groats; beet pulp; pea fiber), a probiotic, and a prebiotic (i.e., inulin), which was expected to impact GI and immune health primarily through fermentation and SCFA production. The third diet contained the fiber-probiotic-prebiotic blend plus ingredients thought to support immune function (i.e., SDAP; yeast fermentation product) via different mechanisms. We hypothesized that the dietary blends would positively shift fecal microbiota populations (greater *Bifidobacterium, Lactobacillus*, and *Faecalibacterium*; lower Proteobacteria, *Clostridium*, and *Fusobacterium*), improve fecal metabolite concentrations (greater SCFA; lower protein catabolites), and enhance immune responses of dogs without negatively impacting stool quality.

Materials and methods

The animal study was conducted at Kennelwood Inc. (Champaign, IL), with all procedures being approved by the Kennelwood, Inc. IACUC prior to experimentation.

Experimental design, animals, and diets

Twelve healthy adult intact English pointer dogs (6 males; 6 females; age = 6.4 ± 2.0 yr; BW = 25.8 ± 2.6 kg) were used in a replicated 3x3 Latin square study design. Each 28-d experimental period consisted of a diet adaptation phase (d 1-22), fecal collection phase (d 23-28), and a blood collection phase (d 28). Dogs were housed individually in pens in a room on a 10 h light: 14 h dark cycle, with gates providing access to individual outdoor pens.

Dogs were fed dry, extruded diets formulated to meet all Association of American Feed Control Officials (AAFCO, 2018) nutrient recommendations for adult dogs at maintenance. Dietary treatments consisted of diets formulated to: 1) contain a low concentration of fermentative substances (control diet; CT); 2) enriched with a fiber-prebiotic-probiotic blend (FPPB); and 3) enriched with a fiber-prebiotic-probiotic blend + immune modulating ingredients (iFFPB). Dogs were fed once a day (access from 10:00 am to 12:00 pm) to maintain BW throughout the study, with ad libitum access to water. Weekly BW and body condition score (BCS) measurements were recorded using a nine-point scale (Laflamme, 1997). Food intake was adjusted on a weekly basis as needed to maintain BW. Ingredient and analyzed chemical composition of the experimental diets are listed in **Table 3.1**.

Sample collection

Following a 22-d diet adaptation phase, fecal samples were collected for 5 d. Feces were collected from the pen floor, weighed, and scored using a 5-point scale scoring system: 1 = very 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; 5 = watery, liquid that can be poured. On the first day of fecal collections, a fresh fecal sample was

collected (within 15 min of defecation) from each dog and processed. Fecal pH was measured immediately upon fresh sample collection using a pH meter (Denver Instrument, Bohemia, NY) with attached electrode probe (Beckman Instruments Inc., Fullerton, CA). Remaining samples were aliquoted accordingly for determination of fecal dry matter (DM), metabolite concentrations (SCFA; BCFA; ammonia; phenol; indole), microbiota populations, IgA and calprotectin.

Fecal samples were placed on aluminum pans in duplicate and dried at 105°C for 2 d for DM determination. For SCFA and BCFA analysis, samples were mixed with 2N hydrochloric acid in 1:1 ratio and stored at -20°C until further analysis. Fecal samples were aliquoted in duplicate and stored at -20°C until phenol and indole analysis. For microbial analyses, fecal samples were aliquoted into four cryovials and stored at -80°C until analyses. Following fresh fecal sampling, all fecal samples were weighed, scored and stored at -20°C until chemical analysis and ATTD calculations.

On the last day of each period (d 28), blood samples were collected from each dog via cephalic venipuncture. Blood was drawn and immediately transferred to appropriate tubes: 1) heparin treated plasma tubes (#366480, Becton Dickinson, Franklin Lakes, NJ) for peripheral blood mononuclear cell (PBMC) collection; 2) K2 EDTA microtainer tubes (#365974, Becton Dickinson, Franklin Lakes, NJ) for complete blood count; and 3) serum tubes with clot activator and gel for serum separation (#368016, Becton Dickinson, Franklin Lakes, NJ) for serum chemistry and oxidative stress marker [superoxide dismutase (SOD); malondialdehyde (MDA)] analyses.

Dietary chemical composition analysis and ATTD calculations

Diets were subsampled from several bags for chemical composition analysis. Total fecal samples collected from d 23-28 were composited and dried at 57°C for 7 d. Fecal and diet samples were ground using a Wiley Mill (model 4, Thomas Scientific, Swedesboro, NJ). DM and organic matter (OM) concentrations were measured according to the Association of Official Analytical Chemists (AOAC, 2006; method 934.01 for DM; method 942.05 for OM). Crude protein content was calculated from total nitrogen values measured by LECO (TruMac N, Leco Corp., St. Joseph, MI; AOAC; method 922.15, 2002). Acid-hydrolyzed fat concentration was determined using methods according to the American Association of Cereal Chemists (AACC; method 30-14, 1983) and (Budde, 1952). Total dietary fiber (TDF) was determined for diet and fecal samples according to Prosky et al. (1985) and AOAC (2002; method 985.29). Gross energy was measured using a bomb calorimeter (Model 6200, Parr Instruments, Moline, IL). Apparent total tract digestibility of nutrients and energy was calculated using the following equation:

$$\% \text{ Digestibility} = \frac{[\text{Nutrient intake (g/d)} - \text{Fecal output (g/d)}]}{\text{Nutrient intake (g/d)}} \text{ x } 100\%$$

Fecal metabolite analysis

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

of phenols and indoles were evaluated using gas chromatography according to Flickinger et al. (2003b) and fecal ammonia concentration was measured according to the method of Chaney and Marbach (1962).

Fecal microbiota analysis

Total fecal DNA was extracted using DNeasy PowerLyzer PowerSoil kits (Qiagen Inc., Carlsbad, CA). Concentration of extracted DNA was quantified using a Qubit 3.0 Fluorometer (Life Technologies, Carlsbad, CA). The quality of extracted DNA was assessed by electrophoresis using agarose gels (E-Gel EX Gel 1%; Invitrogen, Carlsbad, CA). 16S rRNA gene amplicons were generated using a Fluidigm Access Array (Fluidigm Corporation, South San Francisco, CA) in combination with Roche High Fidelity Fast Start Kit (Roche, Indianapolis, IN). The primers 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) that target a 252 bp-fragment of the V4 region of the 16S rRNA gene were used for amplification (primers synthesized by IDT Corp., Coralville, IA) (Caporaso et al., 2012). CS1 forward tag and CS2 reverse tag were added according to the Fluidigm protocol. Quality of the amplicons were assessed using a Fragment Analyzer (Advanced Analytics, Ames, IA) to confirm amplicon regions and sizes. A DNA pool was generated by combining equimolar amounts of the amplicons from each sample. The pooled samples were then size selected on a 2% agarose E-gel (Life Technologies, Carlsbad, CA) and extracted using a Qiagen gel purification kit (Qiagen, Carlsbad, CA). Cleaned size-selected pooled products were run on an Agilent Bioanalyzer to confirm appropriate profile and average size. Illumina sequencing was performed on a MiSeq using v3 reagents (Illumina Inc., San Diego, CA) at the W. M. Keck Center for Biotechnology at the University of Illinois.

Bioinformatics

Sequence data were processed using Quantitative Insights Into Microbial Ecology 2 (QIIME 2.2020.8; Bolyen et al., 2019). Sequences with a quality value ≥ 20 were demultiplexed. Sequences were denoised and assembled into amplicon sequence variants (ASV) using DADA2 (Callahan et al., 2016). Taxonomy was assigned utilizing SILVA_138 reference database with a 99% similarity threshold (Quast et al., 2013). A total of 5,511,994 16S rRNA-based amplicon sequences were obtained, with an average of 83,515 reads per sample. Alpha- and beta-diversity measurements were presented using an even sampling depth of 44,111 sequences per sample. Beta-diversity was calculated using weighted and unweighted UniFrac (Lozupone and Knight, 2005) distance measures and represented using principal coordinates analysis (PCoA) plots.

Fecal IgA and calprotectin analysis

Fecal protein was extracted according to Vilson et al. (2016). Fecal samples (500 mg) were vortexed with 1.5 mL of extraction buffer containing 50 mM-EDTA (ThermoFisher, Waltham, MA) and 100 μg/L soybean trypsin inhibitor (Sigma, St. Louis, MO) in PBS/L percent bovine serum albumin (Tocris Bioscience, Bristol, UK). Phenylmethanesulphonyl fluoride (12.5 μL, 350 mg/L; Sigma, St. Louis, MO) was added into each tube and centrifuged for 10 min. Supernatants were collected for measurements of fecal IgA (E-40A; Immunology Consultants Laboratory, Portland, OR) and calprotectin (MBS030023; MyBiosource, Inc., San Diego, CA) using commercial enzyme-linked immunosorbent assay (ELISA) kits.

Serum chemistry, serum oxidative stress marker, and complete blood count analysis

Serum was isolated by centrifugation at 1,300 x g at 4°C for 10 min (Beckman CS-6R centrifuge; Beckman Coulter Inc., Brea, CA). Blood and serum samples were sent to the

University of Illinois Veterinary Medicine Diagnostic Laboratory for a complete blood count and serum chemistry profile using a Hitachi 911 clinical chemistry analyzer (Roche Diagnostics, Indianapolis, IN). Serum concentrations of lipopolysaccharide binding protein (LBP; MBS093112; MyBioSource, San Diego, CA), SOD (MBS004921; MyBioSource, San Diego, CA), and MDA (MBS2700234; MyBioSource, San Diego, CA) were measured using commercial ELISA kits.

Immune cell populations

Blood was layered over Ficoll Histopaque (Sigma, St. Louis, MO) in a 1:1 volume ratio and centrifuged at 300 x g at 4oC for 30 min to isolate PBMC from the blood. The percentage of antigen presenting cells (APC), natural killer (NK) cells, and T cells were evaluated using a BD LSR flow cytometer (Becton Dickinson, Franklin Lakes, NJ). To determine T cell populations, PBMC were divided into 2 tubes (1×10^6 cells/tube) with one tube as control and another stimulated with cell stimulation cocktail (phorbol 12-myristate 13-acetate, ionomycin, brefeldin A and monensin; eBioscience, San Diego, CA). Both tubes were incubated for 4 h at 37^{o}C in 5% CO2. Following incubation, cells were labeled using surface marker antibodies: anti-CD3-fluorescein isothiocyanate (FITC; MCA1774F; BioRad, Hercules, CA), anti-CD4-allophycocyanin (APC; MCA1038APC; BioRad, Hercules, CA) and anti-CD8-pacific blue (MCA1039PB; BioRad, Hercules, CA). Once labelled, cells were fixed and permeabilized using a buffer solution (00-8222-49; eBioscience, San Diego, CA). Intracellular marker staining was then performed with anti-IFN-γ-phycoerythrin (PE; MCA1783PE; BioRad, Hercules, CA). For NK cell populations, one aliquot of cells (1×10^6 cells/tube) was labeled with anti-CD5-APC antibody (MCA1037APC; BioRad, Hercules, CA). For APC, the cells of interest include B cells

and monocytes presenting major histocompatibility complex class II (MHC-II; MCA1044F; BioRad, Hercules, CA) on the cell surface. One aliquot of PBMC (1×10^6 cells/tube) was stained with anti-CD14-pacific blue (MCA1568PB; BioRad, Hercules, CA), anti-CD21-PE (MCA1781PE; BioRad, Hercules, CA), and anti-MHC-II-FITC antibodies. Flow cytometry data were analyzed using FCS Express 6 Flow Cytometry Software (De Novo Software, Glendale, CA). Gating strategies used to determine immune cell populations are shown in **Figure 3.1**. For NK cells, the population was determined according to Huang et al. (2008).

Immune cell responsiveness to Toll-like receptor (TLR) agonists

In a 96-well plate, PBMC (1×10^6 cells/tube) were seeded. Agonists of TLR2 (100 µg/mL zymosan; tlrl-zyn; Invivogen, San Diego, CA), TLR3 (50 µg/mL polyinosinic–polycytidylic acid sodium salt, poly(I:C); P9582-5MG; Sigma, St. Louis, MO), TLR4 (100 ng/mL LPS; L3024; Sigma, St. Louis, MO) and TLR7/8 (5 µg/mL resiquimod; tlrl-r848; Invivogen, San Diego, CA) were added into assigned wells separately in triplicate. Following 24 h of incubation at 37ºC in 5% CO2, supernatants were collected for measurement of TNF-α concentrations using a commercial ELISA kit (CATA00; R&D systems, Minneapolis, MN).

Statistical analyses

All data were analyzed using a Mixed Models procedure of SAS (version 9.4; SAS Institute, Cary, NC), with treatment as a fixed effect and dog as a random effect. Data were reported as means ± pooled standard error of the mean (SEM) and statistical significance was set as $P < 0.05$ and a trend set as $P < 0.10$.

Results

Food intake, body weight, body condition score and apparent total tract energy and macronutrient digestibility

Of the 12 dogs that were initially enrolled in the study, only 11 were considered for statistical analysis. One dog was removed from the experiment due to an external injury suffered that required antibiotic treatment, so it was excluded from analysis. On average, daily food and caloric intake was 459.6 g/d and 1580 kcal/d, respectively, across all treatment groups. Food intake and caloric intake were lower ($P<0.05$) in dogs fed FPPB and iFPPB compared to those fed CT (**Table 3.2**). ATTD of DM, OM, fat, TDF and energy were lower ($P < 0.01$) in dogs fed FPPB or iFPPB than those fed CT. ATTD of CP was not affected by treatment.

Fecal characteristics and fecal fermentative end-products

Fecal pH was not affected by dietary treatments (**Table 3.3**). However, dogs fed FPPB or iFPPB had lower ($P < 0.0001$) fecal scores (firmer stools) and a higher ($P < 0.0001$) fecal DM % than those fed CT. Many of the fecal metabolites were altered by dietary treatment. Fecal butyrate, isobutyrate, isovalerate, total BCFA, indole and ammonia concentrations were lower ($P < 0.01$) in dogs fed FPPB or iFPPB than those fed CT. Fecal 7-methylindole and calprotectin concentrations were higher ($P < 0.05$) in dogs fed FPPB than those fed CT. Fecal IgA concentrations were higher ($P < 0.01$) in dogs fed FPPB or iFPPB than those fed CT.

Fecal microbiota

Alpha-diversity, which represents species richness and evenness of the microbial community within a sample, was found to be lower ($P < 0.05$) in dogs fed iFPPB than those fed CT (**Figure 3.2A**). Beta-diversity, which represents species richness among samples, did not

differ across dietary treatments when based on both weighted and unweighted UniFrac distance measures represented as PCoA plots (**Figure 3.2B; Figure 3.2C**).

Although alpha- and beta-diversity indexes were not greatly affected by diet, 20 bacterial genera were different among treatments. The predominant bacterial phyla present in all dogs of this study were Firmicutes (57.6% to 59.8%), Fusobacteria (17.5% to 18.6%), Bacteroidetes (15.9% to 17.0%), Proteobacteria (4.9% to 5.9%) and Actinobacteria (0.9% to 1.9%) (**Table 3.4**). At the phyla level, the relative abundance of Actinobacteria was lower ($P < 0.01$) in dogs fed iFPPB than those fed CT. The other bacterial phyla were not affected by diet. At the genus level, the relative abundances of *Catenibacterium*, *Streptococcus*, undefined Lachnospiraceae, *Ruminococcus*, and *Megamonas* were higher ($P < 0.05$), while the relative abundances of *Collinsella* and *Alloprevotella* were lower ($P < 0.05$) in dogs fed FPPB or iFPPB than those fed CT. The relative abundances of *Peptoclostridium*, *Peptococcus* and *Sutterella* were lower ($P < 0.05$) in dogs fed iFPPB than those fed CT, while the relative abundance of *Lactobacillus* was lower ($P < 0.05$) in dogs fed FPPB than those fed CT. The relative abundance of undefined Prevotellaceae was lower ($P < 0.05$) in dogs fed FPPB than those fed CT or iFPPB. The relative abundances of *Turicibacter* and uncultured Lachnospiraceae tended to be lower ($P < 0.10$), while the relative abundance of *Faecalibacterium* tended to be higher ($P < 0.10$) in dogs fed FPPB or iFPPB than those fed CT. The relative abundance of *Prevotella* tended to be higher ($P < 0.10$), while the relative abundances of *Blautia*, *Megasphaera*, *Parasutterella* tended to be lower ($P < 0.10$) in dogs fed FPPB or iFPPB than dogs fed CT.

Blood metabolites, blood cell count, oxidative stress markers and LBP

Serum metabolites were within reference ranges for all dogs, except for alanine aminotransferase (ALT) and triglycerides (**Table 3.5**). Serum ALT exceeded the upper reference

range in dogs fed FPPB or iFPPB, but was highly variable and not different among treatments. Serum triglyceride concentrations were slightly below the reference range in dogs fed FPPB or iFPPB and lower ($P < 0.05$) in dogs fed FPPB or iFPPB than those fed CT. Serum cholesterol concentrations were also lower ($P < 0.05$) in dogs fed FPPB or iFPPB than those fed CT. Serum albumin was higher ($P < 0.05$) in dogs fed iFPPB than those fed CT. Serum sodium was higher ($P < 0.05$) in dogs fed FPPB or iFPPB than those fed CT. Serum albumin:globulin ratio tended to be higher ($P < 0.10$) in dogs fed FPPB or iFPPB than those fed CT.

Blood cell counts were all within the reference ranges (**Table 3.6**), but platelet volume was higher ($P < 0.05$) in dogs fed FPPB or iFPPB than those fed CT. A contrast comparing CT vs. FPPB and iFPPB showed that platelet count tended to be lower ($P < 0.10$) in dogs fed FPPB or iFPPB than those fed CT. A contrast comparing CT vs. FPPB and iFPPB also showed that serum SOD concentrations tended to be lower ($P < 0.10$) in dogs fed FPPB or iFPPB than those fed CT (**Table 3.7**). Serum LPS binding protein concentrations were not altered due to treatment (**Table 3.7**). Serum MDA concentration were below the detectable range (15.6 to 500 µmol/L) indicated by the ELISA kit and are not reported.

Immune cell populations and responsiveness to Toll-like receptor (TLR) agonists

The helper T cell:cytotoxic T cell ratio in unstimulated (control) cells was higher ($P < 0.05$) in dogs fed iFPPB than those fed FPPB or CT (**Table 3.8**). Cytotoxic T cells (% of lymphocytes) in unstimulated (control) cells tended to be higher ($P < 0.10$) in dogs fed FPPB than those fed CT or iFPPB. All other T cell populations were unaffected by dietary treatment. NK cell populations were not altered by dietary treatment (**Table 3.9**). Of the APC populations measured, B cells (% of lymphocytes) were lower ($P < 0.01$) in dogs fed FPPB than those fed

iFPPB. Dogs fed iFPPB also had lower ($P < 0.01$) B cells (% of lymphocytes) than dogs fed CT. Monocyte counts were unaffected by dietary treatment. Cell responsiveness to TLR agonists were unaffected by dietary treatment (**Table 3.10**).

Discussion

The growing interest in the health and well-being of pets by pet owners has fueled the growth of the functional ingredient market. Many of these ingredients are added for their beneficial health effects, but also to increase tag appeal by adding structure-function claims on product labels. When it comes to targeting GI health, the inclusion of dietary fibers, prebiotics, probiotics or postbiotics are common. Those ingredients and others including yeast-based products and SDAP may also support immune health. While functional ingredients are often included and tested on their own, it may be useful to evaluate the effects of these ingredients as a blend because that is commonly how they are used in commercial diets. Therefore, research studies evaluating the physiological effects of these functional ingredient blends may provide useful information beyond what is already known about each individual ingredient.

The objective of the current study was to evaluate the effects of functional blends composed of dietary fibers, prebiotics, probiotics, yeast products and SDAP on ATTD, stool quality, fecal fermentative end-products, fecal microbiota, and immune indices in healthy adult dogs. So that the functional blends could be tested effectively, the control diet was formulated to be a premium diet that provided a low level of substrate for microbial fermentation. The FPPB diet was formulated to contain a blend of fibrous ingredients (i.e., oat groats; beet pulp; pea fiber), a probiotic, and a prebiotic (i.e., inulin) at effective dosages. The iFPPB diet was

formulated to contain the same fiber-probiotic-prebiotic blend plus ingredients thought to support immune function (i.e., SDAP; yeast fermentation product).

Though no differences were observed in the overall food intake (g/d) across treatments, dogs consuming FPPB or iFPPB diets had a reduced caloric intake. This was likely due to the higher fiber content of these diets, which decreased caloric density and may have provided greater satiety (Jackson et al., 1997). ATTD of DM, OM, fat, and TDF was also reduced by FPPB and iFPPB diets, which was also likely due to the higher fiber inclusion level. Previous research has shown a linear decrease ($P < 0.05$) in DM, OM, fat digestibility with increasing dietary beet pulp (0%, 2.5%, 5.0%, 7.5%, 10% and 12.5% inclusion levels) as a source of fiber (Fahey et al., 1990). In that study, a cubic response for nitrogen digestibility was reported, with intermediate to lower digestibility being present at 7.5%, 10% and 12.5% beet pulp treatment levels (Fahey et al., 1990). Fat digestion differed among treatments, but all values were above 90% digestibility, a value that is usually observed in commercially extruded diets (Orr, 1965; Ahlstrom and Skrede, 1998). Even though many others have reported reduced protein ATTD with increasing dietary fiber (Weber et al., 2007; Kröger et al., 2017), that response was not observed in the current study. Protein digestion may be impacted by fiber content, but also may be affected by its quality, as has been reported in ileal-cannulated animals (Cramer et al., 2007).

All dogs remained healthy throughout the study and most of the serum chemistry and hematology values were within reference ranges for healthy dogs. Serum ALT and triglycerides were slightly out of the reference ranges, but no signs of clinical abnormality were observed during the study. For ALT, the increase above the reference range was primarily driven by one dog that had elevated ALT throughout the entire study. Because the dog showed no clinical signs of disease, had a good appetite, and did not show abnormal behaviors, it was retained in the

study. The increased serum albumin in dogs fed iFPPB may be attributed to the SDAP supplementation. A similar effect was reported recently in dogs fed 0% or 12% spray-dried porcine plasma (Andrade et al., 2019). The decreased serum cholesterol and triglyceride concentrations observed in dogs fed FPPB and iFPPB agrees with previous research demonstrating a blood lipid-lowering effect with increased dietary fiber and/or dietary inclusion of β-glucans and scFOS (Diez et al., 1997; Beylot, 2005; Surampudi et al., 2016; Phungviwatnikul, 2020).

Stool quality was improved in dogs fed FPPB or iFPPB diets, with fecal scores being closer to the ideal score (2.5-3.0 on a 5-point scale) than dogs fed CT that had higher (looser) fecal scores. Many of the fecal fermentative end-products were altered by dietary treatments in the current study too. In contrast to our hypothesis, fecal butyrate concentrations were lower in feces of dogs fed the FPPB and iFPPB diets, while the other SCFA were not altered by treatment. This was an unexpected outcome, as many of the fibers, prebiotics and yeast products included in the diet often yield higher SCFA concentrations due to the increased fermentation of undigestible carbohydrates in the colon (Silvio et al., 2000; Strompfová et al., 2012; Panasevich et al., 2013). This surprising outcome may be related to the sample type (i.e., feces) analyzed, which must be considered when interpreting fermentative-end product data. Because SCFA are rapidly absorbed by colonocytes and used for energy, fecal concentrations often do not reflect the amount produced in the gut. The drastic decrease in fecal BCFA, isovalerate, isobutyrate, phenol and ammonia concentrations in dogs fed the FPPB and iFPPB diets were expected. Those metabolites are often decreased in feces of animals consuming increased dietary non-digestible carbohydrates because they promote saccharolytic vs. proteolytic fermentation by gut microbiota (Jackson and Jewell, 2019).

In the current study, greater fecal IgA concentrations were observed in the dogs fed FPPB or iFPPB diets compared to those fed CT. This suggests a beneficial immune response to the functional blend, as secretory IgA plays an important role in maintaining gut immune homeostasis. Secretory IgA provides protection by inhibiting pathogen binding to the intestinal mucosal surface and preventing inappropriate inflammatory responses in the GI tract (Rogier et al., 2014; Zhu et al., 2017). This response is in agreement with several studies that have reported elevated IgA in response to yeast, mannanoligosaccharides, or probiotic treatment in dogs (Swanson et al., 2002a; Middelbos et al., 2007; Delucchi et al., 2014; Lin et al., 2020). Fecal calprotectin concentrations were statistically higher in dogs fed the FPPB diet compared to those fed CT. However, the numerical difference was very small (0.03 ug/g) and the concentrations reported in all three treatments in this study were within the values observed for healthy dogs (Heilmann et al., 2018) so it does not appear to be physiologically relevant.

Many studies have shown that increased intake of dietary fiber, prebiotics and/or probiotics alters the GI microbiota. In agreement with other studies, the predominant bacterial phyla in this study were Actinobacteria, Bacteroidetes, Firmicutes, Fusobacteria and Proteobacteria (Handl et al., 2011; Deng and Swanson, 2015). In this study, we hypothesized that the functional fiber and biotic blends would positively shift the gut microbiota by increasing *Bifidobacterium, Lactobacillus,* and *Faecalibacterium,* while decreasing Proteobacteria, *Clostridium,* and *Fusobacterium*. Even though those specific bacterial taxa were not altered, the relative abundance of *Catenibacterium, Streptococcus,* undefined Lachnospiraceae, *Ruminococcus,* and *Megamonas* were higher in dogs fed iFPPB or FPPB diets than those fed CT. *Catenibacterium,* Lachnospiraceae, *Megamonas* and *Ruminococcus* are core SCFA-producing bacteria that have been shown to be decreased in dogs with IBD (Hidaka et al., 2008; Beloshapka

et al., 2013; Pilla and Suchodolski, 2020; Do et al., 2021; Lee et al., 2021). Unexpectedly, bacterial diversity was lower in dogs fed iFPPB than those fed CT. Although reduced bacterial diversity is often associated with GI disease (Xenoulis et al., 2008; Guard et al., 2015), clinical signs of disease were not observed during this study.

Based on the literature, SDAP and yeast fermentation products were added to the iFPPB diet as immune-modulating agents (Swanson et al., 2002; Grieshop et al., 2004; Middelbos et al., 2007; Peace et al., 2011; Pawar et al., 2017; Andrade et al., 2019; Lin et al., 2019; Moreto et al., 2020; Almeida, 2021). In the current study, blood WBC and TNF-α responses were not altered, as has been reported in other studies (Middelbos et al., 2007; Peace et al., 2011; Lin et al., 2019). The ratio between T helper cells and cytotoxic T cells, however, was greater in dogs fed the iFPPB diet. Arguably this change may have been driven by the numerical increase observed in the number of T helper cells of the control (unstimulated) lymphocytes. A similar observation was made by Echeverry et al. (2021), where yeast cell wall products upregulated the response of T helper-1 and 2 cells in both unchallenged and LPS-challenged B lymphocytes of chickens. B lymphocyte populations were decreased in dogs fed iFPPB or FPPB diets compared to those fed CT in the current study. Supplementation of 6% SDAP in pigs has been shown to reduce the percentage of B lymphocytes as well as macrophages and T cells in ileocolic lymph nodes (Nofrarías et al., 2006). The opposite effect has been noted in the literature, however, where fructooligosaccharide supplementation increased B lymphocyte populations in the Peyer's patches of mice (Manhart et al., 2003). Because our current study only measured circulating lymphocytes, future studies should consider measuring cell populations of the GI mucosa, if possible, as it is the direct immune barrier.

In conclusion, dietary inclusion of functional blends composed of dietary fibers, 'biotics', and/or spray-dried plasma have positive impacts on stool quality, fecal metabolites, and immune function in dogs. These functional blends also help to beneficially modulate gut microbiota by increasing the relative abundance of some of the core SCFA-producing bacterial genera, including *Catenibacterium*, Lachnospiraceae, *Megamonas* and *Ruminococcus*. Although these results may only be attributed to the blend in question – not any single ingredients alone – and were tested in healthy dogs, they appear to support gastrointestinal and immune health of dogs. While the microbial shifts did not correlate with increased fecal SCFA, that may have been due to the sample type (i.e., feces) that is not a good measure of SCFA. Future studies should explore other tools that may provide a more direct and meaningful measurement of SCFA production and/or explore the functional capacity of the microbiota by utilizing shotgun sequencing. Investigating the effects of these fiber, biotic, and immune-modulating blends on geriatric or obese dog populations that are more susceptible to inflammation and gut microbial dysbiosis may also be of interest in future studies.

Tables and figures

Table 3.1. Ingredient and analyzed chemical composition of experimental diets fed to dogs

	Dietary treatments[1]		
	CT	FPPB	iFPPB
Ingredient	---------- %, as-is basis ----------		
Chicken meal, regular ash	20.00	-	-
Deboned chicken slurry	20.00	20.00	20.55
Brewers rice	20.00	-	-
Potato flour	20.00	-	-
Oat groats	-	17.40	14.60
Dried peas	-	13.00	13.00
Menhaden fish meal	-	11.40	11.40
Chicken meal, low ash	-	11.40	11.40
Chicken fat with Naturox[2]	9.54		
Chicken fat	-	5.96	5.96
Dried chicken	5.00	5.00	5.00
Dried plain beet pulp	-	4.00	4.00
Flaxseed meal	-	3.00	3.00
Pea fiber	-	3.00	3.00
Liquid digest[3]	3.00	3.00	3.00
Spray-dried plasma[4]	-	-	2.00
Dried dog digest with Lactosacc probiotic[5]	-	1.20	1.20
Digest[6]	1.00	-	-
Dried brewer's yeast	0.92	0.92	0.92
Yeast fermentation product[7]	-	-	0.25
Potassium chloride	0.19	0.19	0.19
Inulin	-	0.10	0.10
Chelated mineral premix	0.10	0.10	0.10
Choline chloride, 70% dry	0.10	0.10	0.10
Vitamin premix	0.10	0.10	0.10
Dried Naturox	0.05	0.05	0.05
Vitamin E, 50 % dry	-	0.03	0.03
Turmeric powder	-	0.03	0.03
Ascorbic acid[8]	-	0.03	0.03
Chemical composition	-	-	-
Dry matter (DM), %	93.38	93.24	93.22
	---------- %, dry matter ----------		
Crude protein	26.7	33.0	31.8
Acid-hydrolyzed fat	15.7	15.4	14.8
Ash	7.6	9.3	10.2
Total dietary fiber	9.5	17.5	16.5
Insoluble fiber	6.8	12.8	11.1
Soluble fiber	2.7	4.7	5.5

Table 3.1. (Continued)

ME[9], kcal/g	3.7	3.3	3.3

[1]Control diet (CT); a diet containing a fiber-prebiotic-probiotic blend (FPPB); a diet containing a fiber-prebiotic-probiotic blend + immune modulating ingredients (iFPPB).
[2]Naturox (Kemin Industries, Des Moines, IA).
[3]AFB C15065 (AFB International, St. Charles, MO).
[4]Spray-dried plasma (APC Inc, Ankeny, IA).
[5]AFB C28131 (AFB International, St. Charles, MO).
[6]AFB C26017 (AFB International, St. Charles, MO).
[7]Diamond V (Diamond V, Cedar Rapids, IA).
[8]Stay C-35 (DSM Nutritional Products Inc., Parsippany, NJ)[8]Stay C-35 (DSM Nutritional Products Inc., Parsippany, NJ)
[9]Metabolizable energy (ME) = 3.5 kcal/g × crude protein (%) + 8.5 kcal/g × acid-hydrolyzed fat (%) + 3.5 kcal/g × nitrogen free extract (%); nitrogen free extract (%) = 100% - (crude protein % + acid-hydrolyzed fat % + total dietary fiber % + ash %).

Table 3.2. Body weight, body condition score, daily food and energy intake, and apparent total tract macronutrient and energy digestibility of dogs fed experimental diets

	Dietary treatments[1]			SEM	*P*-value	
	CT	FPPB	iFPPB		Treatment	CT vs. FPPB + iFPPB
Body weight, kg	25.5[b]	24.9[a]	25.5[b]	0.958	0.05	0.20
Body condition score[2]	5.5	5.4	5.5	0.136	0.44	0.52
Daily intake, g/day	475.2	454.3	449.2	25.915	0.11	0.04
Energy intake, kcal/day	1758.37[b]	1499.53[a]	1482.44[a]	88.216	< 0.0001	< 0.0001
Digestibility, %						
Dry matter	85.2[b]	74.9[a]	74.9[a]	1.204	< 0.0001	< 0.0001
Organic matter	89.5[b]	81.3[a]	80.8[a]	0.945	< 0.0001	< 0.0001
Crude protein	81.2	80.5	81.5	1.504	0.85	0.92
Acid-hydrolyzed fat	97.7[b]	94.6[a]	95.0[a]	0.460	< 0.0001	< 0.0001
Total dietary fiber	54.0[b]	43.8[a]	39.7[a]	3.090	< 0.01	< 0.01
Energy	89.1[b]	82.0[a]	81.9[a]	0.890	< 0.0001	< 0.0001

[1]Control diet (CT); a diet containing a fiber-prebiotic-probiotic blend (FPPB); a diet containing a fiber-prebiotic-probiotic blend + immune modulating ingredients (iFPPB).
[2]Nine-point body condition score system used (Laflamme, 1997).

Table 3.3. Fecal characteristics and fecal metabolite concentrations of dogs fed experimental diets

	Dietary treatments[1]			SEM	*P*-value	
	CT	FPPB	iFPPB		Treatment	CT vs. FPPB and iFPPB
Fecal characteristics						
pH	6.41	6.33	6.08	0.127	0.13	0.15
Fecal score[2]	4.14 [b]	3.05 [a]	3.14 [a]	0.133	< 0.0001	< 0.0001
Fecal DM (%)	27.32 [a]	31.18 [b]	30.62 [b]	0.601	0.001	< 0.0001
Fecal metabolites	---------- µmol/g DM ----------					
Total SCFA[3]	594.16	606.91	621.29	22.676	0.68	0.46
Acetate	347.35	353.90	375.61	14.237	0.33	0.31
Propionate	172.35	191.75	186.03	9.103	0.29	0.14
Butyrate	74.46 [b]	61.26 [a]	59.65 [a]	3.891	0.02	0.01
Total BCFA[4]	34.93 [b]	20.51 [a]	20.12 [a]	2.940	< 0.01	< 0.01
Isobutyrate	10.98 [b]	6.87 [a]	6.78 [a]	0.770	< 0.0001	< 0.0001
Isovalerate	17.14 [b]	9.94 [a]	10.14 [a]	1.133	< 0.0001	< 0.0001
Valerate	6.81	3.70	3.21	1.986	0.38	0.17
Total P/I[5]	4.84	5.38	3.78	0.647	0.22	0.75
Phenol	0.47	0.28	0.20	0.175	0.55	0.29
Indole	2.64 [b]	1.16 [a]	0.79 [a]	0.294	< 0.01	< 0.0001
7-Methylindole	1.72 [a]	3.94 [b]	2.79 [ab]	0.432	< 0.01	< 0.01
Ammonia	181.81 [b]	143.80 [a]	140.65 [a]	10.728	< 0.01	< 0.01
Fecal IgA, mg/g	2.53 [a]	4.34 [b]	4.59 [b]	0.622	0.01	< 0.01
Fecal calprotectin, ug/g	13.19 [a]	17.15 [b]	16.15 [ab]	1.132	0.05	0.02

[1]Control diet (CT); a diet containing a fiber-prebiotic-probiotic blend (FPPB); a diet containing a fiber-prebiotic-probiotic blend + immune modulating ingredients (iFPPB).
[2]Fecal score: 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; 5 = watery, liquid that can be poured.
[3]SCFA = acetate + propionate + butyrate.
[4]BCFA = valerate + isovalerate + isobutyrate.
[5]P/I = phenols + indoles.

Table 3.4. Predominant bacterial phyla and genera (expressed as percent of total sequences) in feces of dogs fed experimental diets

Phylum	Genus[2]	Dietary treatments[1]			SEM	*P*-value	
		CT	FPPB	iFPPB		Treatment	CT vs. FPPB and iFPPB
Actinobacteria		1.96 [b]	1.49 [ab]	0.87 [a]	0.216	0.01	0.01
	Collinsella	1.56 [b]	0.93 [a]	0.60 [a]	0.171	< 0.01	< 0.01
Bacteroidetes		16.95	15.98	17.01	1.575	0.82	0.78
	Bacteroides	8.78	7.70	9.20	0.986	0.49	0.76
	Alloprevotella	2.25 [b]	1.42 [a]	1.14 [a]	0.299	< 0.01	< 0.01
	Prevotella	3.52	6.01	5.33	1.006	0.15	0.06
	Undefined Prevotellaceae	1.31 [b]	0.66 [a]	1.19 [b]	0.211	0.03	0.07
Firmicutes		57.60	59.79	58.55	2.942	0.84	0.63
	Catenibacterium	0.90	2.61	1.73	0.527	0.06	0.04
	Allobaculum	1.71	1.59	1.38	0.254	0.46	0.35
	Holdemanella	1.58	1.65	1.53	0.294	0.95	0.98
	Turicibacter	0.96	0.29	0.26	0.240	0.08	0.03
	Lactobacillus	16.64 [b]	7.79 [a]	10.73 [ab]	2.426	0.04	0.02
	Streptococcus	2.45 [a]	11.30 [b]	10.66 [b]	1.711	< 0.01	< 0.01
	Undefined Lachnospiraceae	1.85 [a]	2.42 [b]	2.79 [b]	0.237	0.01	< 0.01
	Blautia	4.29	3.63	3.22	0.363	0.12	0.06
	Ruminococcus Gnavus	1.21 [a]	1.92 [b]	1.86 [b]	0.219	0.04	0.01
	Ruminococcus Torques	0.66 [a]	1.17 [b]	1.13 [b]	0.130	0.02	0.01
	Uncultured Lachnospiraceae	1.12	0.82	0.70	0.133	0.07	0.03
	Faecalibacterium	3.29	4.02	5.51	0.745	0.07	0.08
	Peptococcus	0.99 [b]	0.68 [ab]	0.59 [a]	0.137	0.05	0.02
	Peptoclostridium	9.08 [b]	7.13 [ab]	5.89 [a]	0.681	0.01	< 0.01
	Phascolarctobacterium	0.75	0.94	0.83	0.100	0.31	0.21

Table 3.4. (Continued)

	Megamonas	3.10 [a]	5.10 [b]	5.31 [b]	0.795	0.05	0.02
	Megasphaera	1.10	0.44	0.23	0.395	0.21	0.09
Fusobacteria		17.51	17.78	18.59	1.706	0.87	0.72
	Fusobacterium	17.51	17.78	18.59	1.706	0.87	0.72
Proteobacteria		5.90	4.92	4.94	0.576	0.40	0.18
	Anaerobiospirillum	1.29	1.00	1.27	0.276	0.71	0.65
	Parasutterella	0.69	0.37	0.49	0.219	0.12	0.06
	Sutterella	3.43 [b]	2.38 [ab]	2.24 [a]	0.315	0.02	0.01

[1]Control diet (CT); a diet containing a fiber-prebiotic-probiotic blend (FPPB); a diet containing a fiber-prebiotic-probiotic blend + immune modulating ingredients (iFPPB).
[2]All genera with relative abundance >0.5% of total sequences are presented.

Table 3.5. Serum chemistry profiles of dogs fed experimental diets

Item	Reference ranges[2]	Dietary treatments[1]			SEM	*P*-value	
		CT	FPPB	iFPPB		Treatment	CT vs. FPPB and iFPPB
Creatinine, mg/dL	0.5-1.5	0.80	0.79	0.80	0.037	0.93	0.85
BUN[2], mg/dL	6-30	16.18	16.27	16.45	1.065	0.89	0.72
Total protein, g/dL	5.1-7.0	6.28	6.29	6.37	0.132	0.48	0.49
Albumin, g/dL	2.5-3.8	3.05 [a]	3.14 [ab]	3.18 [b]	0.093	0.02	0.01
Globulin, g/dL	2.7-4.4	3.23	3.15	3.19	0.197	0.66	0.44
Albumin:globulin	0.6-1.1	0.98	1.03	1.05	0.068	0.19	0.08
Calcium, mg/dL	7.6-11.4	9.36	9.39	9.43	0.077	0.80	0.59
Phosphorus, mg/dL	2.7-5.2	3.29	3.44	3.39	0.139	0.27	0.13
Sodium, mmol/L	141-152	143.00 [a]	144.00 [b]	144.09 [b]	0.377	0.01	< 0.01
Potassium, mmol/L	3.9-5.5	4.39	4.40	4.33	0.056	0.23	0.49
Sodium:potassium	28-36	32.64	32.91	33.27	0.439	0.25	0.17
Chloride, mmol/L	107-118	109.82	110.00	110.09	0.488	0.79	0.53
Glucose, mg/dL	68-126	92.27	91.09	91.09	2.957	0.89	0.63
ALP[3], U/L	7-92	46.55	44.82	46.91	4.261	0.68	0.76
CALP[3], U/L	0-40	25.64	26.27	28.00	4.082	0.47	0.39
ALT[3], U/L	8-65	54.18	87.82	79.64	27.626	0.63	0.36
GGT[3], U/L	0-7	4.09	4.91	4.09	0.685	0.46	0.53
Total bilirubin, mg/dL	0.1-0.3	0.22	0.17	0.21	0.020	0.24	0.27
CPK[3], U/L	26-310	119.18	123.36	118.82	12.572	0.95	0.89
Cholesterol, mg/dL	129-297	197.45 [b]	150.00 [a]	168.09 [a]	8.540	< 0.0001	< 0.0001
Triglyceride, mg/dL	32-154	43.45 [b]	29.64 [a]	31.36 [a]	4.160	0.02	< 0.01

[1]Control diet (CT); a diet containing a fiber-prebiotic-probiotic blend (FPPB); a diet containing a fiber-prebiotic-probiotic blend + immune modulating ingredients (iFPPB).

[2]Reference ranges were provided from the University of Illinois Veterinary Diagnostic Laboratory.

[3]BUN: blood urea nitrogen; ALP: total alkaline phosphatase; CALP: corticosteroid isoenzyme of ALP; ALT: alanine aminotransferase; GGT: gamma-glutamyltransferase; CPK: creatinine phosphokinase.

Table 3.6. Complete blood count of dogs fed experimental diets

Item	Reference ranges[2]	Dietary treatments[1]			SEM	*P*-value	
		CT	FPPB	iFPPB		Treatment	CT vs. FPPB and iFPPB
Red blood cell. 10^6/µL	5.5-8.5	7.28	7.31	7.26	0.182	0.95	0.94
Hemoglobin, g/dL	12.0-18.0	16.50	16.60	16.53	0.434	0.96	0.84
Hematocrit, %	35.0-52.0	48.26	48.45	48.19	1.105	0.95	0.94
Mean cell volume, fl	58.0-76.0	66.32	66.32	66.42	0.545	0.96	0.89
White blood cell, 10^3/µL	6.00-17.00	8.07	8.58	8.60	0.588	0.48	0.23
Neutrophil, 10^3/µL	NA	64.98	73.27	70.97	3.607	0.44	0.41
Lymphocyte, 10^3/µL	NA	16.69	17.87	20.48	1.672	0.16	0.15
Monocyte, 10^3/µL	NA	6.15	5.28	4.58	0.860	0.45	0.26
Eosinophil, 10^3/µL	NA	3.82	3.52	3.80	0.659	0.91	0.82
MCH[3], pg	20.0-25.0	21.62	22.73	22.80	0.707	0.35	0.15
MCHC[4], g/dL	33.0-38.6	34.18	34.24	34.26	0.212	0.93	0.72
Platelets, 10^3/µL	200-700	351.68	302.55	307.53	32.907	0.12	0.04
Mean platelet volume, fl	NA	11.34 [a]	11.99 [b]	12.23 [b]	0.542	0.01	< 0.01

[1]Control diet (CT); a diet containing a fiber-prebiotic-probiotic blend (FPPB); a diet containing a fiber-prebiotic-probiotic blend + immune modulating ingredients (iFPPB).
[2]Reference ranges were provided by the University of Illinois Veterinary Diagnostic Laboratory.
[3]MCH: mean corpuscular hemoglobin.
[4]MCHC: mean corpuscular hemoglobin concentration.

Table 3.7. Serum superoxide dismutase and lipopolysaccharide (LPS) binding protein concentrations of dogs fed experimental diets

	Dietary treatments[1]			SEM	*P*-value	
	CT	FPPB	iFPPB		Treatment	CT vs. FPPB and iFPPB
Superoxide dismutase, U/mL	323.82	309.19	313.08	11.854	0.14	0.06
LPS binding protein, µmol/L	1.89	1.9373	1.9036	0.120	0.81	0.64

[1]Control diet (CT); a diet containing a fiber-prebiotic-probiotic blend (FPPB); a diet containing a fiber-prebiotic-probiotic blend + immune modulating ingredients (iFPPB).

Table 3.8. T-cell populations of dogs fed experimental diets

	Dietary treatments[1]			SEM	*P*-value	
	CT	FPPB	iFPPB		Treatment	CT vs. FPPB and iFPPB
Control						
Lymphocyte, % of PBMC[2]	12.43	24.67	19.39	5.552	0.34	0.19
T cell, % of lymphocyte	59.83	60.27	63.23	7.387	0.94	0.84
Helper T cell, % of lymphocyte	25.56	30.35	33.62	3.852	0.28	0.16
Cytotoxic T cell, % of lymphocyte	26.56	32.67	23.89	3.777	0.09	0.60
Helper: cytotoxic T-cell ratio	1.27 [a]	1.29 [a]	1.75 [b]	0.408	0.03	0.13
IFN-γ secreting T cell, % of lymphocyte	0.01	0.01	0.02	0.007	0.22	0.33
IFN-γ secreting helper cell, % of lymphocyte	0.01	0.01	0.01	0.004	0.99	0.96
IFN-γ secreting cytotoxic cell, % of lymphocyte	0.00	0.00	0.01	0.003	0.27	0.43
Stimulation[3]						
Lymphocyte, % of PBMC[2]	46.75	49.55	41.08	4.555	0.41	0.80
T cell, % of lymphocyte	60.85	61.35	59.88	4.137	0.97	0.96
Helper T cell, % of lymphocyte	36.84	38.87	39.23	2.556	0.73	0.45
Cytotoxic T cell, % of lymphocyte	29.18	29.86	29.38	3.336	0.93	0.78
Helper: cytotoxic T-cell ratio	1.68	1.68	1.49	0.344	0.65	0.65
IFN-γ secreting T cell, % of lymphocyte	6.49	7.40	4.61	1.328	0.31	0.77
IFN-γ secreting helper cell, % of lymphocyte	2.97	4.26	2.73	0.756	0.33	0.58
IFN-γ secreting cytotoxic cell, % of lymphocyte	2.89	2.62	1.66	0.526	0.15	0.21

[1]Control diet (CT); a diet containing a fiber-prebiotic-probiotic blend (FPPB); a diet containing a fiber-prebiotic-probiotic blend + immune modulating ingredients (iFPPB).
[2]PBMC = peripheral blood mononuclear cells.
[3]Cells were stimulated with cell stimulation cocktail (phorbol 12-myristate 13-acetate, ionomycin, brefeldin A, and monensin) for 4h.

Table 3.9. Natural killer cell and antigen-presenting cell populations of dogs fed experimental diets

	Dietary treatments[1]			SEM	*P*-value	
	CT	FPPB	iFPPB		Treatment	CT vs. FPPB and iFPPB
Natural killer cell, % of lymphocyte	15.23	13.87	14.91	1.809	0.83	0.68
Antigen-presenting cells						
B cell, % of lymphocyte	10.56 [c]	6.72 [a]	8.55 [b]	1.183	< 0.01	< 0.01
B cell, MHC II+, % of B cell[2]	98.66	97.33	98.32	0.977	0.61	0.49
Monocyte, % of white blood cell	4.16	5.17	3.93	1.210	0.75	0.80
Monocyte, MHC II+, % of monocyte[2]	64.15	59.99	58.71	3.821	0.56	0.31

[1]Control diet (CT); a diet containing a fiber-prebiotic-probiotic blend (FPPB); a diet containing a fiber-prebiotic-probiotic blend + immune modulating ingredients (iFPPB).
[2]B cells or monocytes that present major histocompatibility complex class II (MHC II).

Table 3.10. TNF-α concentrations (pg/mL) in cell culture supernatants of dogs fed experimental diets

	Dietary treatments[1]			SEM	*P*-value	
	CT	FPPB	iFPPB		Treatment	CT vs. FPPB and iFPPB
Control	225.56	196.45	108.43	70.736	0.63	0.35
Zymosan	813.54	746.53	966.68	178.350	0.15	0.65
Poly (I:C)[2]	1829.64	1423.21	1204.45	356.930	0.28	0.14
Lipopolysaccharide	875.66	778.81	819.54	198.400	0.82	0.57
R848 (requisimod)	3687.21	3996.15	4622.72	677.640	0.57	0.43

[1]Control diet (CT); a diet containing a fiber-prebiotic-probiotic blend (FPPB); a diet containing a fiber-prebiotic-probiotic blend + immune modulating ingredients (iFPPB).
[2]Poly (I:C): polyinosinic:polycytidylic acid.

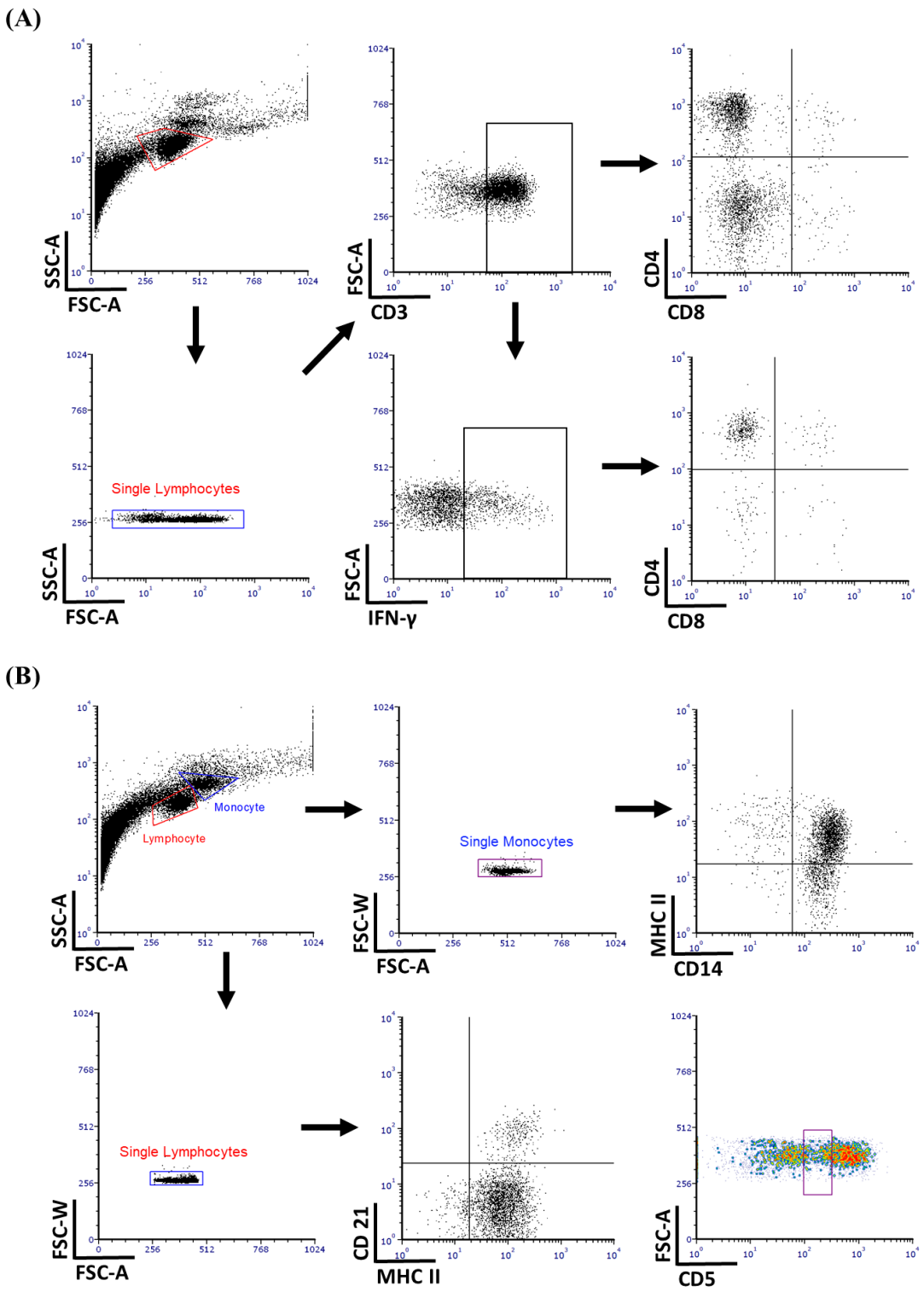

Figure 3.1. Gating strategy used to determine the cell populations of (A) T cells, (B) antigen-presenting cells (B cells and monocytes) and natural killer cells. SSC: side scatter; FSC: forward scatter; -A: the pulse area; -W: the pulse width.

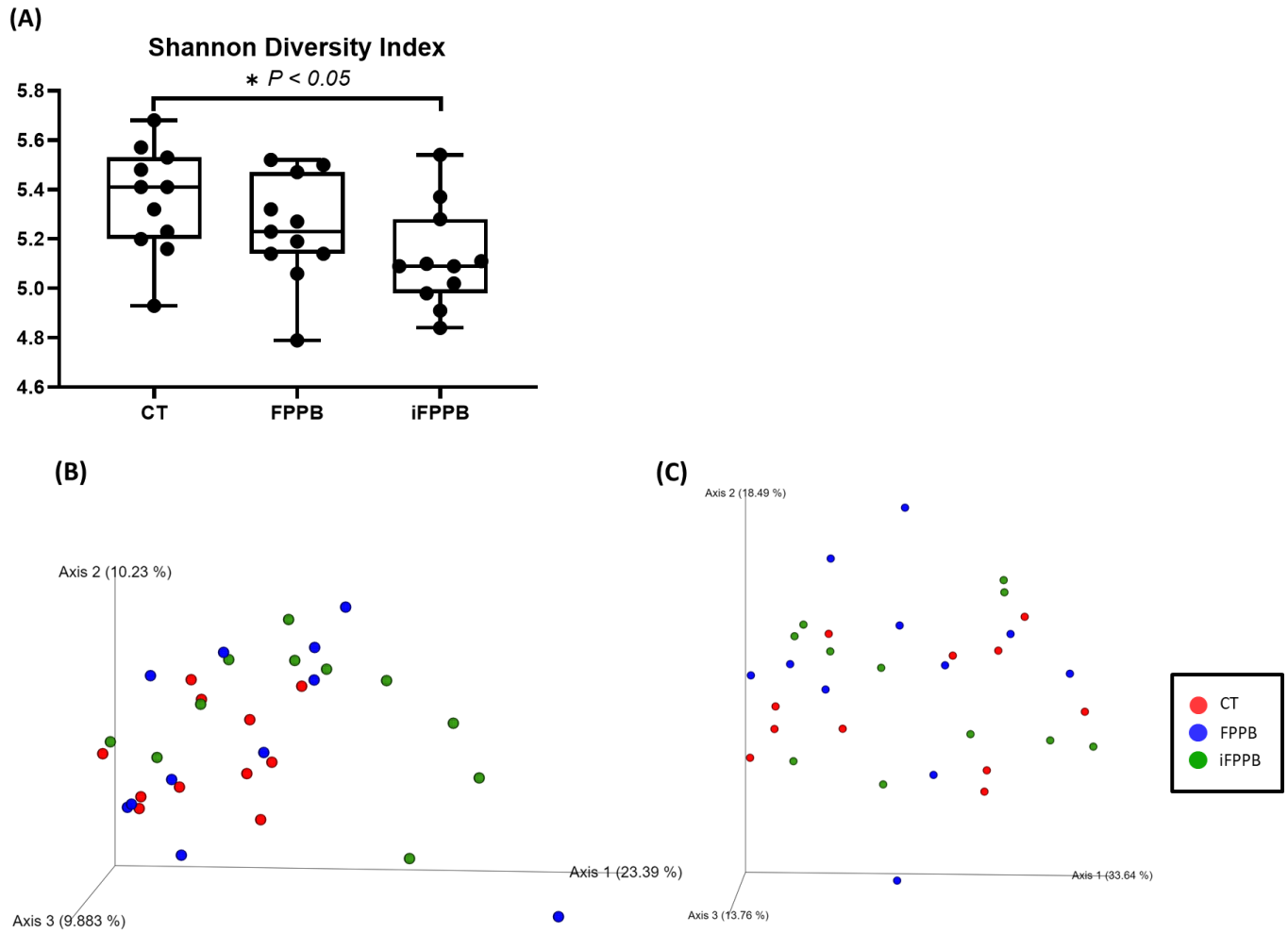

Figure 3.2. Alpha- and beta-diversity measures of fecal samples collected from dogs fed the control diet (CT), a diet containing a fiber-prebiotic-probiotic blend (FPPB), and a diet containing a fiber-prebiotic-probiotic blend + immune modulating ingredients (iFPPB). (A) Alpha-diversity, represented by the Shannon diversity index, suggested that species richness was higher ($P < 0.05$) in dogs fed CT than those fed iFPPB. Principal coordinates analysis plots for unweighted (B) and weighted (C) UniFrac distances of fecal microbial communities were not altered by dietary treatments.

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CHAPTER 4: EFFECTS OF A NOVEL ANIMAL MILK OLIGOSACCHARIDE BIOSIMILAR ON MACRONUTRIENT DIGESTIBILITY, AND GASTROINTESTINAL TOLERANCE, FECAL METABOLITES, AND FECAL MICROBIOTA OF ADULT DOGS

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Abstract

Milk oligosaccharides (MO) are bioactive compounds in mammalian milk that provide health benefits to neonates beyond essential nutrients. GNU100, a novel animal MO biosimilar, was recently tested *in vitro*, with results showing beneficial shifts in microbiota and increased short-chain fatty acid (SCFA) production, but other effects of GNU100 were unknown. Three studies were conducted to evaluate the safety, palatability and gastrointestinal (GI) tolerance of GNU100. In Study 1, the mutagenic potential of GNU100 was tested using a bacterial reverse mutation assay and a mammalian cell micronucleus test. In Study 2, palatability was assessed by comparing diets containing 0% vs. 1% GNU100 in 20 adult dogs. In Study 3, 32 adult dogs were used in a completely randomized design to assess the safety and GI tolerance of GNU100 and explore utility. Following a 2-wk baseline, dogs were assigned to one of four treatments and fed for 26 wk: 0%, 0.5%, 1% and 1.5% GNU100. On wk 2, 4 and 26, fresh fecal samples were collected to measure stool quality, immunoglobulin A, and calprotectin, and blood samples were

collected to measure serum chemistry and inflammatory markers and hematology. On wk 2 and 4, fresh fecal samples were collected to measure metabolites and microbiota. On wk 4, total feces were collected to assess apparent total tract macronutrient digestibility. Although revertant numbers were greater compared to the solvent control in WP2uvrA(pKM101) in presence of metabolic activation (S9) in the initial experiment, they remained below the threshold for a positive mutagenic response in follow-up confirmatory tests, supporting that GNU100 is not mutagenic. Similarly, no cytotoxicity or chromosome damage were observed in the cell micronucleus test. The palatability test showed that 1% GNU100 was strongly preferred ($P < 0.05$; 3.6:1 consumption ratio) over to the control. In Study 3, all dogs were healthy and had no signs of GI intolerance or illness. All diets were well-accepted and food intake, fecal characteristics and metabolite concentrations and macronutrient digestibilities were not altered. GNU100 modulated fecal microbiota, increasing evenness and *Catenibacterium*, *Megamonas*, and *Prevotella* (SCFA producers) and reducing *Collinsella*. Overall, results suggest that GNU100 is palatable and well-tolerated, causes no genotoxicity or adverse effects on health, and beneficially shifts the fecal microbiota, supporting the safety of GNU100 for inclusion in canine diets.

Introduction

Mammalian milk contains high concentrations of structurally diverse, complex carbohydrates in free or conjugated form known as milk oligosaccharides (MO) that act as bioactive compounds. In addition to providing essential nutrients to neonates, there is evidence that MO confer health benefits by serving as an anti-adhesive, an anti-microbial, and helping to modulate immunity, epithelial cell responses, and enhance brain and cognitive function of

neonates (Kunz et al., 2000; Ruiz-Palacios 2003; Eiwegger et al., 2004; Newburg et al., 2005; Newburg, 2009 ; Bode, 2012; Jantscher-Krenn, 2012; Hester et al., 2013; Kavanaugh et al., 2013; Manthey 2014; Comstock et al., 2017; Plaza-Diaz 2018; Kong et al., 2019; Bode, 2019). In addition, MO are known for their prebiotic effect, with in vitro fermentation studies showing fermentation of MO by beneficial microbes that produce short-chain fatty acids (SCFA) (Ward et al., 2006; LoCascio et al., 2010; Asakuma et al., 2011).

There are hundreds of MO that have been identified in mammals, being composed of various monomers, including D-glucose, D-galactose, N-acetylglucosamine, L-fucose and sialic acid (N-acetylneuraminic acid) (Kunz et al., 2000; Kobata, 2010; Bode, 2019). Over 20 MO structures have been identified from dogs through liquid chromatography coupled with mass spectrometry (Macias Rostami et al., 2014; Hughes et al., 2020). Though it has been of great interest to find substitutes of MO that can provide the same health benefits, success has been limited due to the lack of structural diversity and complexity of traditional oligosaccharides [fructooligosaccharides (FOS); galactooligosaccharides (GOS)] as well as a difficulty in large-scale supply (Bode et al., 2016; Thomson, 2018). GNU100 is a source of an animal milk oligosaccharide (AMO) biosimilar that is isolated from hydrolyzed porcine intestinal mucosa. It contains about 30 complex conjugated oligosaccharides composed of O-linked glycans bound to peptidic moieties. The structure of intestinal mucin glycans are similar to MO, in that both have a galactose and N-acetylglucosamine elongated core structure and fucose or sialic acid group terminal chains (Marcobal et al., 2011). Functionally, both mucin glycans and MO have been shown to enrich similar taxa of gut resident microbes, help mitigate perturbations in the gut microbiota and provide host health benefits in mice (Pruss et al., 2020). In addition, mucin

glycans have been shown to provide mucosal immune homeostasis through SCFA production in a rat model (Hino et al., 2020).

Results of a previous in vitro fermentation study showed that when GNU100 was inoculated with canine feces, there was a beneficial shift in microbial composition (increased Bifidobacterium and Lactobacillus; reduction in Escherichia/Shigella and Salmonella) and an increased production in fermentative metabolites [SCFA (acetate; propionate; butyrate) and ammonia] (Oba et al., 2020). Although AMO have been studied using in vitro fermentation assays, to our knowledge, nobody has tested them in dogs. Therefore, we performed three experiments to: 1) assess the genotoxicity of GNU100 by using standard in vitro assays recommended by regulatory authorities to qualify novel ingredient for safe use in food, 2) test GNU100 palatability in dogs, and 3) evaluate primarily the safety and gastrointestinal (GI) tolerance of GNU100 and explore its utility by conducting a 26-wk study in healthy adult dogs. We hypothesized that GNU100 would not demonstrate genotoxic effects, would increase the palatability of food fed to dogs, and would be a safe, well-tolerated ingredient by dogs without having a negative impact on serum chemistry or hematology, fecal characteristics, or nutrient digestibility and by favorably modulating gut microbiota and metabolites.

Materials and methods

Experiment 1: Genotoxicity test

To test the genotoxicity of GNU100, two different *in vitro* assays were conducted. First, a bacterial reverse mutation assay was conducted to evaluate the mutagenic potential of GNU100. A second assay using mammalian cells was done to test cytotoxicity and potential of inducing

micronucleated cells by GNU100. A correction factor was applied to assay dilutions in all tests conducted to account for the purity profile of the test substance.

Bacterial reverse mutation assay

This assay was conducted in accordance with Organisation for Economic Cooperation and Development (OECD) Guideline Test No. 471 (OECD, 1997) using the treat and plate method as described by Ames et al. (1975) and Maron and Ames (1983). The first assay was conducted using *Salmonella typhimurium* tester strains TA98, TA100, TA1535, and TA1537, and *Escherichia coli* tester strain WP2 uvrA(pKM101). The study consisted of two phases conducted in the presence and the absence of metabolic activation (Aroclor 1254-induced rat liver S9-mix, obtained from Trinova Biochem GmbH, Giessen, Germany). The S9 mixture was included at 5% (v/v) in the initial mutation assay and at 10% (v/v) in the confirmatory assay. In the initial assay, the concentrations of GNU100 tested were 0, 52 ,164, 512, 1600, and 5000 µg/plate, while in the second confirmatory phase, the concentrations were 0, 492, 878, 1568, 2800, and 5000 µg/plate. To verify a mutagenic response observed in the second confirmatory phase of the test in the tester strain WP2uvrA(pKM101), an additional experiment was performed using the same concentrations as in the second phase in the presence of 10% (v/v) S9-mix. The negative (vehicle) control and positive controls were evaluated concurrently with the treatment groups. The negative control was cell culture grade water. Positive controls in the absence of S9 metabolic activation included DMSO solutions of sodium azide, 2-nitrofluorene, and methylmethane sulfonate. A positive control in the presence of S9 activation was a DMSO solution of 2-aminoanthracene.

It has been reported that biological materials capable of releasing amino acids can cause increases in the number of revertant colonies that are not related to a mutagenic mode of action and thus give false positive results in this type of assay (Thompson et al., 2005). As GNU100 is a complex of oligosaccharides and peptides that fits this criterion, a "treat and wash" variation of the bacterial reverse mutation test was also conducted with *Escherichia coli* tester strain WP2uvrA with and without rat S9 mix. This method was developed specifically to test peptide- and amino acid-containing materials. The following concentrations of GNU100 were tested: 0, 160, 310, 620, 1200, 2500, and 5000 μg/plate. The negative (vehicle) control was cell culture grade water and was plated in the presence and absence of S9 mix. Positive controls in the absence of S9 metabolic activation was a DMSO solution of 4-nitroquinoline N-oxide. The positive control in the presence of S9 metabolic activation, were a DMSO solution of 2-aminoanthracene and an aqueous solution of cyclophosphamide monohydrate.

For the evaluation of mutagenicity, a test was considered positive (mutagenic) if the total number of revertants in the tester strains TA100 or WP2uvrA was greater than two times the concurrent vehicle control or the total number of revertants in tester strains TA1535, TA1537 or TA98 was greater than three times the concurrent vehicle control.

In vitro mammalian cell micronucleus test

The *in vitro* mammalian cell micronucleus test was conducted in accordance with OECD Guideline test No 487 (OECD, 2010). Prior to treatment, L5178Y TK+/- mouse lymphoma cells (obtained from American Type Culture Collection ATCC, Manassas, USA) were counted and suspended at approximately 3 x 105 cells/mL in the treatment medium (culture medium and 5% inactivated horse serum). Based on a preliminary study, the following concentrations of GNU100

were selected for the main test: 0, 625, 1250, and 2500 μg/mL (the latter being the highest analyzable dose level because higher dose levels induced an important increase in osmolality). Cells were treated with GNU100, vehicle control (sterile water), or positive control for 3 h ("short-term exposure") in either the presence or absence of metabolic activation (Aroclor 1254-induced rat liver S9 mix, obtained from Molecular Toxicology, INC, Boone, NC 28607, USA) or for 24 h ("long-term exposure") in the absence of S9. After short-term exposure in the absence or presence of S9, the test article was removed by washing the cells twice and suspending in culture medium containing 10% inactivated horse serum followed by incubation at 37°C in a humidified atmosphere of 5% CO2/95% air for a recovery period of 24 h. Positive controls in the absence of S9 consisted of 5 μg/mL of colchicine in both the 3-h and 24-h treatment periods and 0.2 and 0.05 μg/mL of mitomycin C in the 3-h and 24-h treatment groups, respectively. In the presence of S9, 3 μg/mL of cyclophosphamide was used. Cells were exposed in 24-well plates, at 37°C in a humidified atmosphere of 5% CO2/95% air. Each treatment was coupled to an assessment of cytotoxicity at the same dose levels. Cytotoxicity was evaluated by determining the population doubling of cells as described by Greenwood et al. (2004). At the completion of the cytotoxicity assessments, cells were washed with culture medium (containing 10% inactivated horse serum) and 1% pluronic acid. Cells were suspended in 49.5% culture medium (containing 10% inactivated horse serum), 50% phosphate buffered saline (PBS) and 0.5% pluronic acid, before being fixed. Following fixation, the cells were kept at 5°C. Cells were prepared on glass slides and air-dried before being stained for approximately 15 min in 5% Giemsa. All slides were encoded for "blind" scoring. Analysis was performed at 1000x magnification. The presence of micronuclei was assessed according to criteria described by Miller et al. (1995). Micronuclei frequencies were analyzed in 1000 mononucleated cells per culture (total of 2000 mononucleated

cells per dose). Number of cells with micronuclei and number of micronuclei per cell were recorded separately for each treated and control culture.

Experiment 2: Palatability test

Twenty beagle dogs were used for a standard 2-d palatability test conducted at Kennelwood Inc. (Champaign, IL). A control diet coated with 5% of a mixed fat source was compared to the same diet coated with 5% mixed fat source + 1% GNU100. Dietary ingredients and guaranteed analysis are presented in **Table 4.1**. Dogs were offered two bowls per day, each containing 400 g of test diets. Food bowls were presented for 30 min each day, and to prevent left-right bias, bowl position was reversed on the second day of test. Total daily consumption and first choice preference were reported for each dog.

Experiment 3: Safety and tolerability study

Animals, experimental design and diets

All animal procedures were approved and conducted at Summit Ridge Farms (Susquehanna, PA). The staff conducting the study were blinded to treatments to avoid any potential bias in the evaluation of general health observations and fecal scores. Prior to the study, a physical examination by a licensed veterinarian was conducted, and blood samples for serum chemistry and hematology measurements were collected to confirm health.

Thirty-two beagle dogs (age =1-8 yr old; 5 male and 27 female beagle dogs; BW = 7.6 ± 2.31 kg) were used in a completely randomized design study. Initially, 36 dogs were acclimated to the control (0%) diet for 14 d prior to study initiation. After the acclimation period, 32 dogs were selected and randomly assigned into one of four dietary treatments (n=8/group) and fed for

26 wk. Dogs were housed in individual cages, with *ad libitum* access to fresh water. Even though dogs were housed individually, they received regular exercise and socialization with humans and other dogs according to kennel standard operating procedures and in line with the USDA Animal Welfare Act.

Dogs were fed dry, extruded diets formulated to meet all Association of American Feed Control Officials (AAFCO, 2019) nutrient recommendations for adult dogs at maintenance. All diets contained poultry by-product meal (low-ash), brewer's rice, poultry fat, corn, vitamin/mineral premixes, a palatant, and the test ingredient (GNU100), and formulated to contain approximately 35% protein, 15% fat, 7% ash, and 5% fiber. Diets were manufactured at Wenger Manufacturing, Inc. (Sabetha, KS). For these diets, Wenger used their large paddle mixer (capacity = ~550-700 kg). For each batch, the appropriate amount of GNU100 was pre-blended into about 50 kg of dry diet mix. The pre-blend was then added to the entire batch. Each batch was mixed for at least 10 to 15 min to ensure complete mixing. Dietary treatments consisted of: 1) control diet (0% GNU100); 2) low dose (0.5% GNU100); 3) medium dose (1.0% GNU100); and 4) high dose (1.5% GNU100). The ingredient and analyzed chemical composition of the diets are listed in **Table 4.2**.

Gnubiotics Sciences SA (Epalinges, Switzerland) provided the test ingredient. GNU100 is a complex of oligosaccharides and peptides isolated from hydrolyzed porcine intestinal mucosa with the typical physicochemical characteristics described in **Table 4.3.** and **Table 4.4**.

Daily food intake, weekly BW, and weekly BCS

Dogs were fed once a day to maintain BW throughout the study. Daily food consumption was recorded to monitor intake, with adjustments made weekly if needed. BW was measured by

personnel at a weekly basis during the study. Body condition scores (BCS; 9-point scale) according to Laflamme (1997) were measured at the beginning and end of the study and weekly during the study.

Fecal sample collection and analyses

Fresh fecal samples were collected on wk 2 and 4 for fecal microbiota composition and fermentative metabolites [SCFA; branched-chain fatty acids (BCFA); phenols and indoles; ammonia], and on wk 2, 4 and 26 for measurement of fecal scores, pH, immunoglobulin A (IgA) and calprotectin. On wk 4, in addition to the fresh fecal samples collected, total fecal samples were collected from each dog for 5 consecutive days to determine apparent total tract macronutrient digestibility. Total feces were weighed and stored in -20°C until later analysis.

Fecal scores were assigned as follows: 1= watery diarrhea; 1.5= diarrhea; 2= moist, no form; 2.5= moist, with some form; 3= moist, formed; 3.5= well formed, sticky; 4= well formed; 4.5= hard, dry; 5= hard, dry, crumbly feces. Fecal pH measurement was taken from the fresh fecal samples immediately and then aliquots of the samples collected were distributed as follows: approximately 4 g aliquots for analysis of phenol and indole were stored in 15 mL conical tubes in duplicates and frozen at -20°C. Fresh fecal aliquots were transferred to sterile 2 mL cryogenic vials (Nalgene, Rochester, NY), frozen immediately on dry ice, and stored at -70°C until microbiota, IgA, and calprotectin analyses. Another aliquot of approximately 5 g of feces were placed in 60 mL Nalgene bottles containing 5 mL of 2N HCl and stored at -20°C until SCFA, BCFA and ammonia analyses. Finally, remaining samples were placed in whirl-pak bag and stored in -20°C for dry matter determination.

Fecal SCFA and BCFA concentrations were determined according to Erwin et al. (1961) using a gas chromatograph (Hewlett-Packard 5890A series II, Palo Alto, CA) and a glass column (180 cm x 4 mm i.d.) packed with 10% SP-1200/1% H3PO4 on 80/100+ mesh Chromosorb WAW (Supelco Inc., Bellefonte, PA). Nitrogen was the carrier with a flow rate of 75 mL/min. Oven, detector, and injector temperatures were 125, 175, and 180°C, respectively. Fecal concentrations of phenol and indole were evaluated using gas chromatography according to Flickinger et al. (2003) and fecal ammonia concentration was measured according to the method of Chaney and Marbach (1962).

Fecal protein was extracted according to Vilson et al. (2016). Fecal samples (250 mg) were vortexed with 750 uL of extraction buffer containing 50 mM-EDTA (ThermoFisher, Waltham, MA) and 100 μg/L soybean trypsin inhibitor (Sigma, St. Louis, MO) in PBS/L percent bovine serum albumin (Tocris Bioscience, Bristol, UK). Phenylmethanesulphonyl fluoride (12.5 μL, 350 mg/L; Sigma, St. Louis, MO) was added into each tube and centrifuged for 10 min. Supernatants were collected for measurements of fecal IgA (E-40A; Immunology Consultants Laboratory, Portland, OR) and calprotectin (MBS030023; MyBiosource, Inc., San Diego, CA) using commercial enzyme-linked immunosorbent assay (ELISA) kits.

Fecal microbiota analysis and bioinformatics

Fecal bacterial DNA were extracted from feces collected according to manufacturer's instructions using the MO BIO PowerSoil Kit (MO BIO Laboratories, Carlsbad, CA). Concentration of extracted DNA were quantified using a Qubit 3.0 Fluorometer (Life Technologies, Grand Island, NY) and DNA quality was assessed using agarose gel electrophoresis. DNA samples were stored at -80°C until samples were analyzed using Pet 16-

sequencing technology. Genomic DNA from fecal samples at 2 wk (control, n = 7; 0.5% GNU100, n = 7 ;1% GNU100, n = 8; 1.5% GNU100, n = 8) and 4 wk (control, n = 6; 0.5% GNU100, n = 7 ;1% GNU100, n = 8; 1.5% GNU100, n = 8) were used for sequencing analysis.

Sequencing analysis was performed by Gnubiotics Sciences SA (Epalinges, Switzerland). In short, targeted PCR-based sequencing approach was used, where unique DNA regions of the 16S rRNA gene were targeted to generate amplicons. Amplicon library was sequenced using MiSeq Illumina platform with a 2 x 150 cycle Illumina sequencing kit (MS-102-2002). Two negative control reactions without the DNA template and two negative control reactions without the amplification primers were included in the run. Fastq files generated by the sequencer were used to de-multiplex and analyze the raw reads, using a publicly available Divisive Amplicon Denoising Algorithm (DADA2) pipeline. Read counts generated by the pipeline were divided by the total sample read count to obtain relative abundances. Microbiota data ordination was done by principal component analysis (PCA), with assessment of differences among microbial profiles of the four groups was done by one-way PERMANOVA (Bray–Curtis similarity distance) using paleontological Statistics (PAST; v3.12) software. Alpha-diversity analyses, including richness (number of amplicon sequence variants), Shannon diversity index (H), and evenness (e^H/S) were calculated, with significant differences being calculated using Mann–Whitney U test in PAST; v3.12. Differences were considered significant with $P < 0.05$ and trends at $P < 0.10$. Predominant bacterial phyla, class and genus were analyzed Mixed Models procedure of SAS 9.3 (SAS Institute, Cary NC).

Diet and apparent total tract digestibility analyses

Test diet subsamples and dried fecal samples collected for digestibility were ground through a Wiley mill (model 4, Thomas Scientific, Swedesboro, NJ) through a 2-mm screen. Dry

matter and organic matter were measured according to the Association of Official Analytical Chemists (AOAC, 2006; method 934.01 for dry matter; method 942.05 for organic matter). Crude protein content was calculated from total nitrogen values measured by LECO (TruMac N, Leco Corp., St. Joseph, MI; AOAC; method 922.15, 2002). Acid-hydrolyzed fat content was determined using methods according to American Association of Cereal Chemists (AACC; method 30-14, 1983) and (Budde, 1952). Total dietary fiber was determined for diet samples according to (Prosky et al., 1985) and AOAC (method 985.29, 2002). Gross energy was measured using a bomb calorimeter (Model 6200, Parr Instruments, Moline, IL). Apparent total tract macronutrient digestibility of nutrients and energy was calculated using the following equation:

$$\% \text{ Digestibility} = \frac{[\text{Nutrient intake (g/d)} - \text{Fecal output (g/d)}]}{\text{Nutrient intake (g/d)}} \text{ x } 100\%$$

Blood sample collection and analyses

On wk 2, 4, and 26, blood samples were collected for serum chemistry, hematology, inflammatory cytokines, immunoglobulin E (IgE), and C-reactive protein (CRP). Up to 8 mL of fasted blood sample was collected via jugular venipuncture. Blood samples were aliquoted so that 6 mL were used for serum separating tubes and 2 mL used for EDTA tubes. Blood collected into serum separating tubes was centrifuged for 15 min at 1,300 × g and stored into cryovials. One was sent to Antech Diagnostics for serum chemistry analysis, with two sent to the University of Illinois for measurement of inflammatory cytokines. Blood samples stored in EDTA tubes were sent to Antech Diagnostics for hematology analysis. Serum concentrations of IgE (ICL, Portland, OR), interleukin-6 (Abcam, Cambridge, MA), tumor necrosis factor-alpha

(R&D Systems, Minneapolis, MN) and CRP (Abcam, Cambridge, MA) were evaluated using commercial ELISA kits.

General health observations

A complete physical examination was given to all dogs by a licensed veterinarian at study initiation and at study completion. Qualified personnel performed clinical observations twice daily. Variables associated with GI tolerance such as abnormal stool quality, evidence of vomiting, abnormal behavior, poor coat quality, poor BCS, were recorded.

Coat and skin evaluation

Skin and coat scores were evaluated prior to study initiation, every 4 wk and at study completion according to Rees et al. (2001): hair: 1 = dull, coarse, dry; 2 = poorly reflective, non-soft; 3 = medium reflective, medium soft; 4 = highly reflective, very soft; 5 = greasy; skin: 1 = dry; 2 = slightly dry; 3 = normal; 4 = slightly greasy; 5 = greasy.

Statistical analysis

All data were analyzed using a Mixed Models procedure of SAS 9.3 (SAS Institute, Cary NC). The main effect of dietary treatment was tested with dogs considered as a random effect. Initially, the interaction between time and treatments effects were tested. Because no interaction effects were observed, those results were excluded from the results and discussion sections. Data normality and homogeneity of variance were tested using SAS. Differences among treatments were determined using a Fisher-protected least significant difference with a Tukey adjustment to control for experiment-wise error. A probability of $P < 0.10$ was accepted as being statistically

significant for safety measures and $P < 0.05$ for other measures. Data were reported as means ± pooled standard error of the mean (SEM).

Results

Experiment 1: Genotoxicity test

Bacterial reverse mutation assay

In the initial test of "treat and plate", no increases in the number of revertants were observed in any of the tester strains treated with GNU100 at any concentration in the absence or presence of S9-mix (5%) (**Table 4.5**). Based on the results of the initial mutation assay, it was determined that the maximum concentration (5000 μg/plate) should be included in the confirmatory assay. In the confirmatory assay of "treat and plate", no increase in the number of revertants was observed in tester strains TA1535, TA1537, TA98, TA100 at any concentration with or without S9 mix (10%) (**Table 4.6**). In tester strain WP2uvrA(pKM101) without S9 mix, GNU100 did not elicit an increase in revertants that reached the threshold for a positive mutagenic response. However, in the presence of S9 mix (10%), GNU100 elicited a concentration-dependent increase in revertants, reaching a 2.3-fold increase at a concentration of 5000 μg/plate (**Table 4.6**). In the additional confirmatory experiment in tester strain WP2uvrA(pKM101) in the presence of S9 mix (10%), GNU100 did not elicit an increase in revertants that reached the threshold for a positive mutagenic response (**Table 4.7**). Based on the equivocal results (one positive and one negative result), the test was considered inconclusive for tester strain WP2uvrA(pKM101). In the additional "treat and wash" assay, a method developed to avoid false positive results induced by peptides and amino acid-containing material, GNU100 did not elicit any increases in revertants in WP2uvrA above the threshold for a positive

mutagenic response at any concentration in the absence or presence of S9 (10%) (**Table 4.7**). In all tests, negative and positive control substances elicited acceptable results that were within the range of historical values.

In-vitro mammalian cell micronucleus test

No cytotoxicity was induced at any of the dosage levels tested, with a lack of any statistically significant or dose-related changes to population doubling. GNU100 did not induce any chromosome damage, or damage to the cell division apparatus in L5178Y TK+/- cells, either in the presence or absence of a rat liver S9 mixture compared to the negative control. GNU100 did not elicit any statistically significant increases in the frequency of micronucleated cells at any concentration. Frequencies of micronucleated cells remained within the historical control range (**Table 4.8**). An incubation time of 24 h was not shown to influence genotoxicity, and the recovery period had no impact, as no significant chromosomal damage was observed. Positive controls induced the expected clastogenic or aneugenic response in L5178Y TK+/- cells. These were different ($P < 0.001$) than the vehicle control and consistent with historical values of positive control responses.

Experiment 2: Palatability test

In the palatability test, a 3.6:1 total consumption ratio was observed for the 1% GNU100 diet vs. the control (0%) diet, showing a significant preference ($P < 0.05$) for the diet containing GNU100. Data collected from both days indicate that the 1% GNU100 diet was consumed first on 31/40 occasions, compared to 9/40 for control diet.

Experiment 3: Safety and tolerability study

General health observations

Thirty-two dogs were initially enrolled into the study. One of the dogs fed the 0.5% GNU100 treatment was removed from the study on d 140 due to chronic food regurgitation. The dog maintained its BW throughout the study and its bloodwork was normal. The staff veterinarian, however, noted that the dog would eat food readily and then regurgitate. Ultrasound scans of the stomach and abdomen did not show any abnormalities. The dog was ultimately diagnosed with megaesophagus and this condition was not due to the treatment diet. All other dogs remained healthy without signs of GI discomfort or intolerance throughout the study. The condition of the skin and hair did not differ among treatments during the experiment, with all dogs having hair that remained in a state described as a medium level of reflectivity and softness and the skin was scored as normal (data not shown).

Food intake, BW and apparent total tract macronutrient and energy digestibility

Average daily food intake was 155 g/d and was not affected by GNU100 supplementation (**Table 4.9**). Dog BW was 7.7 kg on average and was not different among different treatment groups throughout the study. Supplementation of GNU100 did not influence the apparent total tract digestibility of dry matter (79.2%), organic matter (85.1%), crude protein (78.5%), acid-hydrolyzed fat (96.7%), or energy (89.2%) (**Table 4.9**).

Fecal characteristics, metabolites, fecal IgA and calprotectin

Fecal characteristics, including pH, fecal scores and fecal dry matter percentage were not affected by treatment (**Table 4.10**). Similarly, fecal fermentative metabolites were not altered by

treatment. Finally, GNU100 did not alter fecal calprotectin or IgA , except at wk 4 where fecal IgA concentration was greater ($P < 0.05$) in dogs fed 0% GNU100 than those fed 1%.

Fecal microbiota

A total of 18,842,323 high-quality reads were obtained. The overall number of amplicon sequence variants detected was 2,991. The number of reads per sample ranged from 66,045 to 623,381. After calculation of relative abundance per sample, further analyses were performed. Microbial α-diversity measures, which represent species richness and evenness within a sample, indicated that species richness and Shannon diversity index were not affected by treatment. Evenness, however, tended to be greater ($P < 0.10$) in dogs fed 1% GNU100 than those fed 0% GNU100 at wk 4 (**Figure 4.1**).

Six bacterial phyla were detected in fecal samples, with Firmicutes and Fusobacteria being the most predominant (**Table 4.11**). At wk 4, relative abundance of Actinobacteria was higher ($P < 0.05$) in dogs fed 0% GNU100 than those in the other treatment groups. Relative abundance of Actinobacteria was also higher ($P < 0.05$) in dogs fed 1.5% GNU100 than those fed 0.5% GNU100. Relative abundance of Firmicutes was lower ($P < 0.05$) in dogs fed 0.5% than those fed 1.5% GNU100 at wk 4. Relative abundance of Proteobacteria tended to be greater ($P < 0.10$) in dogs fed 1% GNU100 at wk 4.

At the genus level, a total of 118 taxa were detected in the samples, with *Clostridium_XI* being the most abundant genera (**Table 4.12**). Relative abundance of *Catenibacterium* was higher ($P < 0.05$) in dogs fed 1.5% GNU100 than the other treatments at wk 2, while dogs fed 1% or 1.5% had higher ($P < 0.05$) *Catenibacterium* than those fed 0% or 0.5% GNU100 at wk 4. At wk 4, relative abundance of *Collinsella* was lower ($P < 0.05$) in dogs fed 0.5% GNU100

compared to those fed 0% and 1.5% GNU100. Also, dogs fed 1% GNU100 had lower ($P < 0.05$) *Collinsella* than those fed 1.5% GNU100 at wk 4. At wk 2, relative abundance of *Megamonas* was lower ($P < 0.05$) in dogs fed 0% GNU100 than those fed 1.5% GNU100. Relative abundance of *Turicibacter* was lower ($P < 0.05$) in dogs fed 0.5% than those fed 1% or 1.5% GNU100. Relative abundances of *Anaerobiospirillum*, *Peptococcus*, and *Prevotella* tended to be altered ($P < 0.10$) at wk 4.

Serum chemistry profile and blood cell counts

Serum metabolites were unaltered by treatment (**Table 4.13**). All metabolites were within reference ranges for all dogs except for blood urea nitrogen:creatinine ratio, which was slightly higher ($P < 0.05$) than the range in dogs fed 1% GNU100 at wk 2 and 0% and 1.5% GNU100 dogs at wk 4. Cholesterol also tended to be lower ($P < 0.1$) in dogs fed 1.5% GNU100 compared to dogs fed 0% at wk 26. Blood cell count values for all dogs were within reference ranges, and only percent of monocytes were ($P < 0.05$) increased in 1% GNU100 at wk 26 (**Table 4.14**).

Serum IgE, cytokine and CRP

Serum concentrations of IgE and CRP were not altered by treatment (**Table 4.15**). Serum IL-6 and TNF-α concentrations were measured but not reported as values were below than the detectable value (minimum detectable range: 0.9-4.2 pg/mL for TNF-α and 0.1ng/mL for IL-6) using a commercial ELISA kit.

Discussion

The main objective of the present study was to evaluate the palatability, safety and GI tolerance of GNU100 in healthy adult dogs. Moreover, GI functionality was explored. A battery

of genotoxicity *in vitro* studies were conducted to determine the safety of GNU100 as a nutritional ingredient in acceptance with guidelines established by EFSA and FDA. GNU100 was not mutagenic in the bacterial reverse mutation test with or without metabolic activation by a rat liver microsomal fraction. Also, GNU100 was not cytotoxic and did not induce any chromosome damage or damage to the cell division apparatus in mammalian somatic cells *in vitro* with or without metabolic activation. These results are in line with previous genotoxicity studies on MO. For example, no evidence of genetic toxicity and chromosomal aberration was noticed for 3′-fucosyllactose (Pitt et al., 2019) or a mixture of 2′-fucosyllactose and difucosyllactose (Phipps et al., 2018), thus supporting the safe use of such MO as nutritional ingredients. Together with the results obtained from molecules with similar epitopes, our genotoxicity data support a lack of genotoxicity of GNU100.

The pet food industry has grown exponentially over the past couple decades due to the increasing number of households that own companion animals and the preference for premium products. Palatability is one of the most important factors that pet food companies consider when including a new ingredient or producing a new dog food (Li et al., 2020). Evaluating food preference is necessary to determine whether the animals will be satisfied with the new products (Tobie et al., 2015; Alegría-Morán et al.,2019). The inherent animal characteristics, together with the sensorial and nutritional properties of the food, affect the dietary habits of dogs (Hall et al., 2017). In the current study, inclusion of 1% GNU100 in the diet increased palatability. The high content of amino acids, peptides, and glycopeptides of GNU100 may explain the higher palatability or preference of this ingredient in dogs. In fact, a strong preference for protein-rich foods and diets coated with other protein-rich ingredients, including *Saccharomyces cerevisiae* fermentation product (Lin et al., 2019), is often reported.

Testing the gastrointestinal tolerance and measures of general health (serum chemistry; hematology) of animals fed novel ingredients are also of importance. In the current study, all doses of GNU100 were well tolerated, with no signs of gastrointestinal distress and desirable fecal scores, pH, and dry matter percentages being present in dogs fed all treatments. Based on the data of our previous *in vitro* fermentation study of GNU100 using fecal inoculum that suggested moderate fermentation rates that should not drastically affect fecal characteristics in vivo (Oba et al., 2020), this outcome was expected. All serum metabolites and blood cell counts were normal throughout the study, and measures of inflammation (serum CRP; fecal calprotectin) and allergy (serum IgE) were not affected by dietary treatment. These data demonstrate that GNU100 does not cause loose stools, intestinal inflammation, or immune or allergic reactions. Lastly, because nutrient digestibility was unaffected by GNU100 inclusion, these data suggest that the use of GNU100 will not affect nutrient digestibility when used up to 1.5% of the diet.

GNU100 is composed of a diverse and complex mixture of conjugated oligosaccharides, with epitopes similar to that present in AMO. Therefore, its capacity to modulate the canine microbiome was explored in this study. Milk oligosaccharides are non-digestible glycans with frequent sialylation and fucosylation that are key components of animal milk (Boehm & Stahl, 2007; Oliveira et al., 2015; Robinson, 2019). These complex glycans act as prebiotics, anti-adhesives, and anti-microbials and play critical roles in immune cell responses (McKeen et al., 2019). They are selectively utilized as substrates by beneficial gut bacteria and are fundamental to the establishment of a healthier microbiota and promote host health (Robinson, 2019; McKeen et al., 2019). In the current study, GI microbiota were modulated over time with several microbial taxa being changed at wk 2 or wk 4. Also, dogs fed 1% GNU100 at wk 4 tended to

have a higher evenness than at wk 2, providing potential benefits to the animals, as a more diverse microbiome is more resistant to perturbations (Lozupone et al., 2012).

Previous studies have shown that nondigestible fibers and prebiotics such as FOS can modulate microbial communities, increasing the abundance of beneficial bacterial taxa in dogs (Garcia-Mazcorro et al., 2017; Redfern et al., 2017). Changes are not always demonstrated with prebiotics or fibers, however, as dogs fed fiber-prebiotic and saccharin-eugenol blend (Rentas et al., 2020) or the fructooligosaccharide kestose (Ide et al., 2020) did not have a different microbial diversity than those in the control group. In humans, Salli et al. (2019) reported a higher microbial diversity with 2′-fucosyllactose in a simulated infant gut microbiome environment in comparison with GOS and lactose. It has been proposed that differences in concentration, diversity and complexity of glycans may affect the microbiome composition (McKeen et al., 2019). The complexity and diversity of GNU100 could explain the present results.

In the current study, fecal SCFA and BCFA were not affected by dietary treatments even though GNU100 was shown previously to be fermented and increase the production of these metabolites *in vitro* (Oba et al., 2020). Although fecal metabolites are typically measured, there are often discrepancies between the amount measured in feces and the actual amount produced by microbiota in the gut due to rapid absorption and metabolism of SCFA by colonocytes (von Englehardt et al., 1989). Hence the measurement of digesta or circulating metabolites may be of interest in future studies (Müller et al., 2019). Microbiota results indicated that some SCFA-producing taxa were altered in the feces, but more research would be necessary to confirm these effects.

The relative abundance of the *Catenibacterium* was greater at wk 2 in dogs fed 1.5% GNU100 and at 4 wk in dogs fed 1% and 1.5% GNU100. This genus is known to be a SCFA producer (Kageyama and Benno, 2000), with increased dietary fiber being correlated with greater *Catenibacterium* spp. in cats (Hooda et al., 2013; Yang et al., 2013) and dogs (Jarett et al., 2019). Another taxa, *Collinsella*, was lower in dogs fed 0.5% GNU100 compared to controls (0%). Similar results have been reported in dogs after the oral administration of probiotics (Xu et al., 2019). In humans, the genus *Collinsella* has been shown to be enriched in IBS patients (Masoodi et al, 2020) and decreased in patients on a weight-loss diet (Stephens et al., 2018) so a reduction appears to be a beneficial shift. In the current study, *Megamonas* was greater in dogs fed 1.5% of GNU100 in comparison with the controls at wk 2. Similar results have been observed in dogs supplemented with inulin (Beloshapka et al., 2013) or FOS (Hidaka et al., 2008). *Megamonas* is another SCFA (acetate; propionate) producer that has been negatively correlated with BW in dogs (Kieler et al., 2017). Relative abundance of *Peptococcus* and *Prevotella* tended to be higher in dogs fed GNU100, while the relative abundance of *Anaerobiospirillum* was variable. *Prevotella* is a well-known fiber fermentor and SCFA producer that has been associated with gastrointestinal health (Minamoto et al., 2015; Schmidt et al., 2018; Pilla and Suchodolski, 2020), but *Peptococcus* and *Anaerobiospirillum* are not well studied in dogs. The promotion of SCFA producers is in line with a previous *in vitro* fermentation study that demonstrated the fermentation of GNU100 with the concomitant growth of beneficial bacteria and production of SCFA (Oba et al., 2020). Furthermore, O-glycans have been shown to act as a fermentation source to produce SCFA (Yamada et al., 2019; Hino et al., 2020).

This study had many strengths, including the length of the observational period and the number of measurements made. The main aim of this study was to test the safety of GNU100 so

the experimental design (completely randomized design), length of study (6 mo), and animal numbers (n=8/treatment) suggested by AAFCO guidelines were used. Another strength was that this study tested the effects of three GNU100 dosages against a control (0%) group. The study also had a few limitations. Because safety studies require a long intervention period, a completely randomized design was used. This design is ideal for safety testing, but likely limited our ability to observe differences in fecal microbiota and metabolites, which are highly variable among animals. A crossover or Latin square design, which would allow each animal to serve as their own control, likely would have reduced variation and increased statistical power. Also, because the AAFCO protocol requires the study of a healthy animal population, the ability to observe differences is more challenging than a clinical population.

In conclusion, GNU100 was shown to be palatable and its dietary supplementation over a long period of time (6 mo) was well tolerated and did not cause detrimental effects on stool quality or nutrient digestibility. Together with the *in vitro* genotoxicity results, the absence of negative effects on serum chemistry, hematology, and other health biomarkers in the *in vivo* study conducted with healthy dogs, supports the safety of GNU100 for inclusion in canine diets. Finally, GNU100 supplementation favorably impacted the fecal microbiota, notably by promoting SCFA producers and tended to increase diversity. Overall, the results suggest potential benefits on gastrointestinal health of dogs.

Tables and figure

Table 4.1. Guaranteed analysis of the base dog food used for the palatability study

Nutrient	%, as-is
Crude protein, minimum	24
Crude fat, minimum	5
Crude fiber, maximum	7
Ash, maximum	8
Moisture, maximum	10

Ingredients: Ground Yellow Corn, Poultry By-Product Meal, Wheat Flour, Oat Groats, Beet Pulp, White Rice, Pork Meat & Bone Meal, Flaxseed, Brewers Dried Yeast, Potassium Chloride, Vitamins [Choline Chloride, Vitamin E Supplement, Ascorbic Acid, Calcium Carbonate, Riboflavin Supplement, Niacin Supplement, Calcium Pantothenate, Vitamin A Supplement, Vitamin D3 Supplement, Pyridoxine Hydrochloride, d-Biotin, Vitamin B12 Supplement, Thiamine Mononitrate, Folic Acid], Salt, Dried Whey, Minerals [Zinc Sulfate, Manganese Sulfate, Ferrous Sulfate, Magnesium Sulfate, Zinc Proteinate, Ferrous Proteinate, Manganese Proteinate, Copper Sulfate, Magnesium Proteinate, Copper Proteinate, Calcium Iodate], Sodium Selenite, Choline Chloride, Antioxidant.

Table 4.2. Ingredient and chemical composition of experimental diets fed to dogs

	Control, 0% GNU100	Low dose, 0.5% GNU100	Medium dose, 1.0% GNU100	High dose, 1.5% GNU100
Ingredient		---------- %, as-is ----------		
Poultry by-product meal (low-ash)	43.27	43.27	43.27	43.27
Brewer's rice (US #2)	39.77	39.27	38.77	38.27
Poultry fat	8.65	8.65	8.65	8.65
Corn, whole (US #2)	4.81	4.81	4.81	4.81
Palatant	1.92	1.92	1.92	1.92
Test ingredient (GNU100)	0.00	0.50	1.00	1.50
Salt (sodium chloride)	0.48	0.48	0.48	0.48
Potassium chloride, 50% K	0.43	0.43	0.43	0.43
Taurine	0.19	0.19	0.19	0.19
Mineral premix[a]	0.18	0.18	0.18	0.18
Vitamin premix[b]	0.18	0.18	0.18	0.18
Choline chloride	0.13	0.13	0.13	0.13
Chemical composition[c]				
Dry matter, %	93.7	92.6	93.1	93.4
		---------- % dry matter ----------		
Organic matter	93.3	93.2	93.3	93.2
Crude protein	37.2	37.0	35.9	36.2
Acid-hydrolyzed fat	17.6	17.3	15.7	16.8
Total dietary fiber	9.3	8.5	8.1	8.6
Gross energy, kcal/g	5.25	5.22	5.1	5.19

[a]Provided per kg 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.
[b]Provided per kg diet: vitamin A, 5.28 mg; vitamin D_3, 0.04 mg; vitamin E, 120.00 mg; vitamin K, 0.88 mg; thiamin, 4.40 mg; riboflavin, 5.72 mg; pantothenic acid, 22.00 mg; niacin, 39.60 mg; pyridoxine, 3.52 mg; biotin, 0.13 mg; folic acid, 0.44 mg; vitamin B_{12}, 0.11 mg.
[c]Chemical composition analysis was performed from diet subsample at the University of Illinois.

Table 4.3. Physicochemical characteristics of GNU100 used for the study

Parameter	Value
Sensorial	White to yellow powder
Oligosaccharide and peptide complex	42.7% dry matter
Free amino acids	41.7% dry matter
Ash	15.6% dry matter
Moisture	4.0%
pH (2 w/v%, in deionized water at 20°C)	5.6
Molecular weight distribution	
>10,000 Da	0.0%
5,000-10,000 Da	0.2%
2,000-5,000 Da	5.7%
1,000-2,000 Da	38.9%
<1,000 Da	55.3%

Table 4.4. Typical oligosaccharide profile for GNU100 measured by liquid chromatography-tandem mass spectrometry

Mass (g/mol)	Structure[a]
384	Galβ1-3GalNAcol
425	GlcNAcβ1-6GalNAcol
513	NeuAcα2-6GalNAcol
529	NeuGcα2-6GalNAcol
530	Fucα1-2Galβ1-3GalNAcol
587-1	Galβ1-3(GlcNAcβ1-6)GalNAcol
587-2	GlcNAcα1-4Galβ1-3GalNAcol Gal+GlcNAcβ1-6GalNAcol
587-3	Galβ1-4GlcNAcβ1-6GalNAcol
587-4	GlcNAcα1-4Galβ1-3GalNAcol Gal+GlcNAcβ1-6GalNAcol
667	Galβ1-3(6SGlcNAcβ1-6)GalNAcol
675-1	Galβ1-3(NeuAcα2-6)GalNAcol
675-2	NeuAcα2-3Galβ1-3GalNAcol
691-1	Galβ1-3(NeuGcα2-6)GalNAcol
691-2	NeuGcα2-3Galβ1-3GalNAcol
716-1	GlcNAcβ1-3(NeuAcα2-6)GalNAcol
716-2	GalNAcα1-3(NeuAcα2-6)GalNAcol
732	HexNAc-(NeuGcα2-6)GalNAcol
733-1	Fucα1-2(GalNAcα1-3)Galβ1-3GalNAcol
733-2	Fucα1-2Galβ1-4GlcNAcβ1-6GalNAcol
733-3	Fucα1-2Galβ1-3(GlcNAcβ1-6)GalNAcol
813	Fucα1-2Galβ1-3(6S-GlcNAcβ1-6)GalNAcol
821	Fucα1-2Galβ1-3(NeuAcβ2-6)GalNAcol
870-1	GlcNAcβ1-3[Galβ1-4(6S)GlcNAcβ1-6]GalNAcol
870-2	Galβ1-4GlcNAcβ1-3[(6S)GlcNAcβ1-6]GalNAcol
895	Galβ1-3(Fucα1-2Galβ1-4GlcNAcβ1-6)GalNAcol
1016-1	Fucα1-2Galβ1-4(6S)GlcNAcβ1-6[GlcNAcβ1-3]GalNAcol
1016-2	GlcNAcβ1-3[Fucα1-2Galβ1-3(6S-)GlcNAcβ1-6]GalNAcol
1121	Fucα1-2Galβ1-3[Fucα1-2Galβ1-4(6S)GlcNAcβ1-6]GalNAcol

[a]Structures given in table according to the following rules: (i) structure is described clockwise and left-to-right, where reducing end locates rightmost side; (ii) "+" is used for uncertain location; (iii) symbols for monosaccharide residues and derivatives: Fuc = fucose, Gal = galactose, GalNAc = N-acetylgalactosamine, Glc = glucose, GlcNAc = N-acetylglucosamine, Neu5Ac = N-acetylneuraminic acid, Neu5Gc = N-glycoloylneuraminic acid, S = sulfate, Hex = hexose.

Table 4.5. Initial bacterial reverse mutation bacterial assay conducted with GNU100

	S typhimurium TA98		*S typhimurium* TA100		*S typhimurium* TA1535		*S typhimurium* TA1537		*E coli* WP2uvrA(pkM101)	
Concentration µg/plate	-S9[a]	+S9 (5%)	-S9	+S9 (5%)	-S9	+S9 (5%)	-S9	+S9 (5%)	-S9	+S9 (5%)
Negative controls[b]	6 ± 2	10 ± 4	105 ± 10	89 ± 8	6 ± 1	9 ± 2	5 ± 2	8 ± 4	33 ± 4	43 ± 12
52	9 ± 1	6 ± 1	94 ± 22	102 ± 10	5 ± 2	5 ± 2	10 ± 12	13 ± 5	35 ± 9	50 ± 2
164	7 ± 7	12 ± 2	105 ± 7	106 ± 17	8 ± 3	8 ± 6	5 ± 3	11 ± 1	30 ± 9	42 ± 4
512	7 ± 5	7 ± 4	86 ± 8	98 ± 5	5 ± 4	8 ± 7	2 ± 2	7 ± 2	40 ± 5	43 ± 10
1600	7 ± 6	5 ± 2	111 ± 14	118 ± 19	6 ± 2	10 ± 3	3 ± 1	15 ± 5	49 ± 14	61 ± 10
5000	9 ± 5	11 ± 4	125 ± 21	128 ± 13	10 ± 4	11 ± 2	6 ± 4	8 ± 5	57 ± 11	56 ± 6
Positive controls[c]	379 ± 30	156 ± 15	259 ± 36	950 ± 152	93 ± 11	78 ± 6	48 ± 23	115 ± 13	710 ± 136	115 ± 37

[a]-S9: without metabolic activation; +S9: with metabolic activation; 2-AA: 2-aminoanthracene; 2-NF: 2-nitrofluorene; 4NQO: 4-nitroquinoline N-oxide; MMS: methylmethane sulfonate; SA: sodium azide.
[b]Negative controls: -S9: TA98 = 10µg/plate 2-NF; TA100 = 800 µg/plate MMS; TA1535 = 20 µg/plate SA; TA1537 = 30 µg/plate 2NF; WP2uvrA (pKM101) = 2 µg/plate 4NQO.
[c]Positive controls: +S9: TA98 = 1µg/plate 2-AA; TA100 = 15µg/plate 2-AA; TA1535 = 15 µg/plate 2-AA; TA1537 = 10 µg/plate 2-AA; WP2uvrA (pKM101) = 15µg/plate 2-AA.

Table 4.6. Confirmatory and additional bacterial reverse mutation assay conducted with GNU100, with revertant colonies per plate (mean of replicate plates ± standard deviation) reported

	S. typhimurium TA98		*S. typhimurium* TA100		*S. typhimurium* TA1535		*S. typhimurium* TA1537		*E. coli* WP2uvrA(pkM101)		
Concentration, µg/plate	-S9[a]	+S9 (10%)	-S9	+S9 (10%)	-S9	+S9 (10%)	-S9	+S9 (10%)	-S9	+S9 (10%)	+S9 (10%), additional
Negative controls[b]	6 ± 2	17 ± 8	87 ± 12	74 ± 8	8 ± 4	17 ± 8	5 ± 2	3 ± 1	29 ± 6	34 ± 14	45 ± 8
492	11 ± 3	18 ± 2	83 ± 18	81 ± 7	11 ± 1	18 ± 3	2 ± 3	7 ± 1	35 ± 4	49 ± 11	64 ± 9
878	6 ± 3	14 ± 4	88 ± 21	100 ± 4	9 ± 6	17 ± 7	2 ± 2	8 ± 1	48 ± 19	53 ± 12	54 ± 2
1568	8 ± 0	23 ± 7	77 ± 15	97 ± 13	8 ± 1	17 ± 11	3 ± 2	6 ± 2	39 ± 4	57 ± 11	53 ± 12
2800	13 ± 2	12 ± 4	88 ± 6	103 ± 13	11 ± 5	9 ± 2	4 ± 1	7 ± 1	54 ± 10	62 ± 9	64 ± 11
5000	13 ± 2	22 ± 10	92 ± 4	109 ± 19	11 ± 3	13 ± 3	2 ± 2	7 ± 2	49 ± 9	77 ± 5	65 ± 2
Positive controls[c]	792 ± 149	172 ± 13	166 ± 23	759 ± 93	129 ± 17	112 ± 4	65 ± 24	55 ± 9	549 ± 150	175 ± 31	137 ± 19

[a]-S9: without metabolic activation; +S9: with metabolic activation; 2-AA: 2-aminoanthracene; 2-NF: 2-nitrofluorene; 4NQO: 4-nitroquinoline N-oxide; MMS: methylmethane sulfonate; SA: sodium azide.
[b]Negative controls: -S9: TA98 = 10µg/plate 2-NF; TA100 = 800 µg/plate MMS; TA1535 = 20 µg/plate SA; TA1537 = 30 µg/plate 2NF; WP2uvrA (pKM101) = 2 µg/plate 4NQO.
[c]Positive controls: +S9: TA98 = 1µg/plate 2-AA; TA100 = 15µg/plate 2-AA; TA1535 = 15 µg/plate 2-AA; TA1537 = 10 µg/plate 2-AA; WP2uvrA (pKM101) = 15µg/plate 2-AA.

Table 4.7. "Treat and Wash" bacterial reverse mutation assay results (mean revertant colonies per plate ± standard deviation) conducted with GNU100

	E coli WP2uvrA	
Concentration µg/plate	Without metabolic activation	With metabolic activation
Negative control	20 ± 10	34 ± 2
160	25 ± 1	41 ± 7
310	26 ± 7	55 ± 21
620	21 ± 5	42 ± 7
1200	27 ± 6	39 ± 11
2500	31 ± 3	53 ± 17
5000	36 ± 5	54 ± 11
4-nitroquinoline N-oxide, 0.5 µg	943 ± 105	-
Cyclophosphamide monohydrate, 450µg	-	89 ± 17
2-aminoanthracene, 200 µg	-	79 ± 17

Table 4.8. *In vitro* mammalian cell micronucleus test in L5178Y TK$^{+/-}$ cells exposed to GNU100

Concentration (µg/mL)	Mean PD[a] (% of control)	Frequency of micronucleated cells per dose
3-h Treatment + 24-h recovery without metabolic activation		
Vehicle control (water)	100	1.0
625	113	0.5
1250	111	0.5
2500	101	0.5
Mitomycin C (0.2 µg/mL)	83	74.0***
Colchicine (0.5 µg/mL)	#	10.0***
3-h Treatment + 24-h recovery with metabolic activation		
Vehicle control (water)	100	2.5
625	86	2.0
1250	82	3.0
2500	73	3.5
Cyclophosphamide (3 µg/mL)	75	66.0***
24-h treatment + 0-h recovery without metabolic activation		
Vehicle control (water)	100	1.5
625	120	0.5
1250	126	1.0
2500	120	2.5
Mitomycin C (0.05 µg/mL)	83	16.0***
Colchicine (0.5 µg/mL)	#	19.5***

[a]PD: population doubling of cells.

***: # cell concentration at the end of treatment was lower than the cell concentration at the beginning of treatment ($P < 0.001$).

Table 4.9. Mean daily intake, BW, and apparent total tract macronutrient and energy digestibility of dogs supplemented with different dosages of GNU100

	GNU100				SEM	*P*-value
	0%	**0.5%**	**1%**	**1.5%**		
Daily intake, g/day	161.5	158.5	149.6	152.3	12.18	0.89
BW, kg	7.7	7.9	7.7	7.6	0.86	0.99
Initial BW, kg	7.6	7.7	7.7	7.6	0.87	0.99
Final BW, kg	7.6	8.2	7.8	7.7	0.88	0.97
---------- Digestibility[1], % ----------						
Dry matter	78.0	79.6	80.9	78.1	1.48	0.49
Organic matter	84.2	85.5	86.2	84.5	1.07	0.57
Crude protein	77.8	78.9	79.7	77.4	1.53	0.71
Acid-hydrolyzed fat	94.5	94.8	94.4	94.8	0.36	0.74
Gross energy	84.6	85.7	86.1	84.7	1.05	0.70

[1]Digestibility data measured from total feces collected over 5 consecutive days during wk 4.

Table 4.10. Mean fecal characteristics, metabolites, immunoglobulin A (IgA) and calprotectin concentrations of dogs fed GNU100-supplemented diets

Item	Week	GNU100				SEM	*P*-value
		0%	0.5%	1%	1.5%		
pH	2	6.9	7.0	6.9	6.7	0.16	0.75
	4	6.8	7.1	6.9	6.8	0.16	0.55
	26	6.7	6.8	6.8	6.5	0.16	0.43
Score[a]	2	3.1	3.1	3.2	3.3	0.16	0.75
	4	3.2	3.1	3.4	3.2	0.15	0.43
	26	3.3	3.2	3.4	3.4	0.13	0.58
Dry matter, %	2	36.7	34.9	36.2	36.8	1.18	0.68
	4	34.3	35.6	36.7	36.4	1.20	0.55
	26	34.9	34.0	35.1	36.5	0.87	0.23
		---------- µmol/g DM ----------					
Total SCFA[1]	2	270.0	333.0	309.9	296.1	26.71	0.40
	4	295.0	283.0	307.4	281.8	29.60	0.90
Acetate	2	158.1	200.9	180.5	162.7	16.22	0.27
	4	164.5	173.4	179.5	158.5	16.97	0.81
Propionate	2	72.7	94.4	86.7	94.9	7.14	1.00
	4	83.5	79.5	90.1	85.7	9.37	0.85
Butyrate	2	37.3	38.6	42.7	38.5	4.40	1.00
	4	37.1	35.5	37.8	37.6	4.34	0.98
Total BCFA[2]	2	23.9	26.9	27.6	24.3	3.62	0.81
	4	23.1	27.3	23.1	25.1	2.93	0.63
Isobutyrate	2	9.5	10.9	11.0	9.5	1.41	0.76
	4	9.4	11.0	9.1	9.9	1.18	0.62
Isovalerate	2	13.9	15.6	16.1	14.3	2.19	0.82
	4	13.3	15.9	13.4	14.5	1.73	0.62
Valerate	2	0.5	0.5	0.5	0.5	0.05	0.86
	4	0.4	0.5	0.6	0.7	0.08	0.18
Total P/I[2]	2	6.3	5.3	3.7	4.1	1.10	0.41
	4	4.8	5.2	3.8	4.5	1.09	0.83
Phenol	2	3.0	2.0	1.2	1.4	0.72	0.44
	4	2.0	1.6	1.0	2.1	0.77	0.69
Indole	2	3.3	3.4	2.5	2.7	0.46	0.40
	4	2.7	3.5	2.8	2.4	0.50	0.43
Ammonia	2	209.0	223.0	244.2	212.0	27.37	0.72
	4	200.0	214.0	203.0	212.0	17.79	0.82
IgA, mg/g	2	6.1	2.3	2.3	4.4	1.55	0.28
	4	5.6[b]	2.4[ab]	0.7[a]	2.4[ab]	1.19	**0.03**
	26	0.9	1.5	2.4	1.8	0.85	0.90
Calprotectin, µg/g	2	0.12	0.11	0.13	0.12	0.02	0.93
	4	0.14	0.14	0.13	0.15	0.01	0.55

Table 4.10. (Continued)

26	0.10	0.10	0.14	0.13	0.02	0.26

[1]Fecal score: 1 = watery diarrhea; 1.5 = diarrhea; 2 = moist, no form; 2.5 = moist, with some form; 3 = moist, formed;
3.5 = well formed, sticky; 4 = well formed; 4.5 = hard, dry; 5 = hard, dry, crumbly feces.
[2]Short-chain fatty acids (SCFA) = acetate + propionate + butyrate; branched-chain fatty acids (BCFA) = isobutyrate +
isovalerate + valerate; P/I = phenol + indole.
[a-b]Within a row, means with different superscripts differ ($P < 0.05$).

Table 4.11. Predominant bacterial phyla (mean % of total sequences) in feces of dogs fed GNU100-supplemented diets

		GNU100					
Phylum	**Week**	**0%**	**0.5%**	**1%**	**1.5%**	**SEM**	***P*-value**
Actinobacteria	2	0.43	0.44	0.64	0.49	0.15	0.73
	4	0.49[c]	0.15[a]	0.18[ab]	0.43[b]	0.10	**0.04**
Bacteroidetes	2	5.27	6.77	5.78	5.47	1.53	0.91
	4	8.54	13.60	13.89	7.52	16.65	0.92
Deferribacteres	2	0.03	0.02	0.01	0.02	0.01	0.90
	4	0.04	0.05	0.06	0.01	0.02	0.32
Firmicutes	2	79.88	75.48	82.81	83.36	4.62	0.61
	4	74.26[ab]	61.83[a]	63.68[ab]	77.31[b]	4.70	**<.0001**
Fusobacteria	2	11.91	12.65	8.97	8.24	2.46	0.49
	4	13.89	20.36	16.91	12.49	2.66	0.18
Proteobacteria	2	2.13	4.46	1.63	2.33	1.31	0.59
	4	2.56	3.75	5.11	1.97	0.88	0.07

[a-c]Within a row, means with different superscripts differ ($P < 0.05$).

Table 4.12. Predominant bacterial genera (mean % of total sequences) in feces of dogs fed GNU100-supplemented diets

		GNU100					
Genus[1]	**Week**	**0%**	**0.5%**	**1%**	**1.5%**	**SEM**	***P*-value**
Acetanaerobacterium	2	0.17	0.18	0.31	0.34	0.12	0.84
	4	0.30	0.44	0.50	0.45	0.15	0.80
Allobaculum	2	1.35	1.36	1.10	1.39	0.29	0.82
	4	1.34	0.93	1.27	1.46	0.30	0.63
Alloprevotella	2	2.00	2.39	1.52	1.50	0.52	0.59
	4	1.92	3.62	3.21	2.46	0.66	0.32
Anaerobiospirillum	2	0.95	0.75	0.67	0.45	0.45	0.93
	4	1.31	1.24	2.25	0.77	0.39	0.06
Bacteroides	2	2.02	2.86	2.76	1.82	0.79	0.72
	4	6.09	6.18	6.72	2.77	1.37	0.16
Blautia	2	6.69	6.05	8.29	6.66	1.12	0.52
	4	6.15	5.37	5.33	7.10	1.03	0.56
Catenibacterium	2	0.53^{a}	0.097^{a}	0.57^{a}	1.29^{b}	0.21	**0.01**
	4	0.15^{a}	0.12^{a}	0.97^{b}	0.73^{b}	0.25	**0.01**
Clostridium_sensu_stricto	2	0.55	0.50	0.81	0.69	0.19	0.64
	4	0.32	0.20	0.30	0.31	0.09	0.76
Clostridium_XI	2	33.61	27.70	30.72	32.60	3.52	0.66
	4	30.10	21.63	21.99	29.21	3.28	0.15
Clostridium_XlVa	2	0.38	0.26	0.41	0.36	0.06	0.35
	4	0.46	0.32	0.40	0.37	0.07	0.62
Clostridium_XVIII	2	0.46	0.30	0.29	0.28	0.09	0.46
	4	0.39	0.26	0.19	0.23	0.07	0.22
Collinsella	2	0.23	0.28	0.46	0.32	0.11	0.56
	4	0.24^{bc}	0.09^{a}	0.12^{ab}	0.29^{c}	0.05	**0.01**
Escherichia/Shigella	2	0.01	1.28	0.01	0.56	0.64	0.21
	4	0.03	0.25	0.31	0.04	0.17	0.79
Faecalibacterium	2	1.78	1.92	1.63	1.69	0.41	0.96
	4	1.99	1.87	2.51	2.53	0.39	0.52
Fusobacterium	2	11.65	12.26	8.68	8.00	2.37	0.44
	4	13.53	19.65	16.37	12.29	2.58	0.29
Holdemanella	2	0.57	0.41	0.72	1.08	0.25	0.30
	4	0.55	0.45	0.93	0.96	0.26	0.41
Lactobacillus	2	0.44	4.01	1.64	0.57	1.31	0.83
	4	0.75	1.01	0.89	1.18	0.60	0.78
Megamonas	2	0.66^{a}	0.83^{ab}	1.03^{ab}	2.54^{b}	0.50	**0.05**
	4	0.49	1.37	1.96	2.47	0.71	0.11
Parasutterella	2	0.41	0.24	0.19	0.25	0.15	0.82
	4	0.22	0.42	0.59	0.32	0.12	0.19
Peptococcus	2	0.09	0.13	0.34	0.16	0.08	0.15
	4	0.15	0.31	0.39	0.80	0.19	0.10
Phascolarctobacterium	2	0.37	0.42	0.56	0.48	0.09	0.51
	4	0.55	0.86	0.88	0.60	0.13	0.19

Table 4.12. (Continued)

Prevotella	2	0.83	0.78	0.92	1.48	0.40	0.54
	4	0.18	2.57	2.57	1.66	0.69	0.09
Romboutsia	2	4.33	3.92	4.21	4.19	0.63	0.97
	4	3.51	2.39	2.79	3.48	0.42	0.20
Streptococcus	2	1.57	3.41	1.06	2.74	1.85	0.95
	4	0.76	2.89	0.95	1.48	1.03	0.61
Turicibacter	2	1.74^{ab}	0.66^{a}	2.14^{b}	1.87^{b}	0.49	**0.03**
	4	0.91	0.56	1.17	1.22	0.38	0.57

[1]All genera with relative abundance >0.5% of total sequences are presented.
[a-c]Within a row, means with different superscripts differ ($P < 0.05$).

Table 4.13. Serum chemistry profile of dogs fed GNU100-supplemented diets

Item	Week	GNU100				SEM	*P*-value
		0%	0.5%	1%	1.5%		
Total protein (g/dL)	2	5.84	5.93	5.90	6.00	0.18	0.93
	4	5.89	6.00	6.04	6.15	0.18	0.79
	26	5.84	5.94	5.81	5.93	0.15	0.91
Albumin (g/dL)	2	3.28	3.38	3.41	3.46	0.08	0.44
	4	3.25	3.39	3.43	3.48	0.08	0.25
	26	3.24	3.31	3.30	3.35	0.07	0.66
Globulin (g/dL)	2	2.56	2.55	2.49	2.54	0.19	0.99
	4	2.64	2.61	2.61	2.68	0.19	0.99
	26	2.60	2.63	2.51	2.58	0.15	0.96
A/G Ratio	2	1.34	1.38	1.41	1.45	0.11	0.90
	4	1.29	1.35	1.34	1.36	0.10	0.95
	26	1.26	1.31	1.31	1.35	0.08	0.90
AST (U/L)	2	31.8	28.6	32.4	28.4	3.15	0.73
	4	35.6	27.5	32.5	28.9	3.22	0.30
	26	36.5	25.6	37.0	27.3	2.60	0.23
ALT (U/L)	2	68.9	44.1	55.8	53.3	11.75	0.77
	4	92.9	45.5	56.9	55.1	18.47	0.30
	26	114.3	62.0	73.3	58.1	24.87	0.37
Alkaline phosphatase (U/L)	2	34.0	33.9	43.6	36.4	5.44	0.55
	4	33.8	34.8	42.6	35.9	6.05	0.73
	26	37.3	34.1	44.6	40.5	6.26	0.68
GGTP (U/L)	2	4.00	3.38	3.50	3.75	0.37	0.65
	4	3.88	3.63	4.00	4.13	0.30	0.67
	26	4.50	3.57	3.88	4.38	0.33	0.19
Total bilirubin (mg/dL)	2	0.19	0.18	0.19	0.21	0.01	0.28
	4	0.16	0.19	0.19	0.19	0.01	0.52
	26	0.16	0.17	0.14	0.16	0.02	0.60
Urea nitrogen (mg/dL)	2	13.8	14.6	13.8	13.6	0.88	0.84
	4	15.5	15.4	13.9	13.5	0.84	0.24
	26	13.8	12.7	11.8	11.6	0.88	0.30
Creatinine (mg/dL)	2	0.59	0.65	0.55	0.60	0.03	0.26
	4	0.55	0.59	0.54	0.55	0.04	0.81
	26	0.59	0.63	0.58	0.63	0.04	0.65
BUN/creatinine ratio	2	23.8	22.6	27.3	23.0	2.71	0.61
	4	28.9	26.5	26.4	27.5	2.71	0.82
	26	23.5	20.3	21.0	18.9	1.41	0.15
Phosphorus (mg/dL)	2	3.33	3.25	3.19	3.28	0.92	0.98
	4	3.33	3.35	3.33	3.45	0.17	0.94
	26	3.40	3.34	2.91	3.16	0.18	0.22
Glucose (mg/dL)	2	89.4	91.5	89.6	87.6	2.66	0.79
	4	93.0	92.6	90.5	91.5	2.28	0.86
	26	86.6	95.3	88.9	87.5	2.90	0.19

Table 4.13. (Continued)

Calcium (mg/dL)	2	9.51	9.56	9.78	9.54	0.15	0.57
	4	9.58	9.88	9.91	9.76	0.12	0.23
	26	9.66	9.73	9.71	9.61	0.11	0.87
Magnesium (mEq/L)	2	1.48	1.46	1.53	1.50	0.03	0.60
	4	1.48	1.53	1.49	1.54	0.02	0.25
	26	1.54	1.47	1.46	1.51	0.03	0.25
Sodium (mEq/L)	2	148	148	148	148	0.43	0.84
	4	149	149	149	150	0.44	0.34
	26	149	149	149	149	0.41	0.77
Potassium (mEq/L)	2	4.23	4.11	3.98	4.09	0.08	0.19
	4	4.24	4.15	4.19	4.19	0.08	0.89
	26	4.24	4.13	4.08	4.10	0.08	0.45
Chloride (mEq/L)	2	113	113	113	112	0.60	0.99
	4	114	113	113	114	0.63	0.79
	26	113	113	113	114	0.48	0.63
Cholesterol (mg/dL)	2	174	159	165	161	12.22	0.56
	4	163	164	168	155	11.78	0.92
	26	202	176	183	159	11.57	0.08
Triglycerides (mg/dL)	2	45.8	48.6	46.1	40.9	2.16	0.84
	4	46.0	45.9	45.5	40.5	1.85	0.99
	26	45.9	44.0	48.3	42.1	1.50	0.48
CPK(U/L)	2	99.3	103.4	114.8	122.9	8.48	0.99
	4	99.0	102.0	127.9	110.9	10.43	0.22
	26	120.8	87.9	117.6	115.3	10.82	0.16

Table 4.14. Blood cell counts of dogs fed GNU100-supplemented diets

		GNU100				SEM	*P*-value
	Week	**0%**	**0.5%**	**1%**	**1.5%**		
WBC (10^3/mm3)	2	8.83	8.75	8.66	8.61	0.83	0.99
	4	7.51	9.21	7.93	8.55	0.78	0.46
	26	7.11	6.76	7.01	7.65	0.55	0.71
RBC (10^6/mm3)	2	7.09	6.94	7.08	7.24	0.21	0.80
	4	7.41	6.96	7.38	7.25	0.19	0.33
	26	7.23	7.19	7.20	7.36	0.21	0.93
Hemoglobin (g/dL)	2	16.58	16.43	16.66	16.93	0.47	0.90
	4	17.15	16.21	17.10	16.75	0.43	0.40
	26	17.06	16.90	17.33	17.20	0.48	0.93
Hematocrit (%)	2	52.63	52.63	51.75	53.50	1.44	0.86
	4	54.13	51.25	54.75	52.88	1.31	0.27
	26	55.25	54.71	54.88	56.25	1.59	0.90
MCV (µm^3)	2	74.13	75.63	73.25	74.13	0.83	0.26
	4	73.13	73.63	74.13	73.00	0.57	0.50
	26	76.50	76.29	76.25	76.38	0.67	0.99
MCH (µµg)	2	23.39	23.73	23.58	23.41	0.18	0.50
	4	23.21	23.39	23.25	23.13	0.22	0.86
	26	23.69	23.57	24.06	23.41	0.30	0.46
MCHC (g/dl)	2	31.75	31.38	32.25	31.50	0.29	0.17
	4	31.88	32.00	31.50	31.63	0.27	0.56
	26	31.00	31.00	31.50	30.75	0.35	0.49
Absolute	2	57188	48713	58550	62088	9002	0.72
Reticulocytes	4	36875	28113	30175	46800	6391	0.20
	26	45313	33900	46050	61975	5079	0.15
Reticulocytes (%)	2	0.79	0.70	0.81	0.88	0.13	0.82
	4	0.50	0.40	0.39	0.63	0.09	0.25
	26	0.63	0.49	0.64	0.86	0.13	0.26
Platelets (10^3/mm3)	2	338.6	323.0	338.5	334.9	26.35	0.94
	4	336.6	378.1	339.1	357.1	36.81	0.84
	26	346.3	323.3	318.0	351.6	30.98	0.83
Absolute	2	1941	2194	1952	1961	163.4	0.65
Lymphocytes	4	2034	2392	2171	2103	95.67	0.99
	26	1594	1687	1689	1678	131.32	0.95
Lymphocytes (%)	2	22.63	25.50	23.38	23.13	1.63	0.62
	4	27.00	26.75	27.50	25.38	1.61	0.81
	26	23.00	25.29	24.50	22.00	1.55	0.46
Absolute Monocytes	2	366.3	353.4	380.3	343.0	62.13	0.98
	4	282.8	383.5	392.9	339.0	52.98	0.45
	26	331.6	305.9	446.8	301.0	27.78	0.27

Table 4.14. (Continued)

Monocytes (%)	2	3.88	4.00	4.25	3.88	0.37	0.88
	4	3.63	4.25	5.00	3.75	0.45	0.15
	26	4.50[a]	4.57[a]	6.38[b]	3.88[a]	0.59	**0.03**
Absolute Eosinophil	2	309.5	286.3	306.8	355.9	74.52	0.93
	4	315.0	330.9	309.9	420.1	79.29	0.93
	26	296.6	265.6	246.8	338.8	56.99	0.68
Eosinophils (%)	2	3.63	3.50	3.50	3.88	0.73	0.98
	4	4.25	3.75	3.75	4.50	0.83	0.89
	26	4.50	4.14	3.75	4.38	0.89	0.93

Table 4.15. Mean serum immunoglobulin E (IgE) and C-reactive protein (CRP) concentration of dogs fed GNU100-supplemented diets

	Week	GNU100				SEM	*P*-value
		0%	0.5%	1%	1.5%		
IgE, mg/L	2	6.4	5.7	14.2	10.3	3.78	0.44
	4	8.9	6.9	10.3	9.8	0.20	0.67
	26	4.9	6.3	18.0	8.8	4.93	0.41
CRP, mg/L	2	2.2	2.9	3.3	2.1	1.06	0.91
	4	2.8	3.3	2.3	1.3	0.88	0.42
	26	2.6	5.2	4.5	1.9	1.30	0.31

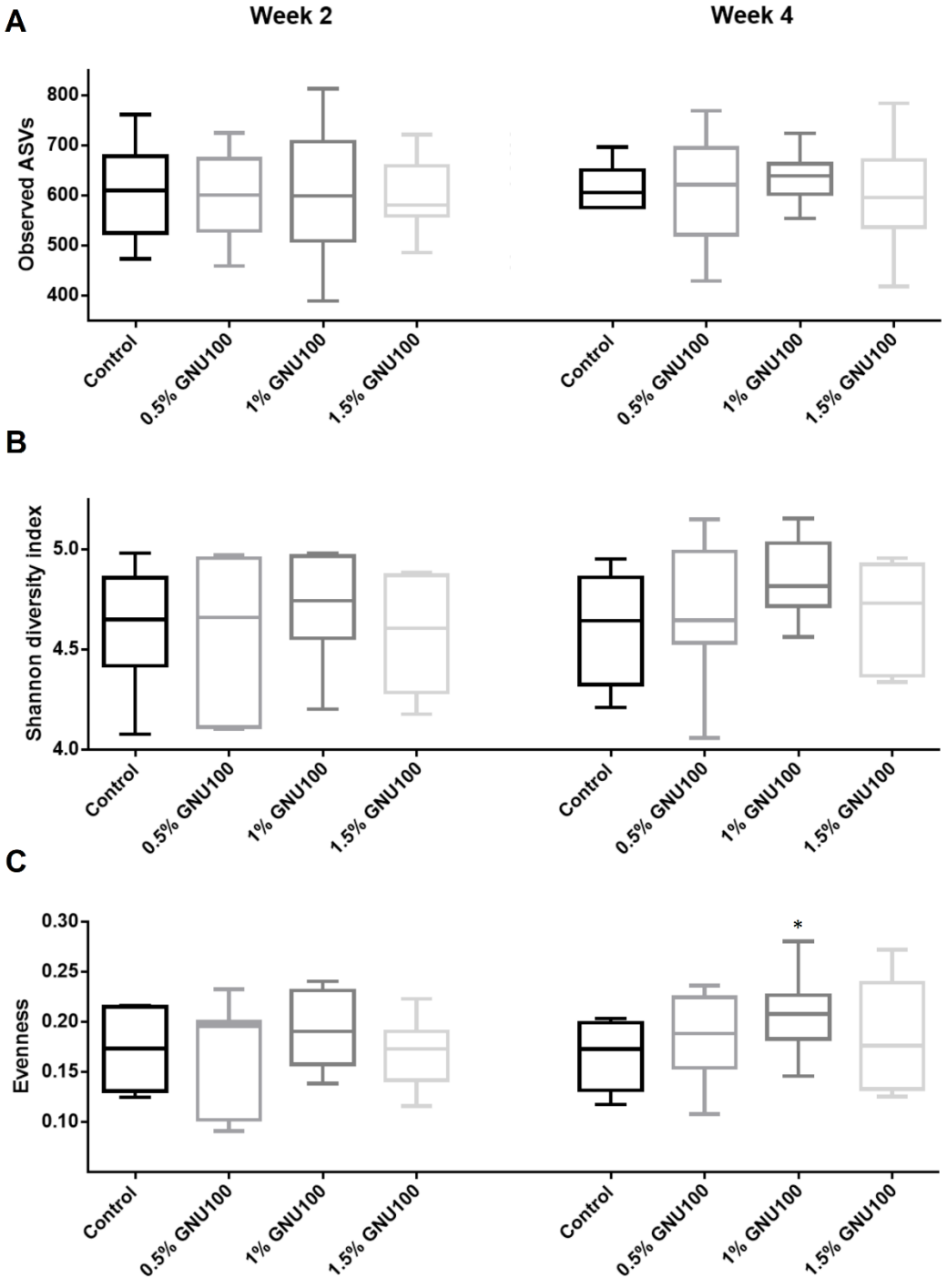

Figure 4.1. Alpha-diversity indices of fecal microbiota from dogs fed GNU100-supplemented diets. **A)** richness (observed amplicon sequence variants), **B)** Shannon diversity index (H), **C)** evenness (e^H/S). *Tended to be different ($P < 0.10$) from 0% GNU100.

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CHAPTER 5: DIETARY ENRICHMENT OF RESISTANT STARCHES OR FIBERS DIFFENTIALLY ALTER THE FELINE FECAL MICROBIOME AND METABOLITE PROFILE

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Abstract

Cats are strict carnivores but possess a complex gastrointestinal (GI) microbial community that actively ferments dietary substrates that are not digested and reach the colon. The GI microbiota responses to dietary inclusion of resistant starches versus fibers have not been tested in cats. Thus, our objective was to evaluate the effects of diets enriched in resistant starch or fibers on the fecal characteristics, microbiome, and metabolite profiles of cats. Twelve healthy adult domestic shorthair cats (age = 9.6±4.0 yr; body weight = 3.9±1.0 kg) were used in a replicated 3x3 Latin square design to test diets that were enriched with: 1) resistant starch (ERS), 2) a fiber-prebiotic-probiotic blend (FPPB), or 3) a fiber-prebiotic-probiotic blend + immune-modulating ingredients (iFPPB). In each 28-d period, 22d of diet adaptation was followed by fecal and blood sample collection. Fecal samples were used for shotgun sequencing and 16S sequencing. In addition, fecal and blood metabolite measurements and white blood cell stimulation was performed to assess immune function. A total of 1,690 bacterial species were identified via shotgun sequencing, with 259 species differing between fiber-rich and ERS treatments. In comparison with fiber-rich treatments that increased diversity and promoted Firmicutes and Bacteroidetes

populations, resistant starch reduced microbial diversity and fecal pH, led to a bloom in Actinobacteria, and modified KO terms pertaining to starch and sucrose metabolism, fatty acid biosynthesis and metabolism, epithelial cell signaling, among others. Resistant starch also differentially modified fecal metabolite concentrations with relevance to gastrointestinal and overall host health (increased butyrate; decreased propionate and protein catabolites - branched-chain fatty acids; phenols and indoles; ammonia) and reduced blood cholesterol, which correlated strongly with microbial taxa and KO terms, and allowed for a high predictive efficiency of diet groups by random forest analysis. Even though domestic cats and other carnivores evolved by eating low-carbohydrate diets rich in protein and fat, our results demonstrate that the feline microbiome and metabolite profiles are highly responsive to dietary change and in directions that are predictable.

Introduction

Domestic cats are carnivores and have traditionally relied on high-protein, high-fat diets containing relatively low fiber concentrations. Despite having a relatively simple gastrointestinal (GI) tract evolved to digest such diets, they possess a rich GI microbial community that actively ferments dietary substrates escaping host digestion. Several hundred bacterial species, predominated by members of Firmicutes (36-50%), Bacteroidetes (24-36%) and Proteobacteria (11-12%), are known to inhabit the cat GI tract (Barry et al., 2012; Jha et al., 2020). The presence of the above-mentioned phyla and their functions differ among individuals depending on living environment, dietary habits, or experimental methods used (Deng et al., 2014), but the drivers of inter-individual GI microbiome variations, their source, and their influence on host physiological processes remain obscure and largely unexplored.

Dietary fibers, prebiotics, and resistant starches (RS) are known to influence host GI microbiota and immune function by promoting carbohydrate fermentation, which leads to increases in short-chain fatty acid (SCFA) production. SCFA have many beneficial effects, including improved gut barrier and immune function (Lührs et al., 2002; Wong et al., 2006; Wang et al., 2012; Arpaia et al., 2013; Furusawa et al., 2013; Kim et al., 2013; Kelly et al., 2013). Other dietary components such as yeast fermentation products and spray-dried plasma (SDP) may serve as immune-modulators using alternative mechanisms (Pérez-Bosque et al., 2006; Mmiddelbos et al., 2007; Stercova et al., 2016; Pawar et al., 2017; Tran et al., 2018; Lin et al., 2019; Lin et al., 2020). These dietary components are included in pet foods, but with little data on their effects in cats.

Moreover, most of the current microbiota data in cats was obtained from 16S rRNA or ITS microbial profiling methods. Although those methods provide useful information regarding the microbial populations, they are unable to provide deeper resolution (species or strain) into the microbial community. Furthermore, there is potential for bias caused during the amplification step for 16S rRNA and ITS microbial profiling methods (Alessandri et al., 2020). Therefore, shotgun sequencing has been used to improve resolution and accuracy in recent years and more human studies have applied these methods to gain understanding of diet-induced changes to the microbiota at the taxonomic level as well as the functional features coming from the gene content data (Lugli et al., 2019). Despite the progress being made in the microbiome field, data in cats are still very limited, with many of the microbiome studies being conducted decades ago, and the impact of diverse diets fed to cats on GI microbial diversity and richness, gene content, and metabolic activity have not been reported. Exploring the microbiota beyond the taxonomic level

and evaluating the effects of diet on gene content and its relation to physiological outcomes improve understanding of the mechanistic insights of how microbes potentially affect feline health.

The primary objective of this study was to explore the fecal microbial community profiles and their functions in domestic cats fed diets enriched in either RS or a combination of dietary fibers, prebiotics, and probiotics utilizing shotgun sequencing. Secondary objectives were to identify significant fecal bacterial taxa-bacterial gene-metabolite changes in response to the different dietary treatments.

Materials and methods

Animals, Diets and Experimental Timeline

The animal study was conducted at Kennelwood, Inc. (Champaign, IL, USA) and all animal procedures for this study were approved by the Kennelwood, Inc. Institutional Animal Care and Use Committee (IACUC). Twelve healthy adult female domestic shorthair cats (age = 9.6 ± 4.0 yr; body weight = 3.9 ± 1.0 kg) were used in a replicated 3x3 Latin square design. All cats were housed in individual pens (54.6 cm W x 64.8 cm L x 68.6 cm H) in an environmentally controlled animal facility. Cats had access to fresh water at all times and were fed once a day to maintain body weight throughout the study. Weekly measurement of body weight and BCS (9-point scale) were recorded prior to feeding (Laflamme, 1997).

Three different extruded, experimental kibble diets were formulated to meet all Association of American Feed Control Officials (AAFCO) nutrient recommendations for adult cats at maintenance (**Table 5.1**) (AAFCO, 2019). All diets contained a fish-based protein source, grains, and chicken fat, but were formulated to contain unique gut microbiota modulators. The first diet was enriched in resistant starch (ERS) and contained potato flour, a starch source that is more

resistant to gelatinization during the extrusion process than grains, providing a source of RS. The second diet [fiber-prebiotic-probiotic blend-containing formula (FPPB)] was formulated to contain a prebiotic (i.e., inulin), a probiotic (i.e., *Lactobacillus acidophilus* and *Enterococcus faecium*), and natural fiber-rich ingredients such as oat groats, beet pulp, and pea fiber. The third diet [fiber-prebiotic-probiotic blend + immuno-modulating ingredient-containing formula (iFPPB)] was formulated similar to FPPB, but with the addition of immunomodulators (i.e., yeast fermentation product and SDP).

Prior to the study, blood samples were collected for serum chemistry and complete blood count measures to confirm health. Each experimental period was 28 d in length, consisting of an adaptation phase from d 1-22, fecal collection phase (e.g., microbiota; metabolites; IgA; pH) from d 23-27, and blood collection on d 28 (e.g., serum chemistry; complete blood count; immune cell functionality).

Blood Collection and Analyses

On d 28 of each experimental period, up to 15 mL of blood was collected via jugular, cephalic, or medial saphenous vein venipuncture for serum chemistry, hematology, and immune assays. Prior to collection, cats were sedated with an intramuscular (IM) injection of a combination of butorphanol tartrate (0.024 mg/kg IM; Torbugesic, Zoetis Inc., USA), dexmedetomidine (0.02 mg/kg IM; Dexdormitor, Zoetis Inc., USA), and ketamine (0.061 mg/kg IM; Zoetis Inc., USA). After blood collection, an injection of the reversal agent for dexmedetomadine, atipamezole hydrochloride (0.2 mg/kg IM; Antisedan, Zoetis Inc., USA), was given. Collected blood samples were immediately placed into appropriate vacutainer tubes: 10 mL in #366480 BD Vacutainer® glass plasma tubes (Becton Dickinson, USA) for immunoassays, 0.5 mL in #365974 BD

Microtainer® Plastic whole blood tubes with K2EDTA additive (Becton Dickinson, USA) for hematology, and 4.5 mL in #367974 BD Vacutainer® Plus plastic serum tube with clot activator and gel for serum separation (Becton Dickinson) for serum chemistry. Serum was isolated by centrifugation at 1,300 × g at 4^{o} C for 10 min (Beckman CS-6R centrifuge; Beckman Coulter Inc., USA). Serum chemistry profile and hematology were analyzed using a Hitachi 911 clinical chemistry analyzer (Roche Diagnostics, USA) at the University of Illinois Veterinary Medicine Diagnostics Laboratory.

Immune cell assays were performed as described by Lin et al. (2019). Briefly, peripheral blood mononuclear cells (PBMC) were separated by layering 10 mL of collected blood over Ficoll Histopaque (Sigma, USA) in a 1:1 volume ratio and centrifuged at 300 x g at 4^{o}C for 30 min. Once PBMC were isolated, the responsiveness of lymphocytes to toll-like receptor (TLR) agonists, including zymosan (TLR2 agonist; 100 µg/mL zymosan; Invivogen, USA), polyinosinic–polycytidylic acid sodium salt (TLR3 agonist; 50 µg/mL polyinosinic–polycytidylic acid sodium salt, poly(I:C); Sigma, USA), lipopolysaccharides (TLR4 agonist; 100 ng/mL LPS; Sigma, USA) and resiquimod (TLR7/8 agonist; 5 µg/mL resiquimod, Invivogen, USA) were assessed by measuring tumor necrosis factor-alpha (TNF-alpha) production. Collected PBMC (1×10^{6} cells/tube) were stimulated in triplicate in a 96-well plate and incubated for 24 h at 37^{o}C in 5% CO_2. Following incubation, collected supernatants were stored in -80^{o}C until measurement of TNF-α using a commercial enzyme-linked immunosorbent assay (ELISA) kit (MyBioSource, San Diego, CA, USA).

Fecal Sample Collection and Metabolite Analyses

During the collection phase, total fecal output was collected, and fecal scores were noted. Total feces excreted during the collection phase were taken from the litter boxes, 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.

During the fecal collection phase, a fresh fecal sample (within 15 min of defecation) was collected for fecal pH, microbiota and metabolite measurement. Fecal pH was measured immediately using an AP10 pH meter (Denver Instrument, USA) equipped with a Beckman Electrode (Beckman Instruments Inc., USA), and then aliquots were collected. Aliquots for phenols and indoles analysis 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 branched-chain fatty acid (BCFA) analyses. An aliquot of fresh feces was immediately transferred to sterile cryogenic vials (Nalgene, USA), frozen in dry ice, and stored at -80^{o}C for microbial analysis. Additional aliquots were used for fresh fecal dry matter (DM) determination and immunoglobulin A (IgA) concentration measurement.

Fecal SCFA (acetate, propionate and butyrate) and BCFA (valerate, isovalerate, isobutyrate) concentrations were determined according to Erwin et al. (1961) using a gas chromatograph (Hewlett-Packard 5890A series II, USA) and a glass column (180 cm x 4 mm i.d.) packed with 10% SP-1200/1% H3PO4 on 80/100+ mesh Chromosorb WAW (Supelco Inc., USA). 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 concentrations of phenols and indoles were evaluated using gas chromatography according to Flickinger et al. (2003) and fecal ammonia concentration was measured according to the method of Chaney and Marbach (1962).

Fecal protein was extracted according to Vilson et al. (2016). Fecal samples (500 mg) were vortexed with 1.5 mL of extraction buffer containing 50 mM-EDTA (ThermoFisher, USA) and 100 μg/L soybean trypsin inhibitor (Sigma, USA) in PBS/L percent bovine serum albumin (Tocris Bioscience, UK). Phenylmethanesulphonyl fluoride (12.5 μL, 350 mg/L; Sigma, USA) was added into each tube and centrifuged for 10 min. The supernatant was collected for measurement of fecal IgA using a commercial ELISA kit (E-20A; Immunology Consultants Laboratory, USA).

Diet and fecal chemical composition and digestibility analyses

Fecal samples used for digestibility analysis were dried at 55°C in a forced-air oven. Diet subsamples and dried fecal samples were ground through a Wiley mill (model 4, Thomas Scientific, USA) through a 2-mm screen. DM and organic matter (OM) content were measured according to the Association of Official Analytical Chemists (AOAC, 2006; method 934.01 for DM; method 942.05 for OM). Crude protein content was calculated from total nitrogen values measured by LECO (TruMac N, Leco Corp., USA; AOAC, 2006; method 922.15). Acid-hydrolyzed fat content was determined using methods according to American Association of Cereal Chemists (AACC, 1983; method 30-14) and Budde, 1952. Total dietary fiber (TDF) was determined for diet and fecal samples according to Prosky et al., 1992 and AOAC (method 985.29). Gross energy was measured using a bomb calorimeter (Model 6200, Parr Instruments, USA). Apparent total tract macronutrient digestibility of nutrients and energy were calculated using the following equation:

$$\%\ Digestibility = \frac{[Nutrient\ intake\ (g/d) - Fecal\ output\ (g/d)]}{Nutrient\ intake\ (g/d)}\ x\ 100\%$$

Fecal DNA Extraction and 16S Amplicon Data Analysis

Total DNA from fecal samples was extracted using Mo-Bio PowerSoil kits (MO BIO Laboratories, Inc., Carlsbad, CA, USA). Concentration of extracted DNA was quantified using a Qubit 3.0 Fluorometer (Life Technologies, USA). 16S rRNA gene amplicons were generated using a Fluidigm Access Array (Fluidigm Corporation, USA) in combination with Roche High Fidelity Fast Start Kit (Roche, USA). The primers 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) that target a 252 bp-fragment of the V4 region of the 16S rRNA gene were used for amplification (primers synthesized by IDT Corp., Coralville, IA, USA) (Caporaso et al., 2010). CS1 forward tag and CS2 reverse tag were added according to the Fluidigm protocol. Quality of the amplicons were assessed using a Fragment Analyzer (Advanced Analytics, USA) to confirm amplicon regions and sizes. A DNA pool was generated by combining equimolar amounts of the amplicons from each sample. The pooled samples were then size selected on a 2% agarose E-gel (Life technologies, USA) and extracted using a Qiagen gel purification kit (Qiagen, USA). Cleaned size-selected pooled products were run on an Agilent Bioanalyzer to confirm appropriate profile and average size. Illumina sequencing was performed on a MiSeq using v3 reagents (Illumina Inc., USA) at the W. M. Keck Center for Biotechnology at the University of Illinois.

Forward reads were trimmed using the FASTX-Toolkit (version 0.0.13) and QIIME 1.9.1 was used to process the resulting sequence data (Caporaso et al., 2010). Briefly, high-quality (quality value $\geq$ 20) sequence data derived from the sequencing process was demultiplexed. Sequences then were clustered into operational taxonomic units (OTU) using UCLUST through a closed-reference OTU picking strategy against the Greengenes 13_8 reference database with a 97% similarity threshold (DeSantis et al., 2006; Edgar, 2010). Singletons (OTU that are observed

fewer than two times) and OTU that have less than 0.01% of the total observation were discarded. An even sampling depth (sequences per sample) was used for assessing alpha- and beta-diversity measures. Beta-diversity was calculated using weighted and unweighted UniFrac distance measures (Lozupone et al., 2005). Statistical analysis was conducted via Statistical Analyses of Metagenomic Profiles (STAMP) software 2.1.3 using ANOVA and Tukey-Kramer multiple comparison tests (Parks et al., 2014). All tests were corrected for multiple inferences using the Benjamini-Hochberg method to control for false discovery rate (FDR). Statistical significance was set at $P < 0.05$.

Fecal DNA Extraction, Shotgun Sequencing, and Data Analyses

Total DNA was extracted with Zymogen Quick-DNA Fecal/Soil Microbe 96 Mag Bead kit (Zymo Research Corp., USA) using Powerbead Pro (Qiagen, USA) plates with 0.5mm and 0.1mm ceramic beads. Extraction controls included were a no template control (water) and a characterized homogenized stool. All samples were quantified with Quant-iT Picogreen dsDNA Assay (Invitrogen, USA). Libraries were prepared with a procedure adapted from the Nextera XT DNA Library Preparation Kit (Illumina, USA). For BoosterShot (Shallow Sequencing, 2 M reads/sample), libraries were sequenced either on an Illumina NovaSeq using single-end 1x100 reads (Illumina, San Diego, CA, USA). Library controls included were a no template control (water) and DNA from a characterized homogenized stool. For quality control, single end shotgun reads were trimmed and processed using Shi7 (Al-Ghalith et al., 2018). The sequences were then aligned to the NCBI RefSeq representative prokaryotic genome collection at 97% identity with BURST using default settings (Al-Ghalith et al., 2017). A total of 70,326,694 reads were mappable and the mean sequencing depth per sample was 2,131,112 (median = 2,124,420) with the standard

deviation of 776,799. Taxa present in < 5% of samples were removed. The resulting taxonomy table was aggregated at higher taxonomy levels.

Kyoto Encyclopedia of Genes and Genomes Orthology groups (KEGG KOs) were observed directly using alignment at 97% identity against a gene database derived from the strain database used above. KOs present in < 5% of samples were removed as part of the quality filtering process. Species richness and Shannon's diversity indices were computed by rarefying samples to various depths starting from 25,000–950,000 sequences per sample and increasing sequence depth by 25,000 reads. One hundred iterations were performed at each depth and the mean values were used as the estimate of these measures in each sample. To investigate the effect of treatment on alpha-diversity, the species richness and Shannon Index were calculated using the rarefaction depth of 950,000. Wilcoxon signed rank test was used to compare the alpha diversity metrics among treatments.

The non-rarefied count data were log-transformed and principal coordinate analysis (PCoA) was performed in R using the Bray-Curtis and Jensen-Shannon distances calculated with the vegan package at the species level (Oksanen et al., 2019). Permutational multivariate analysis of variance (PERMANOVA) was performed using Bray-Curtis distance with 10,000 randomizations to assess the differences in community composition using the vegan package (Oksanen et al., 2019). Differential abundance of bacterial phyla, species, and KO terms between treatments was assessed using a negative binomial generalized linear model (GLM) using the differential expression analysis for sequence count data version 2 (DESeq2) package (Love et al., 2014). Taxa with absolute log2 (fold change [FC]) > 2 and adjusted $P < 0.01$ were considered significant. The adjustment for multiple comparisons was performed using the Benjamini Holchenberg false discovery rate (FDR).

Clustering

Partitioning Around Medoids (PAM) Clustering was performed using the *cluster* package (Maechler et al., 2018). Individuals were clustered into multiple clusters (K = 1 to 5) based on the top two PCoA dimensions obtained using Bray-Curtis distances. Goodness of clustering was assessed using a "gap" statistic with 1,000 bootstrapped replicates.

Random Forest models

Random forest classifiers were constructed using the repeated k-fold cross validation and random search implemented in R-package *caret* (Breiman, 2001; Kuhn, 2020). The model was trained by optimizing the tuning parameters using a 5-fold cross validation repeated 3 times using species as the predictor and accuracy was used to select the optimal model. The performance of the classifiers was assessed by generating area under the receiver operating characteristic curves (AUC) using the R-package *ROCR* (Sing et al., 2005).

Statistical Analyses

Except microbiota analyses, all data were analyzed using the Mixed Models procedure of SAS (version 9.4; SAS Institute, Inc., Cary, NC, USA), with treatment considered as a fixed effect and cat and period considered random effects. Data were tested for normality using the UNIVARIATE procedure of SAS. Differences between treatments were determined using a Fisher-protected least significant difference with a Tukey adjustment to control for experiment-wise error. A probability of $P < 0.05$ was accepted as statistically significant. Reported pooled standard errors of the mean (SEM) were determined according to the Mixed Models procedure of SAS. Correlation analyses

between fecal microbiota and metabolites were assessed by Spearman's rank correlation test in RStudio (version 1.1.463). Significance was set at FDR adjusted $P \leq 0.05$.

Results

Food intake, body weight, body condition score, apparent total tract energy, macronutrient digestibility

During this study, cats were fed to maintain their body weight and body condition score. Average daily food intake across all groups was 58.3 g/d, with food intake being lower ($P < 0.05$) in cats fed ERS than those fed iFPPB, but not different from FPPB (**Table 5.2)**. Caloric intake was lower ($P < 0.05$) in cats fed ERS than those fed FPPB or iFPPB and lower ($P < 0.05$) in cats fed FPPB than those fed iFPPB. Cats fed ERS had slightly lower ($P < 0.05$) body weight compared to those fed FPPB, but not different from iFPPB. Body condition score (BCS) did not differ among dietary treatments. Apparent total tract digestibility of DM, OM, CP, fat, and energy were lower ($P < 0.05$) in cats fed ERS than those fed FPPB or iFPPB (**Table 5.3**). Total dietary fiber digestibility was higher ($P < 0.05$) in cats fed iFPPB than those fed ERS or FPPB. Overall, there were statistical decreases in food intake, body weight, and macronutrient digestibility in cats fed ERS compared with other groups. However, the decrease in body weight had a minimal effect on BCS and no negative health outcomes were observed throughout the study.

Fecal characteristics and metabolites

Fecal characteristics and metabolite concentrations were measured to assess dietary effects on GI tolerance and microbial metabolism. These measurements were strongly affected by diet (**Figure 5.1**). Fecal pH was lower ($P < 0.05$) in cats fed ERS than those fed FPPB or iFPPB (**Table**

5.3). Fecal scores were lower ($P < 0.05$; firmer stools) and fecal DM % were higher ($P < 0.05$) in cats fed iFPPB than those fed ERS. Several fecal metabolites were altered by dietary treatment (**Table 5.3**). Cats fed ERS had lower ($P < 0.05$) fecal propionate, isobutyrate, isovalerate, total phenol and indole, 4-methylphenol, and ammonia concentrations than cats fed FPPB or iFPPB. Fecal butyrate concentrations also differed among treatment groups. Cats fed FPPB and iFPPB had lower ($P < 0.05$) fecal butyrate than cats fed ERS. Fecal total BCFA concentrations were lower ($P < 0.05$) in cats fed ERS than those fed FPPB. Fecal valerate concentrations were lower ($P < 0.05$) in cats fed iFPPB than those fed FPPB. Fecal indole concentrations were lower ($P < 0.05$) in cats fed ERS than those fed iFPPB. Fecal IgA concentrations were lower ($P < 0.05$) in cats fed iFPPB than those fed ERS or FPPB. In summary, fecal characteristics and metabolites were strongly affected by diet, with most of the differences being observed between the ERS diet and FPPB/iFPPB diets.

Immune cell responsiveness to TLR agonists

To assess immune cell responsiveness, TNF-α production was measured from cells stimulated with TLR agonists (**Table 5.4**). TNF-α concentrations of unstimulated control wells were not different among treatment groups. In those stimulated with Poly I:C (TLR3 agonist), TNF-α concentration was higher ($P < 0.05$) in cells from cats fed iFPPB than those fed ERS. In those stimulated with zymosan (TLR2 agonist), TNF-α concentration tended to be higher ($P = 0.06$) in cells from cats fed FPPB or iFPPB than those fed ERS. No differences were observed in cells stimulated with lipopolysaccharide (TLR4 agonist) or R848 (TLR7/8 agonist). Overall, TLR stimulation led to moderate changes in cats fed the test diets.

Serum chemistry profile and blood cell counts

At the end of each experimental period, blood samples were collected for serum chemistry and complete blood cell count analysis to ensure that cats remained healthy throughout the study. Serum metabolites were within reference ranges for all cats, except for glucose, creatinine phosphokinase and sodium to potassium ratio (**Table 5.5**). Glucose was slightly higher than the reference range in cats FPPB or iFPPB, and sodium to potassium ratio was slightly higher in cats fed ERS or iFPPB. Creatinine phosphokinase was higher than the reference range for all treatment groups. Blood urea nitrogen, globulin, and cholesterol concentrations were lower ($P < 0.05$) in cats fed ERS than those fed FPPB or iFPPB. Most of the blood cell counts were within the reference ranges for all cats, except for lymphocytes, monocytes, and eosinophils, which were above the reference ranges for all treatment groups (**Table 5.6**). No statistically significant differences were observed among treatment groups; however, mean cell volume was higher ($P < 0.05$) in cats fed FPPB or iFPPB than those fed ERS. Overall, few serum metabolites and blood counts were affected by diet. Most were within reference ranges and cats remained healthy throughout the study; therefore, these small differences are unlikely to have physiological relevance.

Composition of the feline gut microbiome based on shotgun sequencing

To evaluate the effect of diet on the feline gut microbiome, we performed shotgun sequencing on a total of 33 fecal samples from the 11 cats fed each dietary treatment. After quality control and filtering of low-quality reads, 70,291,067 reads (average 2,130,032 ± 776,926 reads per sample) were aligned to a bacterial database, including a comprehensive list of bacterial reference genomes, and a total of 1,690 bacteria were identified (**Supplementary Figure 1 A-C**).

Three phyla were most prevalent in the gut microbiome of cats regardless of dietary treatment: Actinobacteria, Firmicutes, and Proteobacteria (**Figure 5.2A**). In cats fed ERS, Actinobacteria was the dominant phyla (79% ± 26%), followed by Proteobacteria (13% ± 27%) and Firmicutes (8% ± 7%). The relative abundance of Actinobacteria was higher and that of Firmicutes was lower in cats fed ERS when compared with those fed FPPB or iFPPB (FDR adjusted P = 5.03e-10 and 2.04e-3 respectively). Relative abundance of both Actinobacteria and Firmicutes were intermediate in cats fed FPPB (47% ± 29% and 41% ± 27%, respectively), whereas the relative abundance of Firmicutes was highest (59% ± 18%) and that of Actinobacteria was lowest (30% ± 13%) in cats fed iFPPB. Relative abundance of Proteobacteria remained relatively stable regardless of diet fed (FDR adjusted P = 0.32).

Diet significantly affected α-diversity, as measured by species richness and Shannon's H (P < 0.001, ANOVA). Both measures of α-diversity were lowest in cats fed ERS, intermediate in those fed FPPB, and highest in cats fed iFPPB (P < 0.001, Tukey's HSD test; **Figure 5.2 B; Figure 5.3 A-B**). Neither of the two α-diversity measures nor the gut microbiome composition differed significantly across experimental periods (P = 0.25 and 0.68 for species richness and Shannon's H respectively**,** and P > 0.10, PERMANOVA for composition (**Figure 5.3 C-D**).

Principal coordinates analysis revealed a noticeable shift in the gut microbiome composition among cats fed the three dietary treatments (P = 1e-4, PERMANOVA; **Figure 5.2 C**). The primary principal coordinate axis (PCoA1) was strongly influenced by diet [P = 1.42e-5, Kruskal-Wallis test (KWt)] and it separated cats fed ERS from those fed iFPPB (FDR adjusted P < 1^{e-4}, Dunn's post-hoc test), with those fed FPPB having an intermediate position (FDR adjusted P = 2.5^{e-3}, Dunn's post-hoc test). An association between diet and PCoA2 was not observed (P =

0.14, KWt). Similar results were also observed when using Jensen-Shannon distance to assess bacterial composition (**Figure 5.4 A-D**).

Diet-associated bacterial taxa changes

We used DESeq2 to compare changes in read counts at the species level in cats fed FPPB and/or iFPPB relative to those fed ERS. The relative abundance of 259 bacterial species were different in cats fed FPPB and/or iFPPB compared with those fed ERS (FDR adjusted $P < 0.01$, GLM and absolute log2 fold change >2; **Figure 5.5 A**). A total of 162 species, of which 143 species belonged to the Firmicutes phylum, had a greater abundance in cats fed FPPB and/or iFPPB than those fed ERS, whereas 58 species, of which 26 were from the Actinobacteria phylum, were lower in cats fed FPPB and/or iFPPB than those fed ERS (**Table 5.7 - 5.8**). To test whether the change in abundance was consistent in both FPPB and iFPPB, we compared them individually with ERS and observed that most of the 259 species were also significantly differentially abundant in each comparison (**Figure 5.6 A-B**). Furthermore, only 7 species were significantly different between FPPB and iFPPB (**Figure 5.6 C**). Hierarchical clustering of the relative abundance of the 259 differentially abundant species showed a clear distinction between ERS and FPPB/iFPPB, although the latter did not differentiate into separate clusters (**Figure 5.5 B**).

Of the 259 differentially abundant species, 143 were in the Firmicutes phylum and included 14 species of *Blautia*, 14 species of *Clostridium*, and 5 species of *Lactobacillus* whose relative abundance was increased by FPPB/iFPPB (**Table 5.7**). The FPPB/iFPPB treatments also increased the relative abundances of 13 species in the Bacteroidetes phylum. The Actinobacteria whose relative abundances were depleted by FPPB/iFPPB included 24 species of *Bifidobacterium* (**Table**

5.8). Moreover, some Proteobacteria species belonging to the genera *Campylobacter* and *Helicobacter* also showed depletion in cats fed FPPB/iFPPB relative to those fed ERS.

Composition of the feline microbiota based on 16S rRNA amplicon sequencing

To complement the shotgun analysis and to provide an independent measure of the gut microbiome changes, we performed 16S amplicon sequencing. A total of 4,086,206 high-quality 16S rRNA sequence reads were generated, which were used to calculate α- and β-diversity. Consistent with results from the shotgun data, both α- and β-diversity were different in cats fed ERS compared with those fed FPPB and/or iFPPB. We noted that α-diversity measured using observed OTUs was lower ($P < 0.01$) in cats fed ERS than those fed FPPB and/or iFPPB, while no significant differences in α-diversity were observed between FPPB and iFPPB (**Figure 5.7 A-B**). β-diversity of ERS also differed significantly from FPPB or iFPPB in both unweighted and weighted UniFrac distance measures (**Figure 5.8**).

The three dominant phyla in the shotgun results were also predominant in 16S rRNA results. However, the proportion of phyla varied markedly. In addition to Firmicutes (43.12% to 54.53%), Actinobacteria (12.20% to 40.66%), and Proteobacteria (7.86% to 10.65%), Bacteroidetes (8.03% to 26.24%) were also highly abundant in the 16S results (**Table 5.9**). However, the direction of change at the phyla level was consistent between the two sequencing methods. For example, relative abundances of Bacteroidetes and Firmicutes were lower ($P < 0.05$), while relative abundance of Actinobacteria was higher ($P < 0.05$) in cats fed ERS than those fed FPPB or iFPPB.

The treatment differences were consistent at the genus level as well. Relative abundances of *Bifidobacterium, Lactobacillus, Megasphaera* and *Helicobacter* were higher ($P < 0.05$) and that of *Prevotella, Dialister, Megamonas, Bulleidia,* and *Catenibacterium* were lower ($P < 0.05$) in cats

fed ERS than those fed FPPB or iFPPB. Relative abundances of *Bacteroides, Clostridium, Blautia, Subdoligranulum* and *Eubacterium* all differed ($P < 0.05$) among treatment groups, with cats fed iFPPB having greater levels than those fed FPPB, and cats fed FPPB having greater levels than those fed ERS. Relative abundance of *Collinsella* was higher ($P < 0.05$) and relative abundance of *Mitsuokella* was lower ($P < 0.05$) in cats fed iFPPB than those fed ERS or FPPB. Relative abundance of *Sutterella* was lower ($P < 0.05$) in cats fed ERS than those fed iFPPB. Lastly, the relative abundance of *Succinivibrio* was lower ($P < 0.05$) in cats fed ERS than those fed FPPB.

Bacterial gene abundance, functional modules and enzymes affected by diet

To test the effects of diet on changes in microbial function, we first performed a principal coordinates analysis using the KO terms (**Figure 5.9 A).** The primary principal coordinate axis (PCo1) was not affected by diet ($P = 0.46$, KWt), but PCo2 showed a strong shift due to diet ($P = 1.6^{e-5}$, KWt). Principal coordinate axis (PCo2) scores were higher for FPPB (FDR adjusted $P = 2^{e-3}$, Dunn's post-hoc test) and iFPPB (FDR adjusted $P = 1^{e-4}$, Dunn's post-hoc test) than ERS. However, differences in PCo2 scores between FPPB and iFPPB were marginal ($P = 0.06$). Heatmap representation of the relative abundance of differentially abundant KO terms showed a separate clustering of ERS from iFPPB, while FPPB did not differentiate into a separate cluster (**Figure 5.9 B**), which is consistent with changes in bacterial relative abundances (Figure 3B).

A total of 3,624 KO terms were identified, and after filtering, 212 KO terms were identified as being different among dietary groups using DESeq2. Of these, the relative abundances of 109 KO terms were higher in cats fed ERS compared to those fed FPPB and iFPPB (**Table 5.10**). A total of 103 KO terms had higher abundances in cats fed FPPB or iFPPB relative to ERS (**Table 5.11**). The primary metabolic pathways affected in all dietary treatments were those associated

with carbohydrate metabolism, biosynthesis of amino acids, and metabolism of cofactors and vitamins.

Random forest analysis of shotgun data predicts dietary shifts in bacterial taxonomy

Partition Around Medoids clustering using the top PCo axes (**Figure 5.2**) revealed that cats in this study could be clustered into two clusters. Cluster 1 contained cats fed iFPPB (11 cats) and most of the cats fed FPPB (8 cats), while Cluster 2 represented samples from cats fed ERS (11 cats) and a few from cats fed FPPB (3 cats) (**Figure 5.10**). Furthermore, a random forest classification analysis on shotgun data was able to accurately differentiate the microbial composition between the three diet groups, as ERS and iFPPB cats were predicted as ERS and iFPPB with 100% (11 of 11) and 91% (10 of 11) accuracy, respectively, while the FPPB cats were split between the three groups (3 predicted as ERS, 5 predicted as FPPB, and 3 predicted as iFPPB). These results collectively provide additional evidence demonstrating a clear difference between the gut microbiomes of cats fed ERS and iFPPB.

Diet-microbiome-metabolite relationships

The effects of diet on fecal metabolites showed a separate clustering of cats fed ERS from those fed iFPPB, with those fed FPPB being intermediate but more similar to iFPPB (**Figure 5.11**). Principal component analysis (PCA) indicated that principal component (PC) 1 and 2 explained 24.0% and 12.4% of variability respectively, and PC1 score for ERS was lower ($P < 0.05$) than that of FPPB and iFPPB (**Figure 5.11**). Some of the main metabolites driving the increased PC1 values in FPPB and iFPPB were serum triglyceride concentrations, fecal butyrate concentrations, and fecal scores. On the other hand, fecal pH and fecal ammonia concentrations are some of the

primary factors that decreased PC1 values in ERS. Diet did not have a significant effect on PC2 scores.

Correlations between microbiome measures and metabolites were evaluated using Spearman's rank correlation test (**Figure 5.12 A and B**). The relative abundance of *Bifidobacterium* spp. was negatively correlated ($P < 0.05$) with fecal propionate, isobutyrate, isovalerate, 4-methylphenol, indole, total phenol and indole (total phenols and indoles) and ammonia concentrations, while it was positively correlated ($P < 0.05$) with fecal butyrate concentrations. One exception to these relationships within the *Bifidobacterium* spp. existed, with *Bifidobacterium gallinarum* being negatively correlated ($P < 0.05$) with fecal butyrate concentrations, and positively correlated ($P < 0.05$) with fecal isobutyrate, isovalerate, 4-methylphenol, 3-methylindole, total phenol and indole, and ammonia concentrations. *Bifidobacterium pseudocatenulatum* was also not correlated with any metabolite measured, but *Bifidobacterium sp 12 1 47BFAA* was negatively correlated ($P < 0.05$) with fecal propionate, isobutyrate, isovalerate, indole, total phenol and indole, and ammonia concentrations, and positively correlated ($P < 0.05$) with fecal butyrate concentrations. *Collinsella* spp. were positively correlated ($P < 0.05$) with fecal propionate, isobutyrate, isovalerate, 4-methylphenol and ammonia concentrations, while being negatively correlated ($P < 0.05$) with fecal butyrate concentrations. *Collinsella phocaeensis* was positively correlated ($P < 0.05$) with fecal propionate, isobutyrate, isovalerate, indole and ammonia concentrations. *Collinsella sp 4 8 47FAA* was positively correlated ($P < 0.05$) with fecal propionate, isobutyrate, isovalerate, 4-methylphenol, and ammonia concentrations. *Collinsella tanakaei* was negatively correlated with fecal butyrate and total SCFA concentrations, but positively correlated with fecal isobutyrate, isovalerate, 4-methylphenol and total phenol and indole concentrations. *Slackia piriformis* was positively correlated ($P < 0.05$) with

fecal propionate, isobutyrate, isovalerate, 4-methylphenol, indole, and ammonia concentrations, but negatively correlated ($P < 0.05$) with fecal butyrate concentrations. *Enterococcus faecium* was positively correlated ($P < 0.05$) with fecal isobutyrate, isovalerate, 3 methylindole, total phenol and indole, and ammonia concentrations. *Olsenella scatoligenes* was positively correlated ($P < 0.05$) with fecal valerate and total BCFA concentrations (**Figure 5.12 A**).

Lactobacillus animalis was positively correlated ($P < 0.05$) with fecal propionate, but negatively correlated ($P < 0.05$) with fecal butyrate concentrations. *Streptococcus spp.* were positively correlated ($P < 0.05$) with fecal 3-methylindole concentrations. *Blautia obeum* was positively correlated ($P < 0.05$) with fecal propionate, isobutyrate, and 4-methylphenol concentrations, but negatively correlated ($P < 0.05$) with fecal butyrate concentrations. *Blautia Marseille P3201T* was positively correlated ($P < 0.05$) with fecal isobutyrate, isovalerate and ammonia concentrations. *Blautia wexlerae* was positively correlated ($P < 0.05$) with fecal propionate concentrations. *Ruminococcus gnavus* was positively correlated ($P < 0.05$) with fecal propionate, isobutyrate, isovalerate, indole and ammonia concentrations, but negatively correlated ($P < 0.05$) with fecal butyrate concentrations. *Dorea formicigenerans* was positively correlated ($P < 0.05$) with fecal 4-methylphenol concentrations, while *Fusicatenibacter saccharivorans* was negatively correlated ($P < 0.05$) with butyrate concentrations. *Clostridium hiranonis* was positively ($P < 0.05$) correlated with fecal isobutyrate, isovalerate, indole and ammonia concentrations. *Agathobaculum desmolans* was positively correlated ($P < 0.05$) with fecal propionate, isobutyrate, and 4-methylphenol concentrations, but negatively correlated ($P < 0.05$) with fecal butyrate concentrations. *Subdoligranulum variabile* and *Catenibacterium mitsuokai* were positively correlated ($P < 0.05$) with fecal isobutyrate, isovalerate, 4-methylphenol, total phenol and indole, and ammonia concentrations. In addition, *Subdoligranulum variabile* was

positively correlated ($P < 0.05$) with fecal propionate concentrations, but negatively correlated ($P < 0.05$) with fecal butyrate concentrations. Finally, *Holdemanella biformis* was positively correlated ($P < 0.05$) with fecal isobutyrate and isovalerate concentrations, but negatively correlated ($P < 0.05$) with fecal butyrate concentrations (**Figure 5.12 B**).

Discussion

Functional ingredients targeting gastrointestinal health are increasingly popular dietary inclusions, and may include dietary fibers, prebiotics, probiotics or postbiotics added to commercial pet foods or sold separately as supplements. In addition to GI health, yeast-based ingredients and SDAP are often marketed as improving overall 'immune health.' When considering impacts on potential markers of GI health, many studies have focused on the effects of diet on gut microbiota utilizing 16S rRNA microbial profiling methods. That strategy provides some insights into gut microbial ecology, but often only characterizes bacteria to the genus level. Strategies allowing for a deeper resolution (i.e., to the species level) and the characterization of microbial gene content, which provides more information on functionality and metabolic potential, are needed to expand our understanding of these populations and how they interact with and influence the host. To address these needs, the primary objective of the current study was to characterize and report dietary changes to the fecal metagenome and metabolite profile of healthy adult cats fed diets enriched in RS or dietary fibers and a biotics mixture. Another objective was to identify significant bacterial taxa-bacterial gene-metabolite correlations observed in these cats. Lastly, the effects of diet on the apparent total tract macronutrient digestibility, stool quality and characteristics, fecal fermentative metabolites, and immune indices of the cats were evaluated.

Dietary macronutrient profile (e.g., protein: carbohydrate ratio), dietary fiber amount and type, and the form of food consumed (e.g., raw vs. extruded diets) have been previously shown to alter the feline GI microbiome (Barko et al., 2018; Wernimont et al., 2020). In humans, the dietary protein to carbohydrate ratio quickly and dramatically impacts the GI microbiome (David et al., 2014). Similar dietary shifts alter the GI microbiome and blood metabolome of cats (Hooda et al., 2013; Deng et al., 2014; Deusch et al., 2014). Dietary fibers, prebiotics, and RS also influence stool characteristics and gastrointestinal microbiota in cats (Apanavicius et al., 2007; Barry et al., 2012; Biagi et al., 2013; Shinohara et al., 2020). Yeast-based functional ingredients have been tested in dogs and shown to alter GI microbiota and/or markers of immune function (Swanson et al., 2002; Santos et al., 2017; Lin et al., 2019). SDP is another immune modulator that has been shown to attenuate innate immunity, intestinal barrier function, and reduce intestinal inflammation in rodent models (Pérez-Bosque et al., 2006; Maijó et al., 2012; Pérez-Bosque et al., 2016). Even though SDP is known to be highly palatable and digestible by dogs and cats, it has not been tested for its impact on GI microbiota and immune function in these species (Quigley et al., 2004; Rodriguez et al., 2016).

Non-digestible carbohydrates have been shown to alter aspects of feline metabolism, including altered glucose and amino acid metabolism, and a reduction in uremic toxins (Verbrugghe et al., 2009; Hall et al., 2020a; Hall et al., 2020b). RS is also known to modulate microbiota activity, most notably by increasing butyrate production (McOrist et al., 2011; Peixoto et al., 2018). Although the effects of RS have been extensively studied in humans and rodent models, there are only a few examples in dogs, and its effects on obligate carnivores have not been well studied (Spears et al., 2004; Beloshapka et al., 2014; Peixoto et al., 2018; DeMartino et al., 2020). The consumption of RS has been associated with increased fecal SCFA and is known to

lead to greater butyrate production, including cats (Jackson et al., 2020). This agrees with the increased fecal butyrate observed in cats fed the ERS diet in the current study, which was due to much of the poorly-gelatinized potato starch reaching and being fermented in the colon. In a human study, *Bifidobacterium faecale*/*adolescentis*/*stercoris* and *Ruminococcus bromii* were recognized as being the primary microbial species driving potato starch degradation (Walker et al., 2011; Ze et al., 2012; Venkataraman et al., 2016). The data in the current study agrees, with the relative abundance of *Bifidobacterium adolescentis* and many other *Bifidobacterium* spp. increasing in cats fed the ERS diet compared to the other diet groups. Consumption of RS has also been associated with increased GI IgA concentrations, which are thought to be a butyrate-induced response. This response has been observed in many different mammalian species, including cats in a previous study and those in the current study (Jackson et al., 2020).

In the current study, results of shotgun sequencing were complemented with 16S sequencing data. Over 70 million reads were generated from shotgun sequencing, with over 4 million reads coming from 16S-based sequencing. Both methods showed similar shifts in alpha diversity according to dietary treatment. Although bacterial phyla Actinobacteria, Firmicutes, Proteobacteria, Bacteroidetes and Firmicutes were present in both shotgun and 16S samples, Bacteroidetes and Firmicutes did not predominate in the shotgun dataset like they did in the 16S-based dataset. Such discrepancies in the relative abundance of major bacterial phyla have been reported in human microbiome studies (Ranjan et al., 2016). One possible reason for the discrepancies between sequencing strategies are the primers used to amplify portions of the 16S gene, which could lead to bias in taxonomic representation (Fouhy et al., 2016; Campanaro et al., 2018). Other potential reasons may pertain to the reference databases used, sequence depth, and/or bioinformatics strategies used for gene and taxa annotation.

Although many of the functions of microbial genes and/or activity of bacterial species in the GI tract remain uncharacterized, decades of traditional culture methods and recent microbiome research has shed light on many of the predominant taxa in the GI tract. Firmicutes, for instance, is known to have many carbohydrate fermenters and SCFA producers that ferment dietary fibers and other non-digestible carbohydrates. Many of these bacterial taxa were greater in cats fed the FPPB and iFPPB diets, with corresponding SCFA (acetate; propionate) also being greater. Particularly interesting were the Firmicutes with elevated abundance in the specialized diets of *Lactobacillus* spp. and *Pediococcus* spp., which are known to regulate fermentation and immune responses in mice (Takata et al., 2011). In addition, the relative abundance of *Bacteroides vulgatus*, which has been shown to reduce liposaccharide in the GI tract, was elevated in cats fed the FPPB or iFPPB diets (Yoshida et al., 2018). *Blautia obeum*, which is known to produce lantabiotic peptide, effective against multiple *Clostridium* species and to hydrolyze bile salts, was elevated in cats fed the FPPB or iFPPB diets (Hatziioanou et al., 2017; Gherghisan-Filip et al., 2018; Mullish et al., 2019). In humans, *Holdemanella biformis* is recognized as a tumor-protecting bacteria by reducing tumor cell proliferation, which was reported to be greater in cats fed FPPB or iFPPB diets (Hindson, 2020). The relative abundance of *Olsenella scatoligenes* in the Actinobacteria phylum was depleted in cats fed the FPPB or iFPPB diets relative to ERS. That taxa is known to be a skatole and p-cresol producer (Li et al., 2016). Because fecal phenol and indole concentrations were greater in cats fed FPPB or iFPPB, other bacterial taxa must have contributed to this process.

Enrichment of Actinobacteria, particularly *Bifidobacterium* spp., was observed in the ERS group. *Bifidobacterium* is a commensal bacterium that has been extensively studied in humans for its beneficial effects in treating and preventing GI and immune diseases (Hidalgo-Cantabrana et al., 2017). Though *Bifidobacterium* spp. are carbohydrate fermenters and some (i.e., *B. choerinum*,

B. longum, *B. peseudolongum*, *B. adolescentis*) are known to hydrolyze RS, not all subspecies have that capability. *B. longum* has been extensively studied in human and mice and recognized for its effect in attenuating and preventing inflammatory bowel disease, ulcerative colitis, and Crohn's disease (Jung et al., 2018; Zeng et al., 2018; Jung et al., 2020; Zhang et al., 2021). This bacterial species enhances antioxidant activity to regulate oxidative stress levels, helping attenuate intestinal inflammatory response in mice with experimentally induced colitis model (Abrantes et al., 2020). It also enhances intestinal barrier function by increased epithelial barrier function, and in humans, it has shown to decrease depression scores (Srutkova et al., 2015; Pinto-Sanchez et al., 2017). *Corynebacterium pyruviciproducens* was also enriched in ERS. This member of Actinobacteria is a relatively novel species known as pyruvate producer that has been isolated from different parts of the mammalian body (Tong et al., 2010). *In vitro* studies using human cells have suggested that it acts as an immunoadjuvant by promoting a humoral immune response to pathogens (Tong et al., 2012). *Campylobacter* spp., *Helicobacter* spp. and *Pseudomonas* spp. were enriched in the ERS group, which may have been due to greater protein fermentation in the gut. In many different mammalian species, these Proteobacteria members are linked with infectious GI diseases (Melito et al., 2001). Because many of these strains have been isolated from feces of healthy dogs and cats, their presence does not guarantee disease, but may serve as opportunistic pathogens for the pets or their owners (Stanley et al., 1992; Wieland et al., 2005). Many of these bacterial species have been characterized in culture or in other host species but have been poorly studied in cats. Therefore, more studies dedicated to understanding how changes in specific microbial populations affect the health of cats are needed.

Although KO term abundances were different among cats fed ERS, FPPB, and iFPPB diets, all diets had an enrichment of genes associated with carbohydrate metabolism, biosynthesis of

amino acids, and metabolism of cofactors and vitamins. These results show that although changes in the bacterial community may occur and they may be somewhat predictable between ERS, FPPB, and iFPPB diets, there is a large overlap in terms of functional capacity and metabolic potential. Given the complex nature of the test diets in this study, with each containing different amounts and/or sources of dietary fiber, probiotic, postbiotic and RS, it would be difficult to attribute changes at the KO term level to any of the ingredients. Future studies that test diets differing in a single or a small number of ingredients/nutrients would be needed to make such connections.

In addition to changes in the microbial community, physiological outcomes and immune responses were also measured. All hematological measurements were within ranges of healthy adult cats, with only blood urea nitrogen and cholesterol being lower in cats fed the ERS diet than those fed the FPPB or iFPPB diets which is of questionable clinical significance. The lower blood cholesterol concentrations in ERS-fed cats agrees with other research demonstrating blood-lipid lowering effect of RS in mice fed high-fat diets (Zhou et al., 2019). RS has also been shown to exert a blood urea-lowering effect by enhancing urea nitrogen transfer in rats (Younes et al., 1995). From the measured immune responses, only cells stimulated with Poly I:C were different among groups, with TNF-α being elevated in cells from cats fed the iFPPB diet compared to those coming from cats fed the ERS diet. Poly I:C is a TLR3 agonist, which recognizes dsRNA of viral origin. More research is needed to determine whether the functional ingredients contained in the iFPPB diet enhances response to viral challenge. Overall, small physiological changes were observed throughout the study and all cats remained healthy.

In summary, this study demonstrated that diets containing different dietary fibers and biotic ingredients affect the fecal microbial diversity, metabolite composition, and microbial gene content in cats. By utilizing different sequencing tools, including 16S rRNA- and shotgun-based

sequencing, we identified striking differences among diets fed. Although the sequencing strategies and their resolution of the microbiota populations differed, both reported similar changes in alpha diversity and bacterial taxa shifts due to diet. As expected, increased fecal butyrate and IgA concentrations, but reduced fecal BCFA concentrations were observed in cats fed the high-RS diet. To our knowledge, this is one of the first studies utilizing shotgun sequencing technology to study the metagenome of the feline GI tract. However, the differences in protein content as well as its sources and the changes in several ingredients in the different dietary treatments added another level of complexity in comparing the dietary treatments. Future studies should evaluate diets that are isonitrogenous with fewer macronutrient and ingredient differences so that specific taxa/gene responses may be identified.

Tables and figures

Table 5.1. Ingredient and analyzed nutrient composition of the experimental diets fed to cats

Ingredient	ERS[1]	FPPB	iFPPB
	---------- %, as-is ----------		
Potato flour	21.00	---	---
Chicken meal (regular ash)	20.00	---	---
Menhaden fish meal	---	20.00	20.00
Salmon slurry	20.00	20.00	20.00
Brewers rice	20.58	---	---
Oat groats	---	20.00	16.66
Dried green peas	---	12.91	12.00
Chicken fat (with 5000 ppm Naturox)	9.11	6.00	6.00
Dried egg product	---	5.00	5.00
Dried plain beet pulp	---	3.00	4.00
Flaxseed meal	---	3.00	3.00
Pea Fiber	---	3.00	3.00
Spray dried plasma[2]	---	---	3.00
Probiotic[3]	---	2.30	2.30
Dried salmon	5.00	2.00	2.00
Cat diet palatant[4]	2.00	---	---
Dried brewer's yeast	0.92	0.92	0.92
Menhaden fish oil	---	0.50	0.50
Vitamin premix	0.35	0.35	0.35
Yeast fermentation product[5]	---	---	0.25
Sodium hexametaphosphate	0.20	0.20	0.20
Taurine	0.20	0.20	0.20
Potassium chloride	0.19	0.19	0.19
Inulin	---	0.10	0.10
Mineral premix	0.10	0.10	0.10
Choline chloride (70%, dry)	0.10	0.10	0.10
Dry antioxidant	0.05	0.05	0.05
Vitamin E (50 %, dry)	---	0.03	0.03
Turmeric powder	---	0.03	0.03
Stay C-35 (ascorbic acid)	---	0.03	0.03
Chemical composition			
Dry matter, %	90.6	94.2	95.9
	---------- %, DM ----------		
Crude protein	21.9	28.5	31.6
Acid hydrolyzed fat	9.0	14.1	16.9
Ash	6.3	8.3	8.5
Total dietary fiber	11.2	12.6	16.7
Insoluble fiber	6.1	9.4	11.4
Soluble fiber	5.1	3.2	5.3
Metabolizable energy, kcal/g	3.3	3.5	3.5

Table 5.1. (Continued)

Total starch	50.4	29.9	22.7
Gelatinized starch	35.7	26.3	21.7

[1]Diets enriched in resistant starch (ERS), a fiber-prebiotic-probiotic blend (FPPB), or a fiber-prebiotic-probiotic blend + immuno-modulating ingredients (iFPPB).
[2]Hydrolyzed AP 700 (APC Inc., Ankeny, IA).
[3]LACTO-SACC (Alltech, Nicholasville, KY): yeast culture (live *Saccharomyces cerevisiae* grown on media of ground yellow corn, diastatic malt and cane molasses), dried *Lactobacillus acidophilus* fermentation product, dried *Enterococcus faecium* fermentation product, dried *Aspergillus oryzae* fermentation extract, dried *Trichoderma longibrachiatum* fermentation extract, and dried *Bacillus subtilis* fermentation extract.
[4]AFB international (St. Charles, MO).
[5]Diamond V (Cedar Rapids, IA).

Table 5.2. Body weight, BCS, and food intake of cats fed the experimental diets

	Dietary treatment				*P*-value	
	ERS[1]	**FPPB**	**iFPPB**	SEM	Treatment	ERS vs. FPPB and iFPPB
Body weight, kg	3.78[a]	3.87[b]	3.83[ab]	0.29	0.0025	0.0014
Body condition score[2]	6.4	6.5	6.6	0.49	0.4557	0.3609
Daily intake, g/day	53.6[a]	57.9[ab]	61.6[b]	3.52	0.0010	0.0008
Daily intake, kcal ME/day	199.7[a]	227.0[b]	249.5[c]	13.84	<0.0001	<0.0001

[1] Diets enriched in resistant starch (ERS), a fiber-prebiotic-probiotic blend (FPPB), or a fiber-prebiotic-probiotic blend + immuno-modulating ingredients (iFPPB).
[2] Nine-point body condition score system was used (Laflamme, 1997).

Table 5.3. Feline fecal characteristics and metabolite concentrations and apparent total tract nutrient digestibility of experimental diets

	Dietary treatment			SEM	*P*-value	
	ERS[1]	**FPPB**	**iFPPB**		**Treatment**	**ERS vs. FPPB and iFPPB**
Characteristics						
pH	4.87[a]	5.63[b]	5.97[b]	0.12	<.0001	<.0001
Fecal score[2]	3.6[b]	3.2[ab]	2.7[a]	0.20	0.0001	0.0002
Fecal DM (%)	25.6[a]	28.2[ab]	31.6[b]	1.26	0.0008	0.0013
Digestibility						
Dry matter	72.0[a]	78.4[b]	76.9[b]	1.38	< 0.01	< 0.01
Organic matter	73.8[a]	82.2[b]	81.4[b]	1.305	<.0001	<.0001
Crude protein	68.0[a]	80.3[b]	83.3[b]	1.295	<.0001	<.0001
Acid-hydrolyzed fat	87.7[a]	90.0[b]	91.4[b]	1.076	< 0.01	< 0.01
Total dietary fiber	30.4[a]	30.0[a]	39.3[b]	2.918	0.05	0.25
Energy	74.3[a]	83.1[b]	83.1[b]	1.270	<.0001	<.0001
Metabolites	--------- µmol/g DM ---------					
Total SCFA	554.1	602.6	529.2	35.66	0.3583	0.7859
Acetate	290.0	353.0	329.1	23.36	0.1101	0.0498
Propionate	51.9[a]	125.1[b]	139.8[b]	6.69	<.0001	<.0001
Butyrate	212.2[c]	124.5[b]	60.3[a]	18.40	<.0001	<.0001
Total BCFA	36.3[a]	50.5[b]	41.0[ab]	3.77	0.0417	0.0478
Isobutyrate	1.9[a]	4.5[b]	5.8[b]	0.49	<.0001	<.0001
Isovalerate	3.7[a]	6.6[b]	8.5[b]	0.70	0.0002	0.0001
Valerate	30.7[ab]	39.5[b]	26.8[a]	3.36	0.0370	0.5508
Total P/I[3]	0.05[a]	1.18[b]	1.42[b]	0.21	<.0001	<.0001
4-methylphenol	0.05[a]	0.54[b]	0.89[b]	0.11	<.0001	<.0001
Indole	0.00[a]	0.06[ab]	0.21[b]	0.05	0.0090	0.0254
Ammonia	65.1[a]	105.6[b]	127.3[b]	8.31	<.0001	<.0001
Fecal IgA, mg/g	16.8[b]	13.9[b]	7.9[a]	1.59	0.0002	0.0010

[1]Diets enriched in resistant starch (ERS), a fiber-prebiotic-probiotic blend (FPPB), or a fiber-prebiotic-probiotic blend + immuno-modulating ingredients (iFPPB).

[2]Fecal score: 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; 5 = watery, liquid that can be poured.

[3]P/I = phenols + indoles.

0

Table 5.4. TNF-α concentrations (ng/L) of cell culture supernatants from cats fed the experimental diets

	Dietary treatment				*P*-value	
	ERS[1]	FPPB	iFPPB	SEM	Treatment	ERS vs. FPPB and iFPPB
Control	18.09	16.97	17.56	0.68	0.51	0.33
Zymosan	14.36	17.18	15.42	0.81	0.06	0.06
Poly (I:C)[2]	17.89[a]	19.79[ab]	20.42[b]	0.71	0.05	0.02
Lipopolysaccharide	18.15	18.12	20.62	0.84	0.07	0.25
R848 (requisimod)	15.96	17.84	18.02	1.09	0.35	0.15

[1]Diets enriched in resistant starch (ERS), a fiber-prebiotic-probiotic blend (FPPB), or a fiber-prebiotic-probiotic blend + immuno-modulating ingredients (iFPPB).
[2]Poly (I:C): polyinosinic:polycytidylic acid.

Table 5.5. Serum chemistry profiles of cats fed the experimental diets

Item	Reference ranges[2]	Dietary treatment			SEM	*P*-value	
		ERS[1]	FPPB	iFPPB		Treatment	ERS vs. FPPB and iFPPB
Creatinine, mg/dL	0.4-1.6	1.06	1.07	1.01	0.068	0.5524	0.6923
BUN[3], mg/dL	18-38	18.53[a]	20.85[b]	21.02[b]	1.304	0.0165	0.0048
Total protein, g/dL	5.8-8.0	6.73	7.04	7.03	0.149	0.1350	0.0485
Albumin, g/dL	2.8-4.1	2.93	2.95	2.94	0.088	0.7891	3.1021
Globulin, g/dL	2.6-5.1	3.81	4.09	4.09	0.123	0.0518	0.0166
Albumin:globulin	0.6-1.1	0.77	0.72	0.74	0.032	0.1587	0.0680
Calcium, mg/dL	8.8-10.2	9.12	9.05	8.98	0.117	0.2541	0.1477
Phosphorus, mg/dL	3.2-5.3	7.99	5.17	4.97	2.212	0.5325	0.2682
Sodium, mmol/L	145-157	149.38	149.09	148.18	0.595	0.3423	0.2995
Potassium, mmol/L	3.6-5.3	4.08	4.29	4.07	0.092	0.1842	0.3467
Sodium:potassium	28-36	37.00	34.82	36.64	0.902	0.1705	0.2146
Chloride, mmol/L	109-126	115.75	115.11	115.58	0.571	0.6743	0.5073
Glucose, mg/dL	60-122	107.33	127.34	130.25	17.060	0.4437	0.2313
ALP[3], U/L	10-85	23.89	18.09	27.99	5.401	0.1305	0.1524
ALT[3], U/L	14-71	56.31	64.17	56.31	6.353	0.0727	0.1793
Total bilirubin, mg/dL	0.0-0.3	0.10	0.11	0.099	0.005	0.3608	0.4470
CPK[3], U/L	10-250	421.38	314.45	403.55	109.263	0.8998	0.9842
Cholesterol, mg/dL	66-160	104.39[a]	133.10[b]	143.74[b]	8.819	<.0001	<.0001
Triglycerides, mg/dL	21-166	31.68	28.83	25.49	2.191	0.0679	0.0459

[1]Diets enriched in resistant starch (HRS), a fiber-prebiotic-probiotic blend (FPPB), or a fiber-prebiotic-probiotic blend + immuno-modulating ingredients (iFPPB).

[2]Reference ranges were provided from the University of Illinois Veterinary Diagnostic Laboratory.

[3]BUN: blood urea nitrogen; ALP: total alkaline phosphatase; ALT: alanine aminotransferase; CPK: creatinine phosphokinase.

Table 5.6. Hematology of cats fed the experimental diets

Item	Reference ranges[1]	Dietary treatment			SEM	*P*-value	
		ERS[1]	FPPB	iFPPB		Treatment	ERS vs. FPPB and iFPPB
Red blood cell	5.0-10.0	8.06	8.28	7.70	0.290	0.2938	0.8269
Hemoglobin	8.0-15.0	10.96	11.46	10.71	0.474	0.4220	0.8071
Hematocrit	30.0-45.0	32.03	33.40	31.19	1.358	0.3923	0.8513
Mean cell volume	37.0-55.0	39.64	40.33	40.38	0.658	0.0933	0.0348
White blood cell, 10^6/µL	5.5-19.5	12.94	13.37	11.88	1.051	0.2191	0.6925
Lymphocyte, 10^3/µL	1.7-7.0	2.15	2.41	2.07	0.356	0.6462	0.7711
Monocyte, 10^3/µL	0.0-0.9	0.34	0.39	0.39	0.079	0.8790	0.6191
Eosinophil, 10^3/µL	0.0-0.8	0.60	0.74	0.73	0.151	0.7323	0.4352
MCH[3], pg	13.0-18.0	13.55	13.83	13.85	0.211	0.2280	0.0941
MCHC[3], g/dL	29.0-38.0	34.23	34.32	34.29	0.255	0.9493	0.7564
Platelets, 10^3/µL	300-700	353.27	293.70	350.46	70.371	0.6783	0.6787

[1]Diets enriched in resistant starch (HRS), a fiber-prebiotic-probiotic blend (FPPB), or a fiber-prebiotic-probiotic blend + immuno-modulating ingredients (iFPPB).
[2]Reference ranges were provided from the University of Illinois Veterinary Diagnostic Laboratory.
[3]MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration.

Table 5.7. Bacterial species that were greater in feces of cats fed FPPB or iFPPB than those fed ERS (based on shotgun sequencing)

Phylum	Genus	Species	Log 2 FC	Adjusted *P*
Actinobacteria	***Senegalimassilia***	*Senegalimassilia_anaerobia*	4.1	4.0E-11
	Actinomyces	*Actinomyces_sp._oral_taxon_877*	3.2	1.3E-03
	Slackia	*Slackia_piriformis*	2.7	6.9E-10
	Alloscardovia	*Alloscardovia_sp._HMSC034E08*	2.6	5.1E-03
Bacteroidetes	***Alistipes***	*Alistipes_putredinis*	8.2	2.5E-04
	Bacteroides	*Bacteroides_vulgatus*	7.0	2.6E-10
		Bacteroides_stercoris	6.7	1.1E-10
		Bacteroides_sp._4_3_47FAA	6.7	1.2E-04
		unspecified species	6.1	1.5E-09
		Bacteroides_dorei	5.4	6.5E-04
		Bacteroides_sp._HMSC068A09	4.8	1.7E-03
		Bacteroides_ovatus	4.4	3.0E-03
		Bacteroides_thetaiotaomicron	4.4	5.5E-03
	Parabacteroides	*Parabacteroides_merdae*	6.5	1.9E-06
		unspecified species	5.7	2.2E-04
		Parabacteroides_distasonis	4.4	2.4E-03
	Odoribacter	*Odoribacter_laneus*	4.1	1.1E-03
Firmicutes	***Aerococcus***	*Aerococcus_urinaeequi*	25.7	1.8E-16
		unspecified species	23.1	1.7E-18
		Aerococcus_viridans	8.0	3.4E-04
	Acetivibrio	*Acetivibrio_ethanolgignens*	3.8	4.9E-11
	Absiella	*Absiella_dolichum*	2.3	1.8E-03
	Anaeromassilibacillus	*Anaeromassilibacillus_sp._Marseille-P3371*	3.9	2.4E-08
	Anaerosporobacter	*Anaerosporobacter_mobilis*	2.3	1.5E-03
	Agathobaculum	*Agathobaculum_desmolans*	2.2	1.5E-03
	Anaerotruncus	*Anaerotruncus_sp._G3(2012)*	3.9	5.2E-06
		Anaerotruncus_colihominis	3.0	1.2E-07
	Angelakisella	*Angelakisella_massiliensis*	3.6	4.0E-11

Table 5.7. (Continued)

Anaerotignum	*Anaerotignum_lactatifermentans*	2.6	6.9E-04
Anaerostipes	*Anaerostipes_hadrus*	2.3	5.8E-04
	Anaerostipes_sp._3_2_56FAA	2.7	5.5E-06
Bacillus	*Bacillus_coagulans*	4.6	2.3E-08
	Bacillus_smithii	2.6	7.4E-03
Bariatricus	*Bariatricus_massiliensis*	4.4	2.0E-04
Bittarella	*Bittarella_massiliensis*	3.9	5.6E-04
Blautia	*[Ruminococcus]_torques*	5.8	4.5E-08
	Blautia_hydrogenotrophica	5.1	1.5E-08
	unspecified species	4.1	9.2E-11
	Blautia_wexlerae	4.1	6.1E-11
	Blautia_sp._Marseille-P2398	4.1	2.5E-15
	Blautia_obeum	3.9	1.1E-13
	Blautia_schinkii	3.4	1.3E-12
	Blautia_sp._Marseille-P3087	3.4	9.7E-13
	Blautia_sp._Marseille-P3201T	3.2	9.2E-06
	[Ruminococcus]_gnavus	2.9	1.4E-09
	Blautia_producta	2.8	5.0E-10
	Blautia_sp._SF-50	2.7	5.2E-06
	Blautia_hansenii	2.6	2.0E-06
	Blautia_sp._KLE_1732	2.5	1.0E-03
Butyricicoccus	*Butyricicoccus_pullicaecorum*	5.5	6.4E-12
Butyrivibrio	*Butyrivibrio_crossotus*	3.3	4.4E-03
Catonella	*Catonella_morbi*	5.0	2.6E-06
Clostridioides	*Clostridioides_difficile*	2.1	4.2E-04
Clostridium	*Clostridium_sp._ATCC_BAA-442*	4.0	2.4E-05
	Clostridium_sp._ASF502	4.0	2.6E-07
	Clostridium_sp._KLE_1755	3.8	2.3E-04
	Clostridium_sp._M62/1	3.6	2.1E-06
	Clostridium_sp._AT4	3.5	2.1E-13
	Clostridium_sp._SS2/1	3.2	7.9E-06

Table 5.7. (Continued)

	Clostridium_sp._ATCC_29733	3.0	5.3E-03
	Clostridium_cochlearium	3.0	1.1E-03
	Clostridium_novyi	2.8	3.0E-03
	Clostridium_sp._HMSC19A11	2.6	7.1E-08
	Clostridium_polynesiense	2.6	8.3E-04
	Clostridium_sp._Marseille-P3244	2.5	6.9E-03
	Clostridium_sp._SN20	2.4	1.1E-03
	Clostridium_phoceensis	2.2	1.1E-03
Coprobacillus	*Coprobacillus_sp._D7*	3.1	5.6E-07
	Coprobacillus_sp._8_2_54BFAA	2.3	1.0E-04
	Coprobacillus_sp._29_1	2.3	9.6E-05
	Coprobacillus_sp._3_3_56FAA	2.1	2.4E-03
Coprococcus	*Coprococcus_eutactus*	2.5	1.3E-09
	Coprococcus_sp._HPP0048	2.4	4.2E-09
Dorea	*Dorea_sp._5-2*	3.1	9.3E-07
	Dorea_longicatena	2.1	3.8E-09
Eisenbergiella	*Eisenbergiella_tayi*	3.5	6.3E-06
Emergencia	*Emergencia_timonensis*	3.3	6.5E-07
Enterococcus	*Enterococcus_sp._3G6_DIV0642*	18.8	2.9E-08
	Enterococcus_sp._HMSC069A01	5.3	1.6E-04
	Enterococcus_sp._HMSC065H03	4.9	5.1E-03
	Enterococcus_gilvus	3.9	7.0E-03
	Enterococcus_sp._10F3_DIV0382	3.8	6.7E-03
	Enterococcus_gallinarum	3.5	9.4E-03
Eubacterium	*Eubacterium_plexicaudatum*	4.5	1.9E-07
	unspecified species	4.4	8.4E-07
	Eubacterium_limosum	3.4	3.7E-09
	Eubacterium_ventriosum	3.1	2.9E-07
	Eubacterium_sp._YI	3.0	2.4E-03
	Eubacterium_sp._3_1_31	3.0	1.8E-04
	[Eubacterium]_hallii	2.8	6.1E-11

Table 5.7. (Continued)

Faecalicatena	*Faecalicatena_contorta*	2.1	2.8E-04
Faecalicoccus	*Faecalicoccus_pleomorphus*	3.2	5.1E-10
Faecalitalea	*Faecalitalea_cylindroides*	4.3	4.2E-08
Flavonifractor	*Flavonifractor_plautii*	3.1	5.5E-08
Fournierella	*Fournierella_massiliensis*	4.2	9.2E-18
Fusicatenibacter	*Fusicatenibacter_sp._2789STDY5834925*	4.6	1.1E-07
Gemmiger	*Gemmiger_formicilis*	3.2	1.5E-09
Geobacillus	*unspecified species*	2.7	2.3E-03
Geobacillus	*Geobacillus_sp._WCH70*	2.6	4.9E-03
Hespellia	*Hespellia_stercorisuis*	2.9	4.3E-05
Holdemanella	*Holdemanella_biformis*	5.5	1.6E-19
Holdemania	*Holdemania_sp._Marseille-P2844*	2.9	8.1E-04
	Holdemania_massiliensis	2.5	1.0E-05
	Holdemania_filiformis	2.4	1.8E-03
Hungatella	*Hungatella_hathewayi*	2.5	2.6E-07
Ileibacterium	*Ileibacterium_massiliense*	2.4	7.8E-03
Intestinimonas	*Intestinimonas_butyriciproducens*	3.9	3.4E-05
	Intestinimonas_massiliensis	3.4	4.2E-04
Lachnoclostridium	*Lachnoclostridium_sp._YL32*	4.6	2.4E-07
	[Clostridium]_saccharolyticum	4.0	1.7E-09
	[Clostridium]_lavalense	3.9	1.4E-04
	unspecified species	3.5	2.2E-15
	Lachnoclostridium_phocaeense	3.4	9.7E-08
	[Clostridium]_glycyrrhizinilyticum	2.8	7.6E-08
	[Clostridium]_bolteae	2.8	1.0E-10
	[Clostridium]_clostridioforme	2.7	4.2E-12
	[Clostridium]_symbiosum	2.7	9.6E-05
	[Clostridium]_scindens	2.0	1.1E-04
Lachnospira	*Lachnospira_pectinoschiza*	3.8	3.3E-04
Lactobacillus	*Lactobacillus_animalis*	8.5	1.1E-11
	Lactobacillus_murinus	8.3	6.5E-10

Table 5.7. (Continued)

	unspecified species	5.8	1.1E-09
	Lactobacillus_rogosae	3.5	4.7E-04
	Lactobacillus_aviarius	2.9	2.6E-03
Leuconostoc	*Leuconostoc_citreum*	5.3	7.4E-03
Mageeibacillus	*Mageeibacillus_indolicus*	3.4	7.8E-03
Massilioclostridium	*Massilioclostridium_coli*	3.0	1.1E-03
Merdimonas	*Merdimonas_faecis*	3.5	7.8E-11
Mordavella	*Mordavella_sp._Marseille-P3756*	3.1	3.5E-08
Murdochiella	*Murdochiella_vaginalis*	3.1	2.4E-03
Negativibacillus	*Negativibacillus_massiliensis*	4.0	1.8E-12
Neglecta	*Neglecta_timonensis*	2.5	1.2E-03
Oscillibacter	*unspecified species*	6.0	1.0E-07
Pediococcus	*Pediococcus_acidilactici*	7.4	8.7E-09
	Pediococcus_inopinatus	3.5	6.3E-04
	Pediococcus_damnosus	3.2	2.7E-04
Phocea	*Phocea_massiliensis*	4.9	6.8E-05
Provencibacterium	*Provencibacterium_massiliense*	2.8	2.3E-07
Pseudoflavonifractor	*Pseudoflavonifractor_capillosus*	3.8	1.8E-12
	Pseudoflavonifractor_sp._Marseille-P3106	3.3	4.5E-04
Roseburia	*Roseburia_hominis*	4.2	4.2E-11
	Roseburia_inulinivorans	2.9	6.3E-12
Ruminiclostridium	*[Clostridium]_leptum*	4.1	1.6E-04
Ruminococcus	*Ruminococcus_faecis*	6.7	3.5E-07
	Ruminococcus_bicirculans	3.6	1.1E-04
	Ruminococcus_sp._AT10	2.8	5.9E-15
	Ruminococcus_lactaris	2.3	4.3E-07
Ruthenibacterium	*Ruthenibacterium_lactatiformans*	2.9	1.4E-04
Sharpea	*Sharpea_azabuensis*	2.4	4.9E-03
Solobacterium	*Solobacterium_moorei*	4.6	5.1E-14
Streptococcus	*Streptococcus_canis*	6.1	1.6E-03

Table 5.7. (Continued)

		Streptococcus_entericus	5.2	1.1E-03
		Streptococcus_agalactiae	5.0	3.4E-07
		Streptococcus_parauberis	4.1	6.1E-08
		Streptococcus_parasanguinis	3.7	2.8E-07
		Streptococcus_mitis	3.6	9.3E-03
		Streptococcus_intermedius	3.4	9.3E-03
	Subdoligranulum	*Subdoligranulum_sp._4_3_54A2FAA*	3.5	1.5E-05
		Subdoligranulum_variabile	3.2	6.4E-16
	Traorella	*Traorella_massiliensis*	3.2	1.2E-04
Proteobacteria	***Escherichia***	*unspecified species*	3.8	3.5E-03
Tenericutes	***Mycoplasma***	*Mycoplasma_hominis*	3.9	4.9E-03

Table 5.8. Bacterial species that were lower in feces of cats fed FPPB or iFPPB than those fed ERS (based on shotgun sequencing)

Phylum	Genus	Species	Log 2 FC	Adjusted *P*
Actinobacteria	***Actinomyces***	*Actinomyces_europaeus*	-3.3	1.4E-07
	Atopobium	*Atopobium_minutum*	-2.2	8.3E-04
	Bifidobacterium	*Bifidobacterium_pseudolongum*	-2.0	7.3E-04
		Bifidobacterium_callitrichos	-2.5	2.6E-07
		Bifidobacterium_stellenboschense	-2.5	4.1E-06
		Bifidobacterium_choerinum	-2.8	3.0E-13
		Bifidobacterium_sp._TRE_H	-3.1	1.7E-05
		Bifidobacterium_saguini	-3.2	3.0E-08
		Bifidobacterium_sp._12_1_47BFAA	-3.3	2.7E-08
		Bifidobacterium_scardovii	-3.4	8.8E-09
		Bifidobacterium_boum	-3.4	3.6E-07
		Bifidobacterium_catenulatum	-3.5	4.5E-09
		Bifidobacterium_longum	-3.5	4.1E-09
		Bifidobacterium_sp._TRE_D	-3.6	1.5E-08
		Bifidobacterium_aesculapii	-3.6	4.2E-12
		Bifidobacterium_dentium	-3.6	9.4E-12
		Bifidobacterium_sp._AGR2158	-3.7	1.8E-05
		Bifidobacterium_sp._TRE_1	-3.7	7.2E-10
		Bifidobacterium_reuteri	-3.7	5.0E-08
		Bifidobacterium_bifidum	-3.8	9.2E-15
		Bifidobacterium_angulatum	-3.8	2.8E-12
		Bifidobacterium_breve	-3.9	1.5E-11
		Bifidobacterium_kashiwanohense	-3.9	7.1E-12
		Bifidobacterium_cuniculi	-4.6	4.0E-24
		Bifidobacterium_adolescentis	-4.7	2.9E-14
		Bifidobacterium_thermophilum	-5.9	2.4E-23
	Corynebacterium	*Corynebacterium_pyruviciproducens*	-2.1	1.4E-06
	Olsenella	*Olsenella_profusa*	-2.1	4.8E-05
		Olsenella_sp._KH1P3	-2.5	8.6E-03

Table 5.8. (Continued)

		Olsenella_sp._kh2p3	-2.9	1.5E-05
		Olsenella_scatoligenes	-2.9	1.5E-04
		Olsenella_sp._KH3B4	-3.0	5.1E-06
		Olsenella_sp._Marseille-P2300	-3.3	5.4E-06
		Olsenella_umbonata	-3.8	1.3E-07
	Rhodococcus	*unspecified species*	-2.9	5.1E-06
Firmicutes	***Acidaminococcus***	*Acidaminococcus_intestini*	-4.6	2.2E-06
	Anaerocolumna	*Anaerocolumna_jejuensis*	-6.9	2.1E-19
	Clostridium	*Clostridium_sp._7_3_54FAA*	-3.3	4.7E-12
		Clostridium_puniceum	-4.3	7.2E-04
	Eubacterium	*Eubacterium_pyruvativorans*	-5.3	1.2E-04
	Fontibacillus	*Fontibacillus_panacisegetis*	-4.9	4.3E-09
	Lachnoclostridium	*[Clostridium]_aerotolerans*	-5.9	9.8E-13
	Lactobacillus	*Lactobacillus_agilis*	-3.1	8.2E-03
		Lactobacillus_acetotolerans	-4.1	3.2E-03
		Lactobacillus_ruminis	-5.0	6.9E-07
	Levyella	*Levyella_massiliensis*	-2.5	1.9E-04
	Mitsuokella	*Mitsuokella_jalaludinii*	-4.0	5.3E-03
		Mitsuokella_multacida	-6.3	3.1E-06
	Mobilibacterium	*Mobilibacterium_timonense*	-5.6	1.4E-04
	Mogibacterium	*Mogibacterium_timidum*	-8.1	5.4E-04
	Paenibacillus	*unspecified species*	-4.4	2.1E-13
		Paenibacillus_xylanexedens	-4.5	4.3E-06
	Pelosinus	*Pelosinus_fermentans*	-5.1	1.6E-11
	Selenomonas	*Selenomonas_bovis*	-4.1	3.8E-04
Proteobacteria	***Campylobacter***	*Campylobacter_upsaliensis*	-4.0	5.4E-04
		Campylobacter_helveticus	-4.2	3.7E-04
	Helicobacter	*Helicobacter_winghamensis*	-4.3	2.5E-03
	Pseudomonas	*Pseudomonas_putida*	-2.5	4.9E-03

Table 5.9. Fecal bacterial phyla and genera (% of total sequences) affected by diet as evaluated using 16S rRNA sequencing

		Dietary treatment				*P*-value	
Phyla	Genus	HRS[1]	FPPB	iFPPB	SEM	Treatment	HRS vs. FPPB and iFPPB
Actinobacteria		40.66[b]	13.66[a]	12.20[a]	2.10	<.0001	<.0001
	Bifidobacterium	23.38[b]	6.91[a]	4.65[a]	1.496	<.0001	<.0001
	Collinsella	1.92[a]	3.39[a]	5.28[b]	0.582	< 0.01	< 0.01
Bacteroidetes		8.03[a]	21.06[b]	26.24[b]	1.898	<.0001	<.0001
	Bacteroides	0.34[a]	2.02[b]	3.98[c]	0.351	<.0001	<.0001
	Prevotella	7.66[a]	18.05[b]	20.39[b]	1.528	<.0001	<.0001
Firmicutes		43.12[a]	54.53[b]	53.16[b]	2.43	< 0.01	< 0.01
	Enterococcus	0.12	0.29	2.33	1.178	0.46	0.24
	Lactobacillus	6.37[b]	2.48[a]	1.58[a]	1.317	< 0.01	< 0.01
	Streptococcus	0.19	7.61	1.25	2.346	0.13	0.29
	Clostridium	0.95[a]	1.48[b]	2.64[c]	0.264	< 0.01	< 0.01
	Blautia	0.38[a]	3.5[b]	5.87[c]	0.544	<.0001	<.0001
	Ruminococcus	0.23	1.12	1.51	0.197	<.0001	<.0001
	Subdoligranulum	0.12[a]	1.09[b]	1.49[c]	0.135	<.0001	<.0001
	Acidaminococcus	2.29	1.82	1.70	0.221	0.13	0.05
	Dialister	1.76[a]	4.28[b]	4.77[b]	0.396	<.0001	<.0001
	Megamonas	0.43[a]	3.43[b]	3.69[b]	0.445	<.0001	<.0001
	Megasphaera	24.35[b]	11.65[a]	8.61[a]	1.046	<.0001	<.0001
	Mitsuokella	2.93[b]	2.14[b]	0.41[a]	0.633	0.01	0.01
	Bulleidia	0.08[a]	2.82[b]	3.09[b]	0.374	<.0001	<.0001
	Catenibacterium	0.54[a]	3.81[b]	3.82[b]	0.605	< 0.01	<.0001
	Eubacterium	0.28[a]	1.10[b]	2.18[c]	0.251	<.0001	<.0001
Proteobacteria		8.15	10.65	7.86	2.08	0.58	0.66
	Sutterella	1.10[a]	1.38[ab]	2.18[b]	0.334	0.04	0.07
	Helicobacter	1.31[b]	0.33[a]	0.39[a]	0.225	< 0.01	< 0.01
	Anaerobiospirillum	1.72	1.04	1.79	0.414	0.30	0.49
	Succinivibrio	0.01[a]	4.62[b]	2.57[ab]	1.191	0.02	0.01

[1]Diets enriched in resistant starch (ERS), a fiber-prebiotic-probiotic blend (FPPB), or a fiber-prebiotic-probiotic blend + immuno-modulating ingredients (iFPPB).

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Table 5.10. Kyoto Encyclopedia of Genes and Genomes Orthology (KO) terms that were greater in cats fed ERS than those fed FPPB or iFPPB[1]

KO terms	Description	Log 2 FC*	Adjusted *P***
K01200	pullulanase	-2.92	1.29E-07
K01218	mannan endo-1,4-beta-mannosidase	-2.81	3.19E-06
K18567	MFS transporter, DHA1 family, purine base/nucleoside efflux pump	-2.78	1.67E-06
K01858	myo-inositol-1-phosphate synthase	-2.73	6.61E-08
K07442	tRNA (adenine57-N1/adenine58-N1)-methyltransferase catalytic subunit	-2.72	2.65E-08
K12583	phosphatidylinositol alpha 1,6-mannosyltransferase	-2.68	6.13E-07
K17829	crotonyl-CoA reductase	-2.66	1.98E-06
K01182	oligo-1,6-glucosidase	-2.64	5.09E-07
K07692	two-component system, NarL family, response regulator DegU	-2.64	2.98E-07
K01057	6-phosphogluconolactonase	-2.60	3.70E-06
K03453	bile acid:Na+ symporter, BASS family	-2.59	1.22E-08
K00240	succinate dehydrogenase / fumarate reductase, iron-sulfur subunit	-2.58	3.31E-08
K03929	para-nitrobenzyl esterase	-2.57	3.62E-07
K11263	acetyl-CoA/propionyl-CoA carboxylase, biotin carboxylase, biotin carboxyl carrier protein	-2.56	4.45E-07
K16649	rhamnopyranosyl-N-acetylglucosaminyl-diphospho-decaprenol beta-1,3/1,4-galactofuranosyltransferase	-2.55	3.71E-07
K03707	thiaminase	-2.54	6.50E-06
K18968	diguanylate cyclase	-2.54	3.46E-07
K02205	arginine/ornithine permease	-2.54	2.22E-06
K05794	tellurite resistance protein TerC	-2.51	1.50E-06
K18205	non-reducing end beta-L-arabinofuranosidase	-2.49	1.81E-09
K07230	periplasmic iron binding protein	-2.45	1.77E-06
K03338	5-dehydro-2-deoxygluconokinase	-2.38	8.90E-07
K13571	proteasome accessory factor A	-2.37	1.67E-06
K00936	two-component system, sensor histidine kinase PdtaS	-2.37	4.01E-07
K01760	cysteine-S-conjugate beta-lyase	-2.37	3.77E-08
K07259	serine-type D-Ala-D-Ala carboxypeptidase/endopeptidase (penicillin-binding protein 4)	-2.36	3.73E-07
K15531	oligosaccharide reducing-end xylanase	-2.34	1.12E-06
K13288	oligoribonuclease	-2.34	1.07E-07
K16856	ureidoglycolate lyase	-2.34	1.22E-08
K00324	H+-translocating NAD(P) transhydrogenase subunit alpha	-2.33	2.70E-08
K14215	trans,polycis-decaprenyl diphosphate synthase	-2.33	2.19E-06
K00702	cellobiose phosphorylase	-2.33	4.05E-07
K03658	DNA helicase IV	-2.32	8.22E-07
K07183	two-component system, response regulator / RNA-binding antiterminator	-2.32	8.41E-07

Table 5.10. (Continued)

K20814	Pup amidohydrolase	-2.32	1.38E-06
K06221	2,5-diketo-D-gluconate reductase A	-2.32	8.80E-11
K16147	starch synthase (maltosyl-transferring)	-2.32	2.58E-06
K07503	endonuclease	-2.31	3.79E-06
K01997	branched-chain amino acid transport system permease protein	-2.30	1.42E-10
K07407	alpha-galactosidase	-2.30	1.80E-05
K01897	long-chain acyl-CoA synthetase	-2.30	4.30E-08
K18907	GntR family transcriptional regulator, regulator for abcA and norABC	-2.30	4.32E-08
K10805	acyl-CoA thioesterase II	-2.27	2.17E-08
K01704	3-isopropylmalate/(R)-2-methylmalate dehydratase small subunit	-2.27	9.33E-08
K07777	two-component system, NarL family, sensor histidine kinase DegS	-2.25	4.26E-06
K18473	acetoacetyl-[acyl-carrier protein] synthase	-2.25	5.28E-06
K03772	FKBP-type peptidyl-prolyl cis-trans isomerase FkpA	-2.25	2.32E-07
K01894	glutamyl-Q tRNA(Asp) synthetase	-2.24	7.69E-08
K00657	diamine N-acetyltransferase	-2.24	1.99E-08
K13051	L-asparaginase / beta-aspartyl-peptidase	-2.24	9.93E-08
K15524	mannosylglycerate hydrolase	-2.24	7.45E-08
K00674	2,3,4,5-tetrahydropyridine-2,6-dicarboxylate N-succinyltransferase	-2.23	4.36E-08
K00705	4-alpha-glucanotransferase	-2.23	3.82E-08
K05364	penicillin-binding protein A	-2.23	4.66E-07
K00018	glycerate dehydrogenase	-2.23	8.11E-07
K01703	3-isopropylmalate/(R)-2-methylmalate dehydratase large subunit	-2.22	7.23E-08
K00886	polyphosphate glucokinase	-2.21	5.90E-06
K07053	3',5'-nucleoside bisphosphate phosphatase	-2.21	4.19E-08
K19285	FMN reductase (NADPH)	-2.21	1.93E-05
K08301	ribonuclease G	-2.21	5.42E-09
K11734	aromatic amino acid transport protein AroP	-2.20	5.73E-08
K17235	arabinooligosaccharide transport system permease protein	-2.20	5.86E-07
K13940	dihydroneopterin aldolase / 2-amino-4-hydroxy-6-hydroxymethyldihydropteridine diphosphokinase	-2.18	2.73E-06
K08289	phosphoribosylglycinamide formyltransferase 2	-2.18	1.14E-08
K03817	ribosomal-protein-serine acetyltransferase	-2.17	9.46E-09
K02006	cobalt/nickel transport system ATP-binding protein	-2.17	1.47E-06
K03307	solute:Na+ symporter, SSS family	-2.17	9.81E-10
K02523	octaprenyl-diphosphate synthase	-2.16	2.51E-09
K00254	dihydroorotate dehydrogenase	-2.16	2.68E-08
K07768	two-component system, OmpR family, sensor histidine kinase SenX3	-2.16	2.47E-07
K01673	carbonic anhydrase	-2.16	8.22E-07

Table 5.10. (Continued)

K16918	acetoin utilization transport system permease protein	-2.16	5.83E-07
K01996	branched-chain amino acid transport system ATP-binding protein	-2.15	4.63E-11
K00990	uridylyltransferase	-2.14	5.82E-08
K13243	c-di-GMP-specific phosphodiesterase	-2.14	4.58E-05
K04518	prephenate dehydratase	-2.14	4.38E-06
K13892	glutathione transport system ATP-binding protein	-2.13	5.67E-10
K15584	nickel transport system substrate-binding protein	-2.13	9.86E-08
K02483	two-component system, OmpR family, response regulator	-2.13	3.36E-10
K00851	gluconokinase	-2.12	7.19E-07
K20866	glucose-1-phosphatase	-2.12	9.89E-10
K17318	putative aldouronate transport system substrate-binding protein	-2.12	3.37E-06
K09016	putative pyrimidine permease RutG	-2.12	1.92E-08
K07757	sugar-phosphatase	-2.11	1.75E-07
K03575	A/G-specific adenine glycosylase	-2.11	1.30E-08
K07778	two-component system, NarL family, sensor histidine kinase DesK	-2.11	1.78E-05
K04772	serine protease DegQ	-2.10	9.55E-08
K01664	para-aminobenzoate synthetase component II	-2.09	1.17E-08
K01621	xylulose-5-phosphate/fructose-6-phosphate phosphoketolase	-2.08	8.90E-06
K16148	alpha-maltose-1-phosphate synthase	-2.06	8.90E-06
K01681	aconitate hydratase	-2.05	2.08E-08
K01207	beta-N-acetylhexosaminidase	-2.05	3.00E-09
K01761	methionine-gamma-lyase	-2.05	9.27E-07
K00766	anthranilate phosphoribosyltransferase	-2.04	2.93E-11
K00982	[glutamine synthetase] adenylyltransferase / [glutamine synthetase]-adenylyl-L-tyrosine phosphorylase	-2.04	7.83E-08
K08884	serine/threonine protein kinase, bacterial	-2.04	1.45E-06
K01077	alkaline phosphatase	-2.03	3.16E-08
K09810	lipoprotein-releasing system ATP-binding protein	-2.03	3.10E-10
K05349	beta-glucosidase	-2.02	3.64E-07
K01854	UDP-galactopyranose mutase	-2.02	1.80E-08
K06155	Gnt-I system high-affinity gluconate transporter	-2.02	3.79E-06
K07282	gamma-polyglutamate biosynthesis protein CapA	-2.02	2.20E-06
K01494	dCTP deaminase	-2.02	1.73E-08
K07243	high-affinity iron transporter	-2.02	3.33E-06
K03587	cell division protein FtsI (penicillin-binding protein 3)	-2.02	8.72E-09
K03727	ATP-dependent RNA helicase HelY	-2.01	1.03E-06
K01739	cystathionine gamma-synthase	-2.01	9.51E-07
K01696	tryptophan synthase beta chain	-2.00	4.92E-09
K01903	succinyl-CoA synthetase beta subunit	-2.00	2.71E-06

Table 5.10. (Continued)

[1]Diets enriched in resistant starch (ERS), a fiber-prebiotic-probiotic blend (FPPB), or a fiber-prebiotic-probiotic blend + immuno-modulating ingredients (iFPPB).
*FC: fold change.
**P values were adjusted using the false discovery rate.

Table 5.11. Kyoto Encyclopedia of Genes and Genomes Orthology (KO) terms that were greater in cats fed FPPB or iFPPB than those fed ERS[1]

KO terms	Description	Log 2 FC*	Adjusted *P***
K03529	chromosome segregation protein	2.01	2.95E-07
K12573	ribonuclease R	2.04	1.06E-05
K07568	S-adenosylmethionine:tRNA ribosyltransferase-isomerase	2.04	4.16E-06
K03427	type I restriction enzyme M protein	2.04	2.98E-08
K03572	DNA mismatch repair protein MutL	2.08	3.11E-03
K03722	ATP-dependent DNA helicase DinG	2.08	9.43E-07
K02111	F-type H+/Na+-transporting ATPase subunit alpha	2.08	5.72E-08
K07668	two-component system, OmpR family, response regulator VicR	2.09	8.42E-09
K11749	regulator of sigma E protease	2.10	6.82E-06
K02837	peptide chain release factor 3	2.11	3.43E-06
K11085	ATP-binding cassette, subfamily B, bacterial MsbA	2.12	1.35E-06
K02622	topoisomerase IV subunit B	2.12	4.69E-04
K03495	tRNA uridine 5-carboxymethylaminomethyl modification enzyme	2.12	5.59E-04
K00963	UTP--glucose-1-phosphate uridylyltransferase	2.12	3.11E-05
K01223	6-phospho-beta-glucosidase	2.12	9.82E-07
K04083	molecular chaperone Hsp33	2.13	1.98E-04
K03151	tRNA uracil 4-sulfurtransferase	2.13	2.43E-06
K01534	Zn2+/Cd2+-exporting ATPase	2.16	1.65E-04
K02601	transcription termination/antitermination protein NusG	2.19	9.81E-09
K01893	asparaginyl-tRNA synthetase	2.22	4.02E-08
K11069	spermidine/putrescine transport system substrate-binding protein	2.23	1.28E-04
K00864	glycerol kinase	2.23	1.11E-05
K02112	F-type H+/Na+-transporting ATPase subunit beta	2.24	2.35E-07
K03569	rod shape-determining protein MreB and related proteins	2.25	1.49E-06
K03555	DNA mismatch repair protein MutS	2.25	4.82E-04
K03546	DNA repair protein SbcC/Rad50	2.25	1.87E-03
K16786	energy-coupling factor transport system ATP-binding protein	2.26	2.78E-04
K07010	putative glutamine amidotransferase	2.26	1.82E-06
K09458	3-oxoacyl-[acyl-carrier-protein] synthase II	2.27	3.58E-05
K06131	cardiolipin synthase A/B	2.28	7.12E-04
K01443	N-acetylglucosamine-6-phosphate deacetylase	2.29	1.02E-04
K02621	topoisomerase IV subunit A	2.29	4.81E-03
K01963	acetyl-CoA carboxylase carboxyl transferase subunit beta	2.32	2.35E-05
K00847	fructokinase	2.33	3.41E-07
K03446	MFS transporter, DHA2 family, multidrug resistance protein	2.33	6.61E-05
K18908	multidrug efflux pump	2.34	1.17E-08
K05565	multicomponent Na+:H+ antiporter subunit A	2.34	1.94E-07

Table 5.11. (Continued)

K07462	single-stranded-DNA-specific exonuclease	2.36	3.79E-09
K03621	phosphate acyltransferase	2.38	1.31E-07
K20141	2-oxoglutarate carboxylase small subunit	2.38	1.01E-05
K01809	mannose-6-phosphate isomerase	2.41	1.74E-06
K02819	trehalose PTS system EIIBC or EIIBCA component	2.43	1.45E-06
K19077	two-component system, OmpR family, sensor histidine kinase GraS	2.43	3.91E-07
K01232	maltose-6'-phosphate glucosidase	2.47	8.73E-09
K02794	mannose PTS system EIIAB component	2.48	1.83E-12
K00645	S-malonyltransferase	2.48	1.04E-05
K02082	D-galactosamine 6-phosphate deaminase/isomerase	2.48	9.91E-06
K03169	DNA topoisomerase III	2.50	8.69E-13
K01990	ABC-2 type transport system ATP-binding protein	2.50	1.53E-12
K13292	phosphatidylglycerol---prolipoprotein diacylglyceryl transferase	2.51	1.45E-08
K01478	arginine deiminase	2.57	6.42E-08
K02824	uracil permease	2.59	1.42E-04
K03581	exodeoxyribonuclease V alpha subunit	2.60	2.33E-05
K00566	tRNA-uridine 2-sulfurtransferase	2.61	1.00E-07
K16013	ATP-binding cassette, subfamily C, bacterial CydD	2.62	3.06E-03
K07636	two-component system, OmpR family, phosphate regulon sensor histidine kinase PhoR	2.65	5.42E-12
K00648	3-oxoacyl-[acyl-carrier-protein] synthase III	2.65	6.35E-06
K06213	magnesium transporter	2.66	4.33E-09
K03294	basic amino acid/polyamine antiporter, APA family	2.70	1.85E-05
K03491	lichenan operon transcriptional antiterminator	2.71	1.12E-07
K19789	DNA repair protein RadD	2.72	1.52E-06
K01738	cysteine synthase	2.74	1.92E-08
K03311	branched-chain amino acid:cation transporter	2.80	3.34E-08
K02099	AraC family transcriptional regulator, arabinose operon regulatory protein	2.87	1.09E-05
K05846	osmoprotectant transport system permease protein	2.88	5.28E-06
K09693	teichoic acid transport system ATP-binding protein	2.94	1.80E-08
K09698	nondiscriminating glutamyl-tRNA synthetase	2.96	4.71E-08
K04041	fructose-1,6-bisphosphatase III	2.97	1.42E-10
K20118	glucose PTS system EIICBA or EIICB component	2.97	1.83E-12
K19005	lipoteichoic acid synthase	2.98	1.14E-08
K02761	cellobiose PTS system EIIC component	3.01	3.37E-11
K03710	GntR family transcriptional regulator	3.06	4.55E-10
K07652	two-component system, OmpR family, sensor histidine kinase VicK	3.08	3.94E-09
K05823	N-acetyldiaminopimelate deacetylase	3.14	5.36E-07
K05847	osmoprotectant transport system ATP-binding protein	3.15	3.16E-07
K00625	phosphate acetyltransferase	3.17	6.01E-10
K12555	penicillin-binding protein 2A	3.17	4.48E-10

Table 5.11. (Continued)

K04086	ATP-dependent Clp protease ATP-binding subunit ClpL	3.20	7.36E-05
K04094	methylenetetrahydrofolate--tRNA-(uracil-5-)-methyltransferase	3.22	6.87E-10
K18682	ribonucrease Y	3.28	9.55E-12
K16323	purine nucleoside transport protein	3.33	2.59E-04
K03697	ATP-dependent Clp protease ATP-binding subunit ClpE	3.34	4.69E-08
K16898	ATP-dependent helicase/nuclease subunit A	3.37	4.40E-12
K15986	manganese-dependent inorganic pyrophosphatase	3.40	3.43E-13
K11144	primosomal protein DnaI	3.55	1.66E-13
K08724	penicillin-binding protein 2B	3.66	8.29E-06
K06286	septation ring formation regulator	3.66	1.22E-06
K19220	peptidoglycan DL-endopeptidase CwlS	3.69	1.02E-11
K11632	bacitracin transport system permease protein	3.77	1.57E-13
K16899	ATP-dependent helicase/nuclease subunit B	3.79	1.83E-09
K17473	sigma-54 dependent transcriptional regulator, dga operon transcriptional activator	3.80	7.00E-08
K07456	DNA mismatch repair protein MutS2	3.82	1.24E-09
K09697	sodium transport system ATP-binding protein	3.82	4.14E-14
K00687	penicillin-binding protein 2B	3.92	5.82E-07
K03328	polysaccharide transporter, PST family	3.97	1.84E-08
K18940	two-component system, OmpR family, sensor histidine kinase ArlS	4.04	1.69E-09
K01533	P-type Cu2+ transporter	4.88	1.41E-04
K03315	Na+:H+ antiporter, NhaC family	5.16	3.81E-20
K00756	pyrimidine-nucleoside phosphorylase	5.48	1.44E-16
K01361	lactocepin	5.62	1.60E-09
K09952	CRISPR-associated endonuclease Csn1	8.09	3.75E-15
K00689	dextransucrase	9.03	6.01E-10
K12953	cation-transporting P-type ATPase F	11.63	2.61E-15

[1]Diets enriched in resistant starch (ERS), a fiber-prebiotic-probiotic blend (FPPB), or a fiber-prebiotic-probiotic blend + immuno-modulating ingredients (iFPPB).
*FC: fold change.
**P values were adjusted using the false discovery rate.

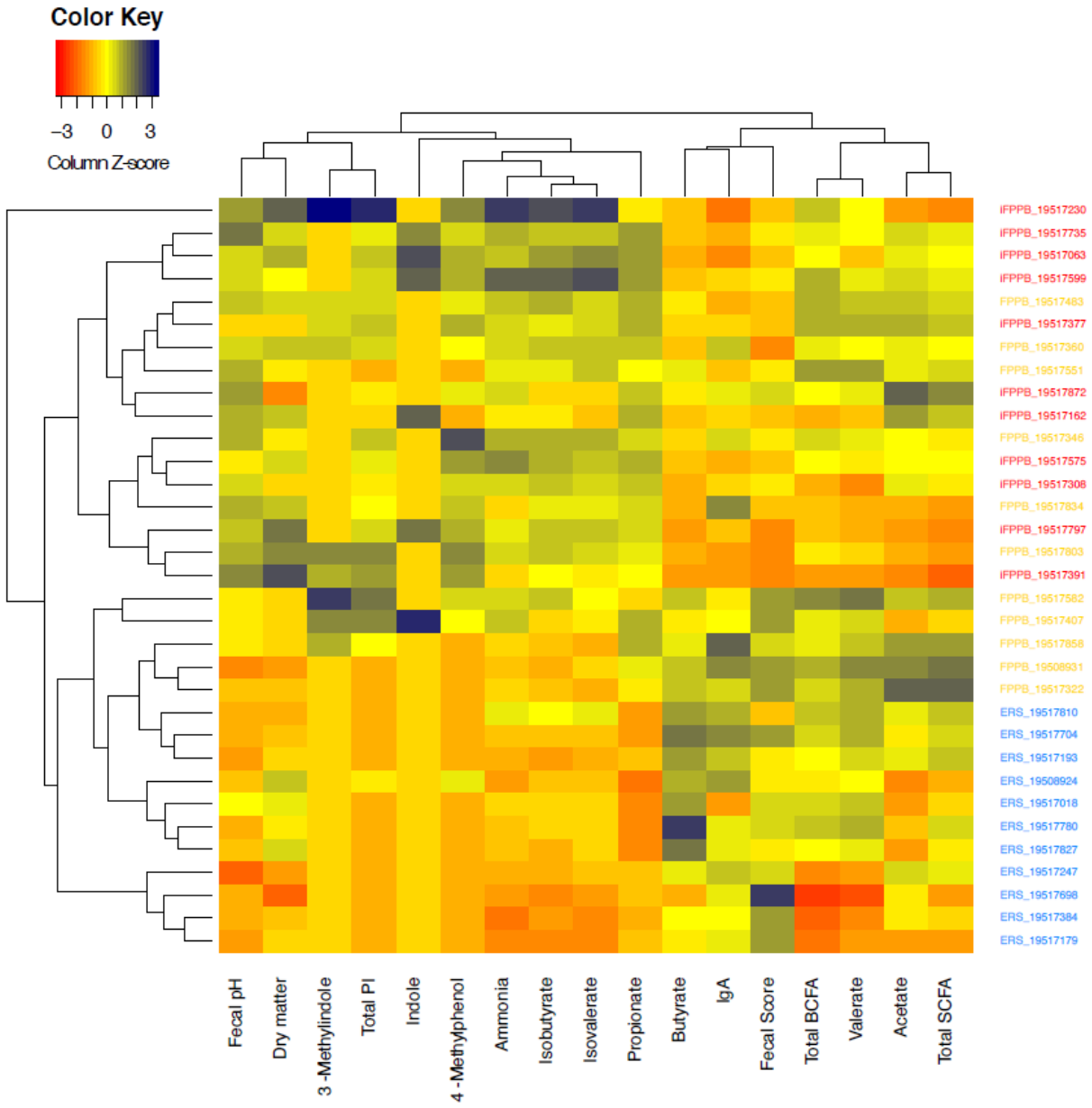


Figure 5.1. Heatmap showing that fecal characteristics and metabolite concentrations were different among diet groups, with those fed ERS (blue text) clustering separately from those fed FPPB (yellow text) and iFPPB (red text).

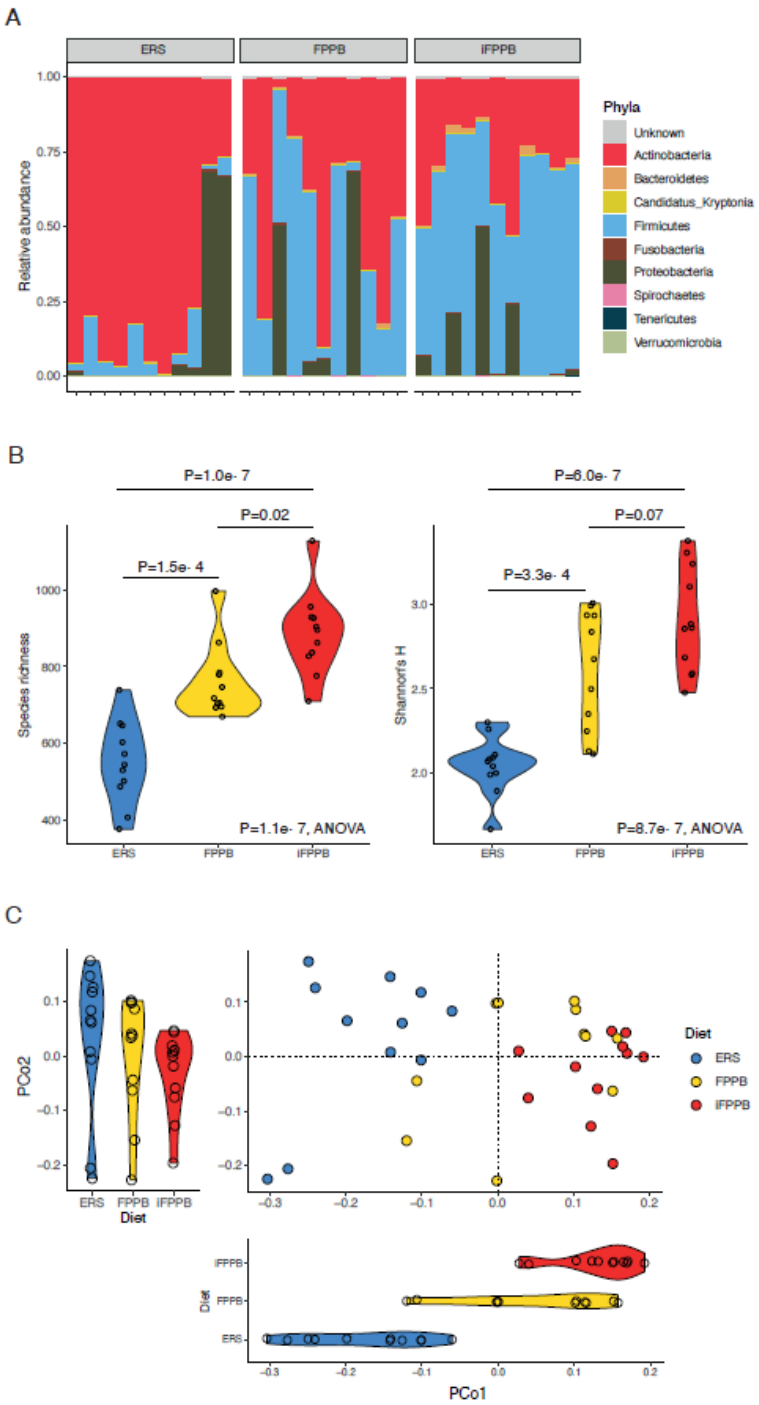


Figure 5.2. Shift in bacterial species between the diet groups. **(A)** Relative abundances at the phylum level and diet groups. Actinobacteria is the most abundant phyla in diet group A while Firmicutes dominate in group C. Both phyla are present at similar proportions in group B. **(B)** Alpha diversity differences between the diet groups. **(C)** Principal Coordinate Analysis (PCoA) of species-level gut microbiomes using Bray-Curtis distances. PERMANOVA was used to assess the association between diet and period with the gut microbiome composition. Kruskal-Wallis test was used to assess association between diet and the top two axes (PCo1, PCo2) followed by Dunn's post-hoc test to evaluate the difference between the dietary groups.

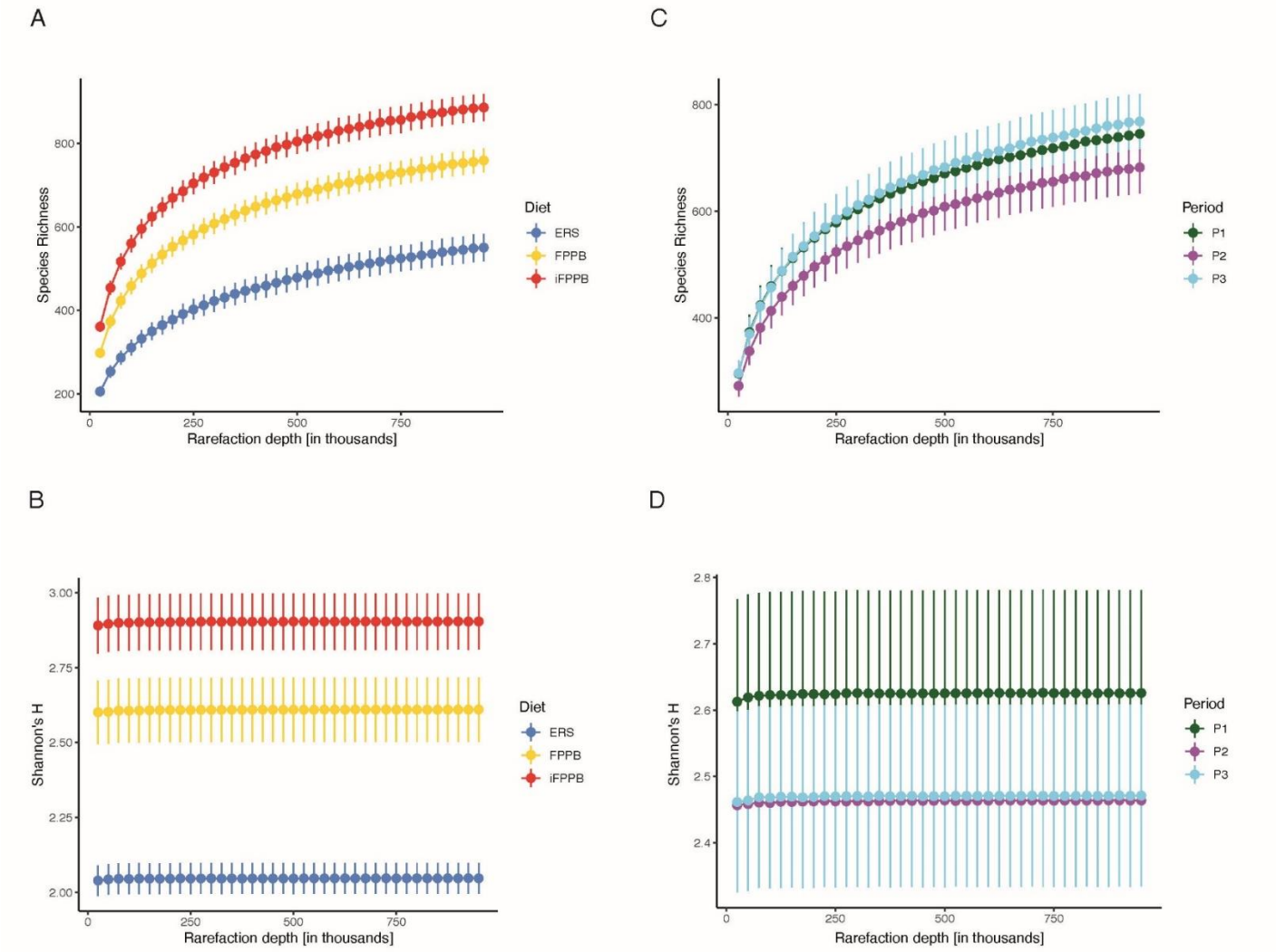


Figure 5.3. Rarefaction curves showing alpha diversity differences between diet groups. **(A, B)** Differences in Species Richness and Shannon's diversity index between the three diet groups. **(C, D)** No difference in these measures were observed between the periods during which the different diets were administered.

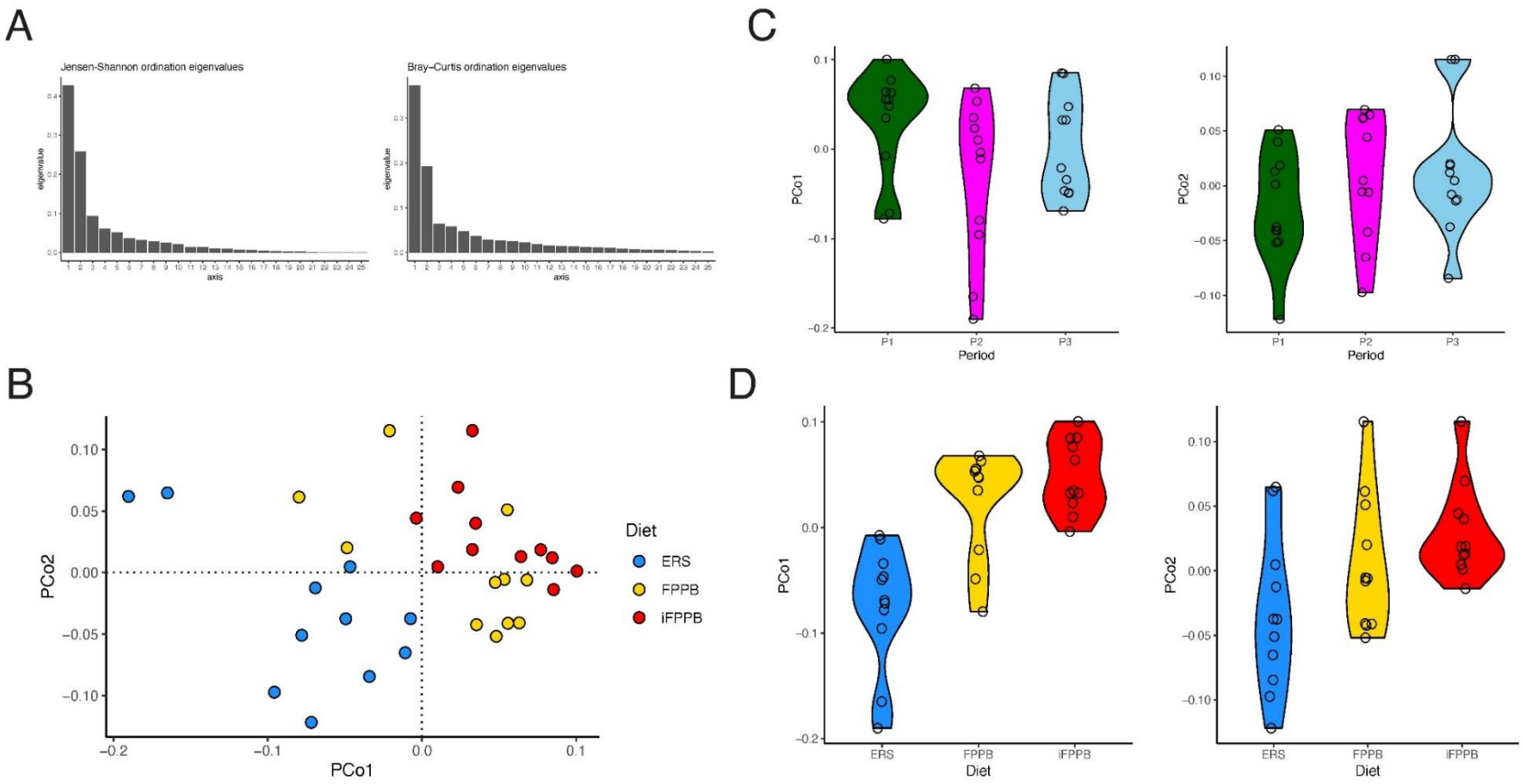


Figure 5.4. Effect of diet on the gut microbial composition **(A)** Screeplot showing the eigenvalues obtained from PCoA using Bray-Curtis (left) and Jensen-Shannon distances (right). **(B-D)** Principal Coordinate Analysis (PCoA) of species-level gut microbiomes using Jensen-Shannon distances. PERMANOVA was used to assess the association between diet and period with the gut microbiome composition. Kruskal-Wallkis test was used to assess association between diet and the top two axes (PCo1, PCo2) followed by Dunn's post-hoc test to evaluate the difference between the dietary groups. **(B)** A PCoA plot using Jensen-Shannon distance revealed significant association between diet groups (P=0.0001, PERMANOVA). Period was also significant in this case but with much weaker significance (P=0.034, PERMANOVA). **(C)** No difference in PCo1 or PCo2 between the periods (P=0.12 and 0.41, respectively, Kruskal-Wallis test). **(D)** Diet was associated with both PCo1 and PCo2 (P=1.7e-4 and 0.03, respectively, Kruskal Wallis test).

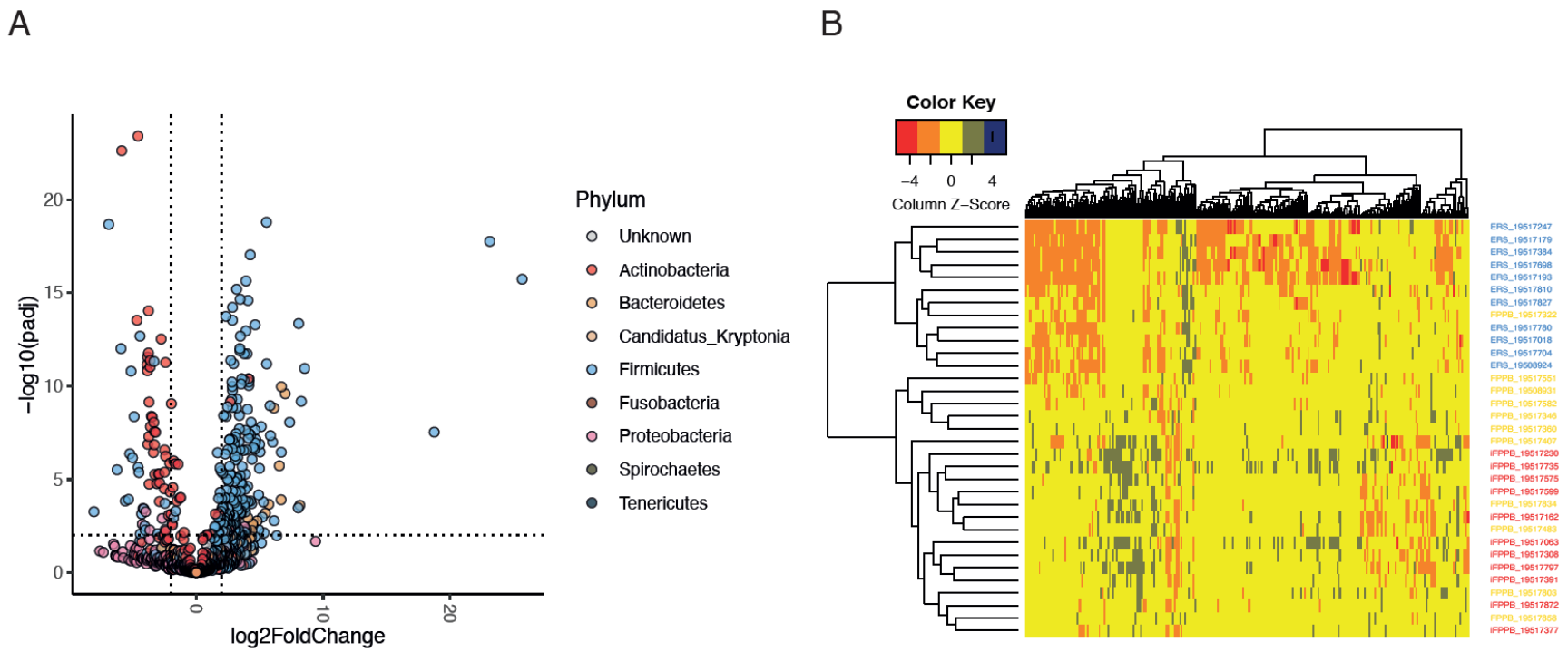


Figure 5.5. Bacterial species associated with diet. **(A)** A volcano plot showing differential abundance of 259 bacterial species between diets. Each dot is a bacterial species and dots are colored by phylum. Positive values in x-axis represent species that increased in abundance in the FPPB and/or iFPPB diets relative to ERS and negative values in the x-axis represent species that decreased in abundance in these diets relative to the ERS diet. The horizontal dotted line represents significance threshold of FDR adjusted P-value <0.01 obtained from DESeq2 and the two horizontal line differentiate the species with log2 fold change in abundance. Species with FDR adjusted P values <0.01 and absolute log2 fold change > 2 were considered statistically significant. **(B)** Heatmap showing differences in relative abundance of differentially abundant bacterial species between the diet groups.

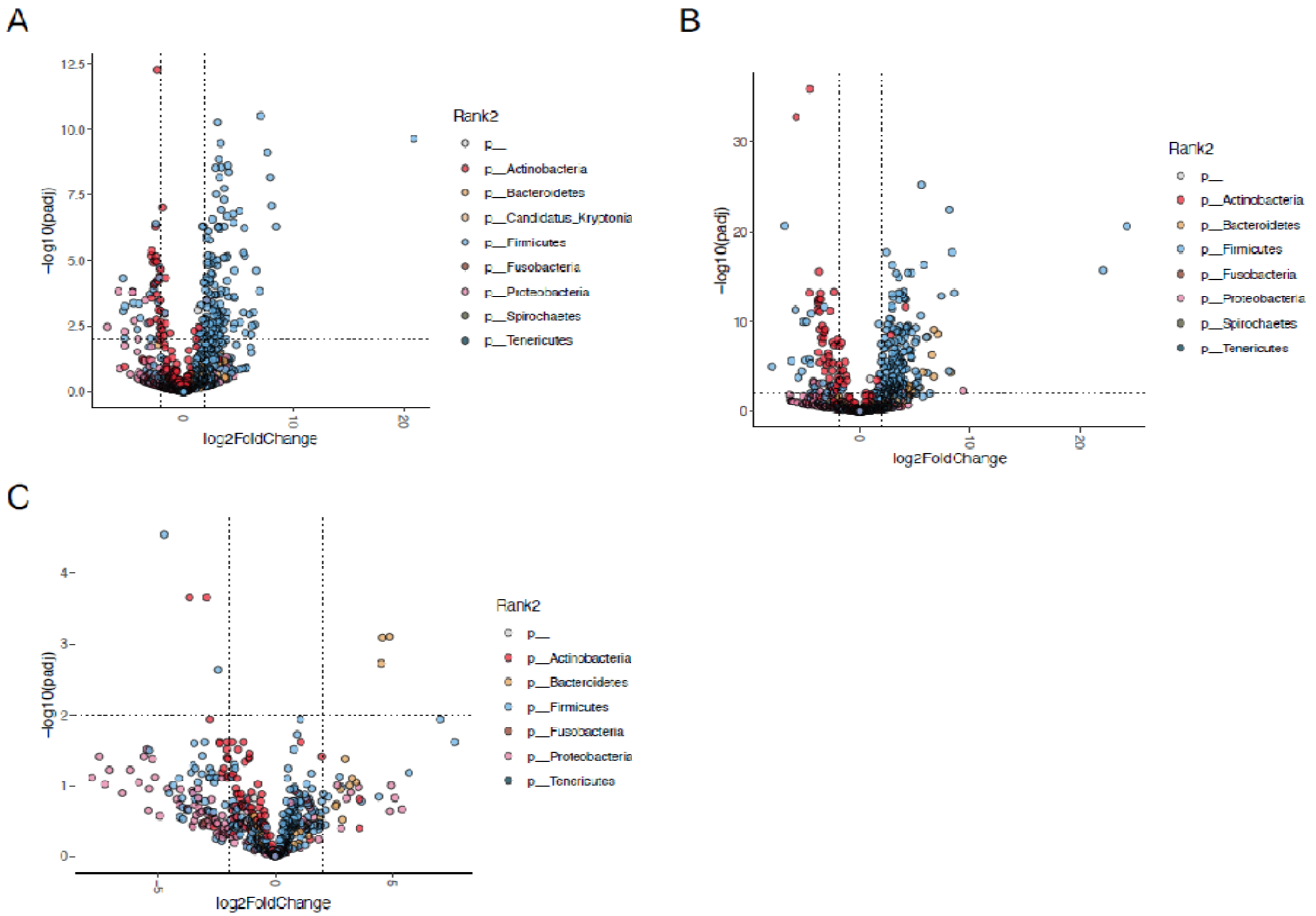
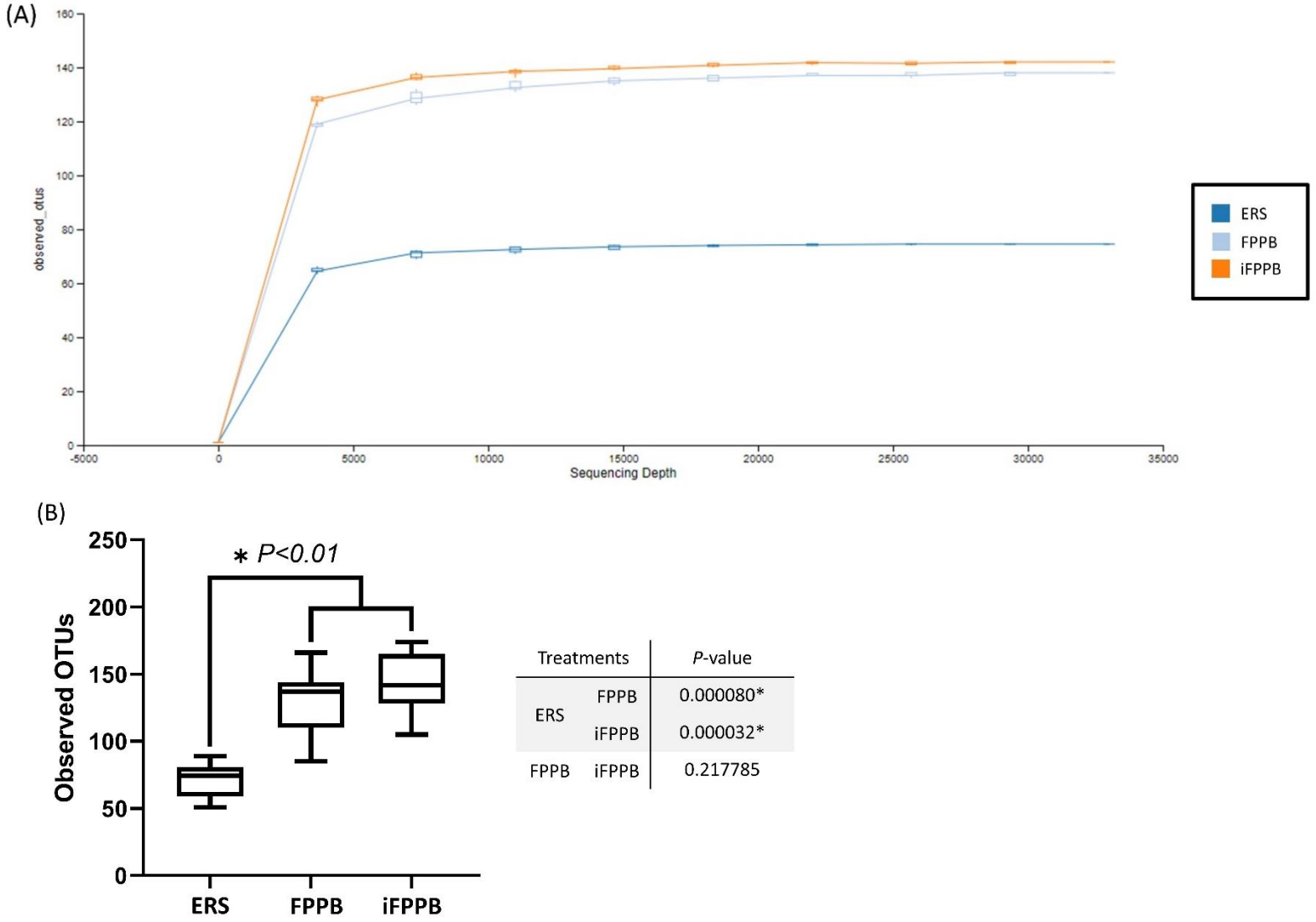


Figure 5.6. Volcano plot showing differential abundance of bacterial species between diets. Each dot is a bacterial species and dots are colored by phylum. Positive values in x-axis represent species that increased in abundance in the FPPB and/or iFPPB diets relative to ERS and negative values in the x-axis represent species that decreased in abundance in these diets relative to ERS. The horizontal dotted line represents significance threshold of FDR adjusted P-value <0.01 obtained from DESeq2 and the two horizontal lines differentiate the species with log2 fold change in abundance. Species with FDR adjusted P values <0.01 and absolute log2 fold change > 2 were considered statistically significant. ERS vs FPPB only **(A)**, ERS vs iFPPB only **(B)**. Comparison of FPPB vs iFPPB revealed only a handful of differentially abundant species **(C)**.



Treatments		*P*-value
ERS	FPPB	0.000080*
	iFPPB	0.000032*
FPPB	iFPPB	0.217785

Figure 5.7. Alpha-diversity measures of fecal microbiota communities of cats fed experimental diets. **(A)** Rarefaction curve of alpha diversity measures expressed in number of observed OTU. **(B)** Alpha diversity measures expressed in boxplots.

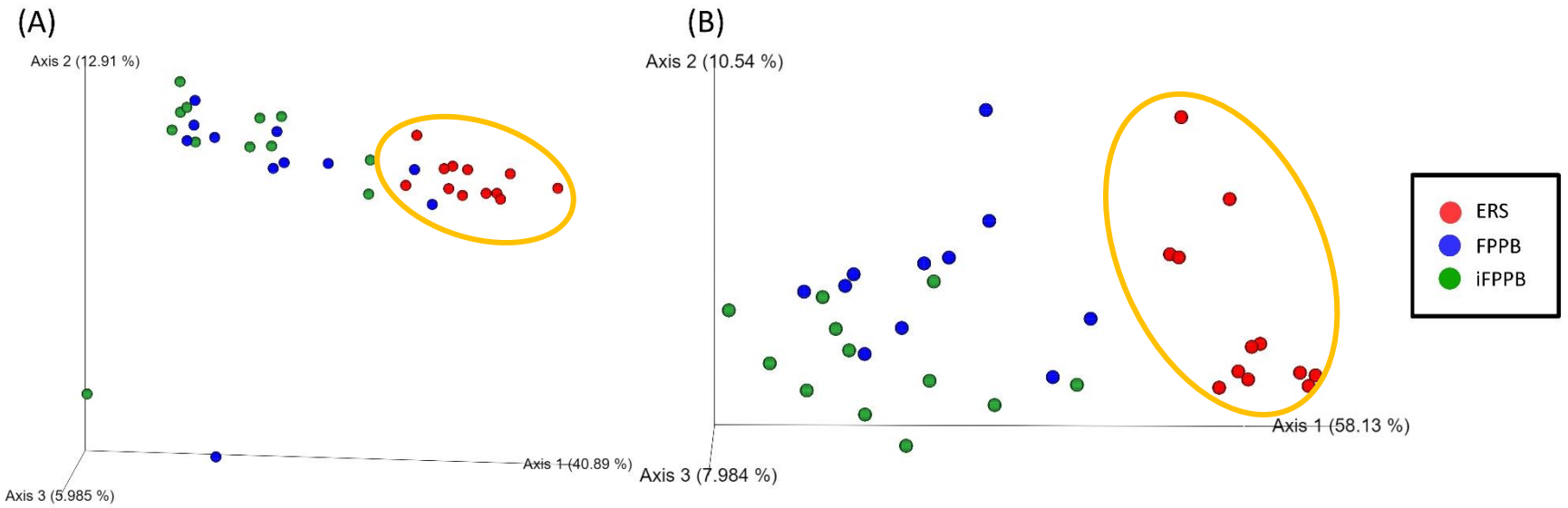


Figure 5.8. Principal coordinates analysis plots of **(A)** unweighted UniFrac distances of fecal microbial communities of cats fed experimental diets. The ERS group (circled in yellow) showed a differential clustering (P<0.01) from FPPB and iFPPB groups. **(B)** Weighted UniFrac distances of fecal microbial communities of cats fed experimental diets. The ERS group (circled in yellow) showed a differential clustering (P<0.01) from FPPB and iFPPB groups. FPPB and iFPPB also showed a smaller yet differential clustering between the two groups.

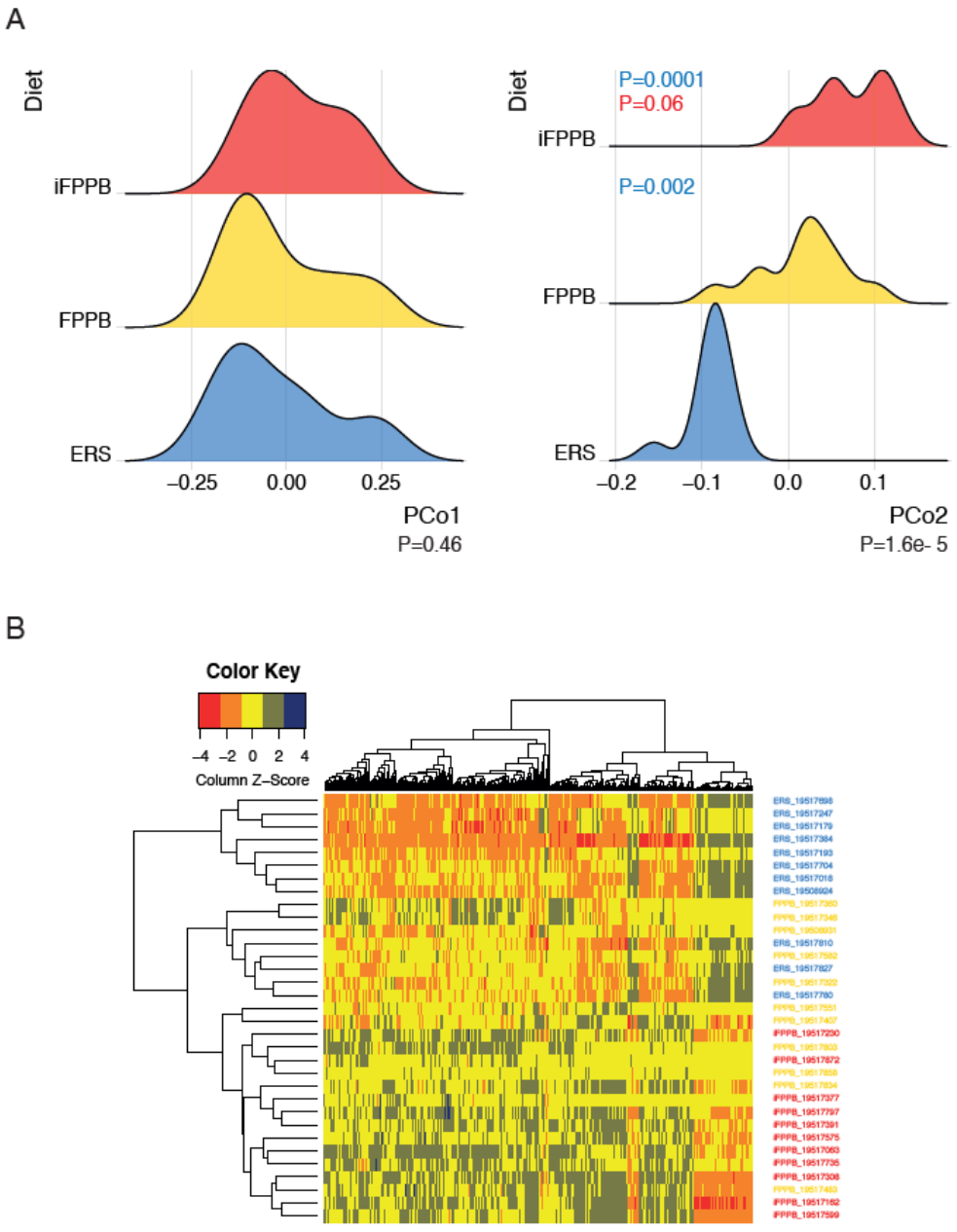


Figure 5.9. PCoA performed using KO terms showed significant shift in microbial functions between the diet groups, indicating gut bacterial functions changed in response to diet. Kruskal-Wallis test was used to assess association between diet and the top two PCo axes followed by Dunn's post-hoc test to evaluate the difference between groups. **(A)** No significant differences between the diet groups were observed along the PCo1 (P>0.05, Kruskal-Wallis test). However, strong shifts along the PCo2 was observed between the diet groups (P=1.6e-05, Kruskal-Wallis test). PCo2 scores were higher in iFPPB and FPPB groups (FDR adjusted P-values=0.0001 and 0.002, respectively, Dunn's post-hoc test) but they did not differ significantly between the iFPPB and FPPB groups (P=0.06, Dunn's post-hoc test). **(B)** Heatmap showing differences in relative abundance of differentially abundant KO terms between the diet groups.

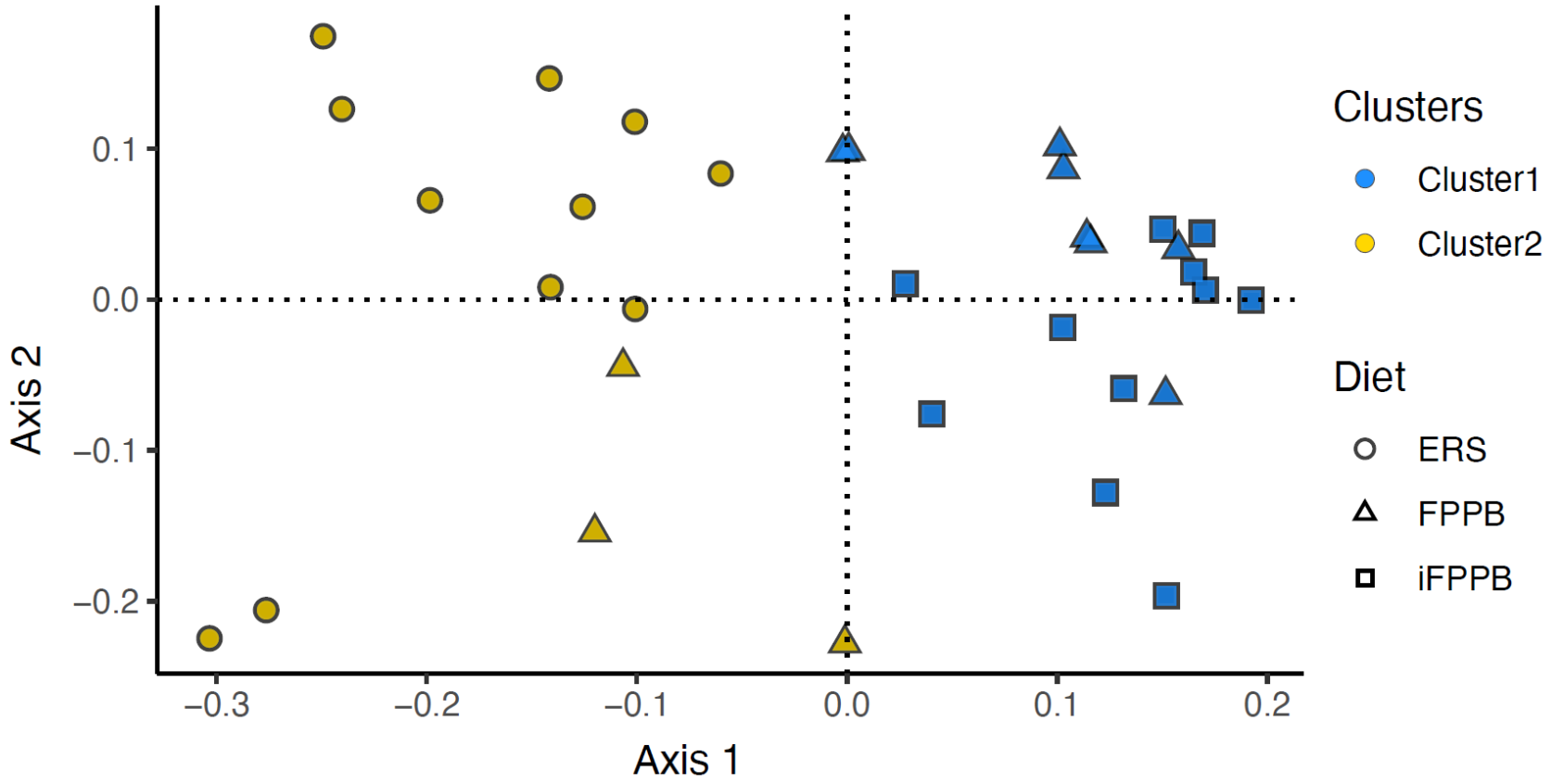


Figure 5.10. Partition Around Medoids clustering revealed two clusters, with Cluster 1 containing primarily FPPB and iFPPB diet groups and Cluster 2 containing primarily the ERS group.

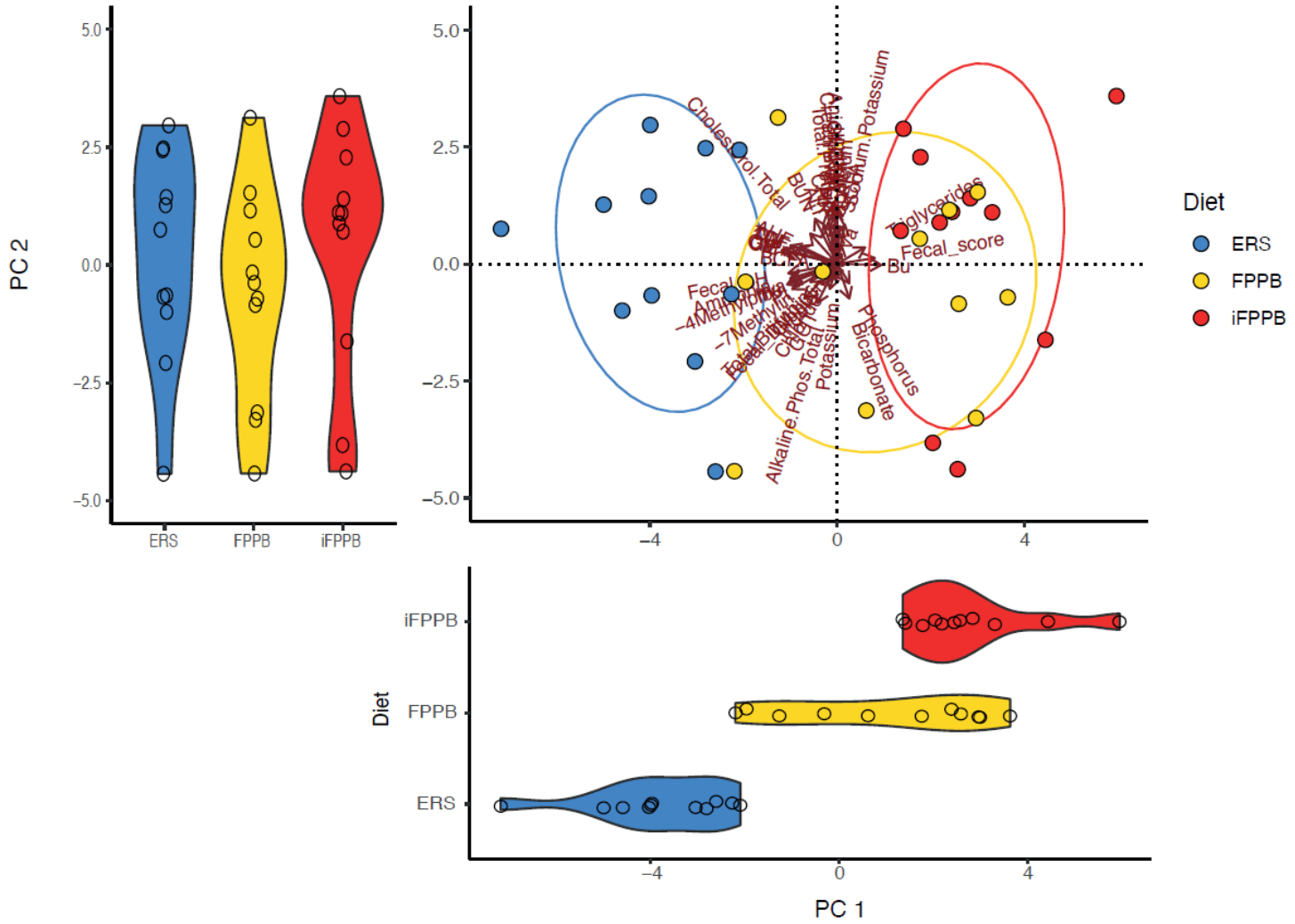


Figure 5.11. Principal component analysis of metadata, showing that serum triglyceride concentrations, fecal butyrate concentrations, and fecal scores were the primary variables driving principal component 1 in cats fed FPPB and iFPPB and fecal pH and fecal ammonia concentrations were the primary variables driving principal component 1 in cats fed ERS.

Figure 5.12 A

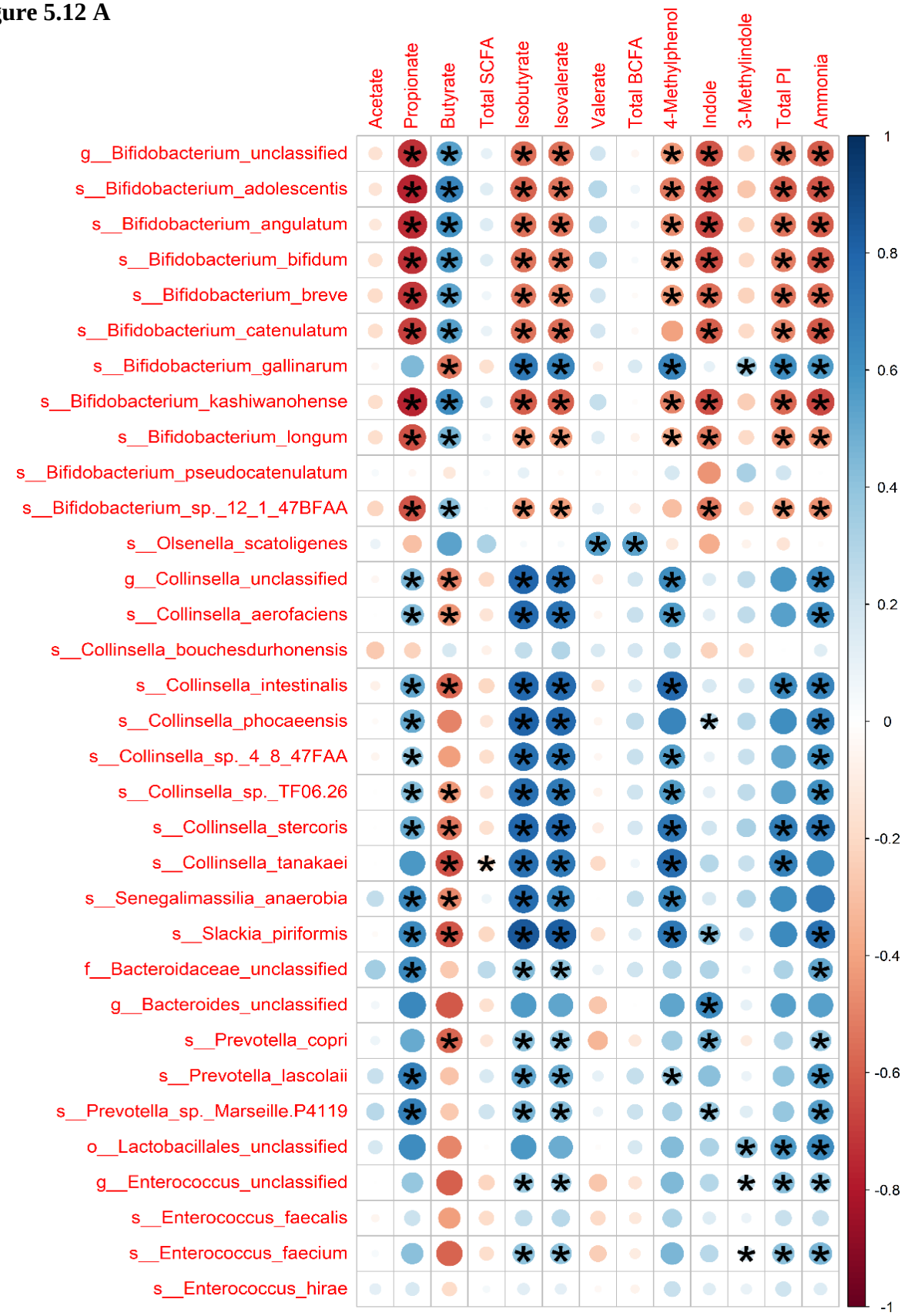

Acetate
Propionate
Butyrate
Total SCFA
Isobutyrate
Isovalerate
Valerate
Total BCFA
4-Methylphenol
Indole
3-Methylindole
Total PI
Ammonia
g__Bifidobacterium_unclassified
s__Bifidobacterium_adolescentis
s__Bifidobacterium_angulatum
s__Bifidobacterium_bifidum
s__Bifidobacterium_breve
s__Bifidobacterium_catenulatum
s__Bifidobacterium_gallinarum
s__Bifidobacterium_kashiwanohense
s__Bifidobacterium_longum
s__Bifidobacterium_pseudocatenulatum
s__Bifidobacterium_sp._12_1_47BFAA
s__Olsenella_scatoligenes
g__Collinsella_unclassified
s__Collinsella_aerofaciens
s__Collinsella_bouchesdurhonensis
s__Collinsella_intestinalis
s__Collinsella_phocaeensis
s__Collinsella_sp._4_8_47FAA
s__Collinsella_sp._TF06.26
s__Collinsella_stercoris
s__Collinsella_tanakaei
s__Senegalimassilia_anaerobia
s__Slackia_piriformis
f__Bacteroidaceae_unclassified
g__Bacteroides_unclassified
s__Prevotella_copri
s__Prevotella_lascolaii
s__Prevotella_sp._Marseille.P4119
o__Lactobacillales_unclassified
g__Enterococcus_unclassified
s__Enterococcus_faecalis
s__Enterococcus_faecium
s__Enterococcus_hirae
1
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
-1

Figure 5.12 B

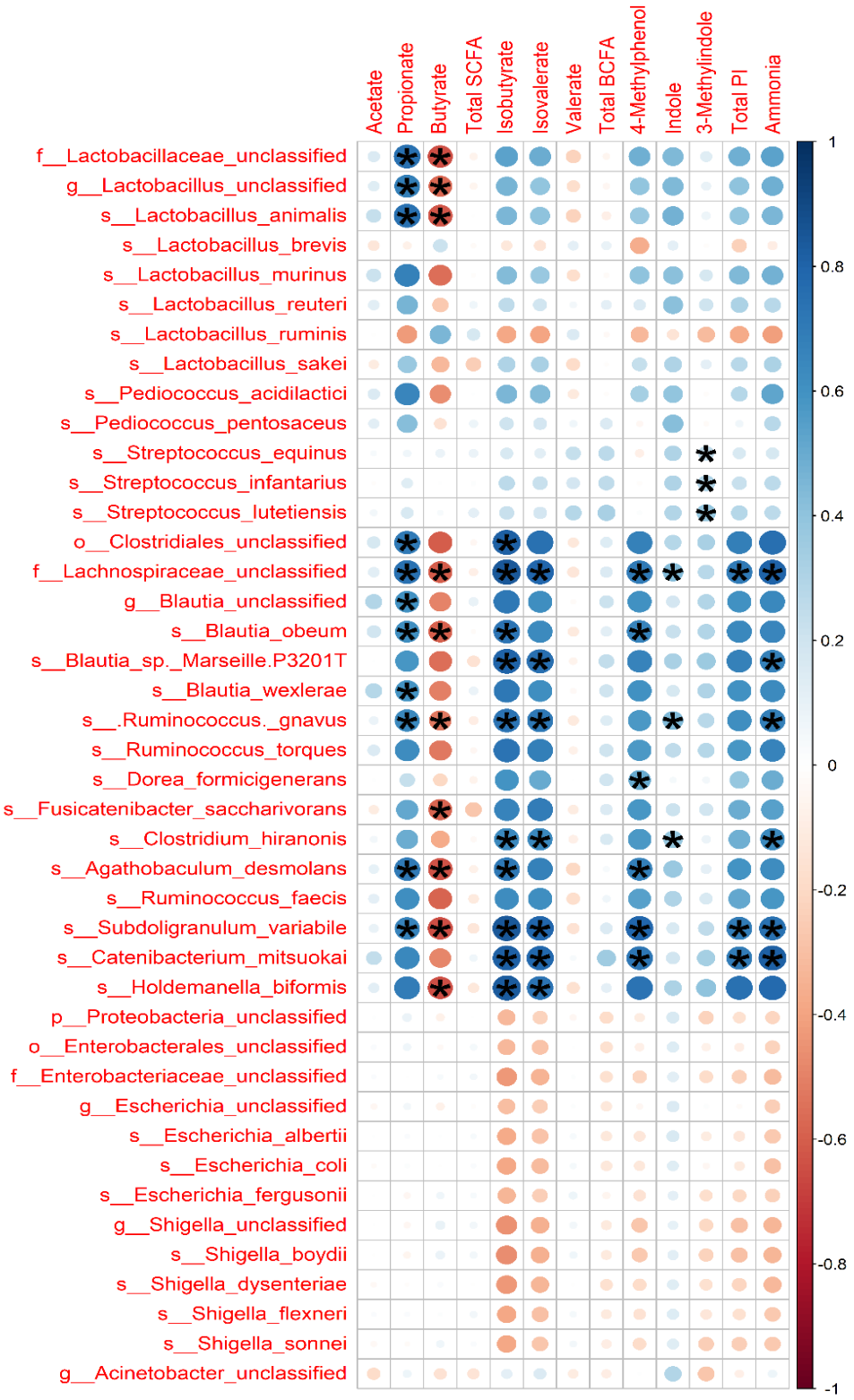


Figure 5.12. Correlation plot between fecal microbial species and fecal metabolites. The X and Y axes are the metabolites and species, respectively. R values are represented by different colors (blue: positive; red: negative). Significant correlations (p adj < 0.05) are indicated by *.

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CHAPTER 6: SUMMARY

The relationship between pets and owners has evolved over the past several decades, with now pets being considered a member of the family. This has changed how owners feed their animals. Fulfilling the basic nutritional needs is no longer acceptable, as owners now look for diets that contain functional ingredients that may help boost health and longevity. This change in mindset has helped fuel the search for novel ingredients that are able to provide health benefits. Gastrointestinal (GI) health and immune health are some of the most common target areas and many different options have been identified, including dietary fibers, prebiotics, probiotics, and postbiotics. Some of the ingredient options like milk oligosaccharides (MO) and yeast fermentation products may fall under these categories, but that classification is not absolutely necessary as long as they provide benefits. Though most of these ingredients are and have been evaluated individually to assess efficacy at an adequate inclusion level, these ingredients are often added to diets as blends in the pet food industry. Therefore, this thesis included the study of fiber-probiotic-prebiotic blends to evaluate their effectiveness in improving gastrointestinal and immune health indices.

The first experiment (Chapter 3) tested the effects of diets containing blends of fibers, 'biotics', and/or spray-dried plasma on apparent total tract digestibility (ATTD), stool quality, fecal microbiota and metabolites, and immune health outcomes of healthy adult dogs. The control diet (CT) was formulated to be a premium diet that provided a low level of substrate for microbial fermentation. The second diet (FPPB) contained a blend of fibrous ingredients (i.e., oat groats; beet pulp; pea fiber), a probiotic, and a prebiotic (i.e., inulin), which was expected to impact GI and immune health primarily through fermentation and SCFA production. The third diet (iFPPB) contained the fiber-probiotic-prebiotic blend plus ingredients thought to support

immune function [i.e., spray-dried animal plasma (SDAP); yeast fermentation product] via different mechanisms. We hypothesized that the dietary blends would positively shift fecal microbiota populations (greater *Bifidobacterium*, *Lactobacillus*, and *Faecalibacterium*; lower Proteobacteria, *Clostridium*, and *Fusobacterium*), improve fecal metabolite concentrations [greater short-chain fatty acids (SCFA); lower protein catabolites], and enhance immune responses of dogs without negatively impacting stool quality and ATTD. Dry matter (DM), organic matter, fat, fiber, and energy ATTD were decreased, fecal scores were lower (firmer stools), and fecal DM percentage was higher in dogs fed FPPB or iFPPB than those fed CT. Serum triglyceride and cholesterol concentrations were lower in dogs fed FPPB or iFPPB than those fed CT. Fecal protein catabolites (isobutyrate, isovalerate, indole, ammonia) and butyrate were lower, while fecal immunoglobulin A (IgA) was higher in dogs fed FPPB and iFPPB than those fed CT. Fecal microbiota populations were affected by diet, with alpha diversity being lower in dogs fed iFPPB and the relative abundance of 20 bacterial genera being altered in dogs fed FPPB or iFPPB compared to CT. The circulating helper T cell:cytotoxic T cell ratio was higher in dogs fed iFPPB than those fed CT. Circulating B cells were lower in dogs fed FPPB than those fed iFPPB, and lower in dogs fed iFPPB than those fed CT. Results of the first study suggest that feeding a fiber-prebiotic-probiotic blend may be beneficial to canine health, including improved stool quality, beneficial shifts to fecal microbiota and metabolite profiles, reduced blood lipids, and increased fecal IgA.

The second study (Chapter 4) evaluated the safety and beneficial effects of a novel MO biosimilar (GNU100) in healthy adult dogs. Three experiments were conducted to evaluate the safety, palatability and GI tolerance of GNU100. In Experiment 1, the mutagenic potential of GNU100 was tested using a bacterial reverse mutation assay and a mammalian cell micronucleus

test. In Experiment 2, palatability was assessed by comparing diets containing 0% vs. 1% GNU100 in adult dogs. In Experiment 3, 32 adult dogs were used to assess the safety, GI tolerance, and beneficial effects of GNU100 (0%, 0.5%, 1%, and 1.5% of diet) over 26 wk. We hypothesized that GNU100 would not demonstrate genotoxic effects, would increase the palatability of the food, and would be a safe, well-tolerated ingredient that favorably modulated gut microbiota and metabolites without having a negative impact on serum chemistry or hematology, fecal characteristics, or nutrient digestibility. Safety was confirmed in the initial experiment, with no mutagenic or cytotoxic effects being observed. The palatability test showed that 1% GNU100 was strongly preferred (3.6:1 consumption ratio) over to the control diet. In Experiment 3, all dogs remained healthy and had no signs of GI intolerance or illness. All diets were well-accepted and food intake, fecal characteristics and metabolite concentrations and macronutrient digestibilities were not altered. GNU100 slightly modulated the fecal microbiota, increasing microbial evenness, increasing relative abundance of *Catenibacterium*, *Megamonas*, and *Prevotella* (SCFA producers), and reducing relative abundance of *Collinsella*. Overall, the results suggest that GNU100 is palatable and well-tolerated, causes no genotoxicity or adverse effects on health, and beneficially shifts the fecal microbiota, supporting the safety of GNU100 for inclusion in canine diets. These studies were important in obtaining the approval for use of GNU100 in the U.S. pet food industry.

The objective of the third study (Chapter 5) was to compare the fecal microbial populations and their functions in domestic cats fed diets enriched in either resistant starch (RS) or blends dietary fibers, prebiotics, probiotics, and/or immune modulators using 16S-based and shotgun sequencing. Secondary objectives were to identify significant fecal bacterial taxa-bacterial gene-metabolite shifts and associations in response to the dietary treatments. Three

different diets were designed, including those enriched in: 1) resistant starch (ERS), 2) a fiber-prebiotic-probiotic blend (FPPB), or 3) a fiber-prebiotic-probiotic blend + immune-modulating ingredients (iFPPB). Because they are metabolized by different microbial taxa, we hypothesized that the RS vs. fiber-prebiotic-probiotic interventions would differentially modify fecal microbiota populations, fecal metabolite concentrations, and immune responses of cats. A total of 1,690 bacterial species were identified via shotgun sequencing, with 259 species differing between fiber-rich and ERS treatments. In comparison with fiber-rich treatments, that increased diversity and promoted Firmicutes and Bacteroidetes populations, RS reduced microbial diversity and fecal pH, led to a bloom in Actinobacteria, and modified KO terms pertaining to starch and sucrose metabolism, fatty acid biosynthesis and metabolism, epithelial cell signaling, among others. RS also differentially modified fecal metabolite concentrations, with relevance to GI and overall host health. The ERS diet led to increased butyrate concentrations, decreased propionate and protein catabolite (branched-chain fatty acids; phenols and indoles; ammonia) concentrations, and reduced blood cholesterol concentrations. Because those changes were correlated strongly with microbial taxa and KO terms, it allowed for a high predictive efficiency of diet groups by random forest analysis. Our results demonstrate that the feline microbiome and metabolite profiles are highly responsive to dietary change and in directions that are predictable.

Collectively, the research conducted was successful in testing various functional ingredients individually or as a blend, with each resulting in noticeable changes to the composition and activity of the GI microbial population. Although the objectives were met in each case, there were limitations to each study and not all changes were as hypothesized. First, although our studies evaluated functional ingredients at adequate dosages, the use of blends in two of the studies (Chapter 3 and Chapter 5) made it impossible to attribute any specific health

effects to a specific ingredient. While these diets are very applicable to the industry, other researchers may not benefit from the results as much as they may if individual ingredients had been studied. Second, in Chapters 3 and 5, the functional ingredients in focus were not the only dietary adjustments made. These diet formulations added another level of complexity when it came to comparing the results among treatments. This was particularly difficult in Chapter 5, whereby the intended control diet was rich in RS, which greatly modulated the fecal microbiota and metabolites. This dietary issue resulted in a comparison of RS vs. a fiber-prebiotic-probiotic blend rather than comparision with a premium diet with low-fermentative activity. Lastly, the mixed or limited outcomes observed in our studies, particularly in regard to immune responses, may be due to the animal models used. Many of the functional ingredients tested for immune function in livestock animals are tested using immune challenge models. Although our studies showed positive immune and gut microbiota changes due to treatment (Chapter 3), it is possible that a stronger response may have been observed in geriatric, obese, or stressed dog and cat populations that are more susceptible to GI upset, microbial dysbiosis, and inflammation. Other experimental conditions may be of interest and better suited for the testing of ingredients in future studies. Additionally, recent studies in humans have identified a potential relationship between host circadian rhythm, gut microbiota populations, and health and disease. Therefore, accounting for the time of day and the compositional and functional structure of the gut microbiota of dogs and cats might be of interest in the future.