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Balance of saccharolysis and proteolysis underpins improvements in stool quality induced by adding a fiber bundle containing bound polyphenols to either hydrolyzed meat or grain-rich foods

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ABSTRACT

Dietary fiber is a key component in gastrointestinal health maintenance partly due to its fermentation by the gut microbiome. The food-dependent effects of a novel fiber bundle added to hydrolyzed meat (HM) or grain-rich (GR) foods in healthy dogs (n = 16) or those with chronic enteritis/gastroenteritis (n = 16) were examined. Addition of fiber to either food improved stool quality in dogs regardless of health status; microbiome diversity of dogs with chronic enteritis/gastroenteritis became more similar to healthy dogs. The abundance of bacteria mediating beneficial saccharolytic processes (eg, Lachnospiraceae) significantly increased on addition of fiber to the GR food, while those mediating detrimental proteolytic catabolism (eg, Desulfovibrionaceae) significantly decreased. Fiber addition to the HM food led to significant changes in saccharolytic/proteolytic bacteria. Higher levels of free saccharides in feces upon fiber addition to either food indicated increased saccharolysis. Fiber addition to the GR food decreased levels of fecal free amino acids, indicating decreased proteolysis. Addition of fiber decreased fecal pH for both foods but likely by different mechanisms: addition of fiber to the HM food led to increased straight-chain short-chain fatty acids (SCFAs) and no significant change in proteolytic branched-chain SFCAs, while in the GR food, fiber mainly led to decreased proteolytic branched-chain SFCAs. Other postbiotics related to intestinal health were consistently altered when fiber was added to either food. Plant-derived bioactive molecules were enriched in feces from dogs fed either food with added fiber, which could account for the observed modulation of the canine gut microbiome and shifts in metabolic capacity.

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Canine; fiber; metabolome; endocannabinoid; saccharolysis; putrefaction

Introduction

The domestic canine microbiome has been characterized, and it bears similarities to both its divergent cousin, the wolf,[1] and to other mammals such as humans.[2,3] Like the microbiome of other organisms, the canine hindgut microbiome plays a role in digestion of food and energy,[4] and its composition can change[5] and function can be impaired during aging processes.[6] Macronutrient content[4,7,8] and food type[9] both influence microbiome composition in canines.

An important role of the gut microbiome in gastrointestinal health is to ferment dietary substrates to the straight-chain short-chain fatty acids (SCFAs) acetate, propionate, and butyrate with a concomitant decrease in the colonic pH.[9] These molecules act as a link between gut microbes and the immune system to confer anti-inflammatory effects in the intestine.[10] In addition, butyrate is used as a primary source of energy for colonocytes.[11] Lower pH may also inhibit the growth of pathogenic bacteria.[12] In contrast, branched-chain SCFAs (2-methylpropionate, 2-methylbutyrate, 3-methylbutyrate) are derived from proteolysis of protein in the colon and subsequent putrefaction of the branched chain amino acids valine, isoleucine, and leucine, respectively, and may be proxy markers of proteolytic generation of metabolites harmful to health (eg, inflammatory or uremic toxins). Similarly, polyamines are derived from putrefaction of dietary lysine and arginine. On balance, compounds generated by microbial production of metabolites (postbiotics) can have physiological benefits (eg, SCFAs), but some metabolites can be detrimental and contribute to inflammatory gastrointestinal

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disease (eg, proteolytic products such as p-cresol sulfate).[10,13] Herein, we generally refer to as proteolysis those processes where dietary protein polymers are hydrolyzed to amino acid monomers, making the amino acids available for catabolic putrefaction.

Fibers are known to exhibit numerous biological activities related to gastrointestinal health due to the chemical diversity of fiber paired with selective microbial fermentation in the hindgut of monogastrics. Furthermore, fiber has the capacity to entrap plant secondary metabolites with biological activity (eg, bioactive molecules such as polyphenols). Both fiber and polyphenols are documented to provide benefits to gastrointestinal physiology but are poorly digested and metabolized in the upper gastrointestinal tract. The dietary supply of bioactive molecules has different effects on gastrointestinal physiology depending on the mode of delivery; a complex, unpurified matrix has been shown to delay release from the stomach and provide the least impact on protein digestibility.[14] In the hindgut, plant secondary metabolites appear to be metabolized in concert with dietary fiber to influence microbial postbiotic production.[15]

Out of many studies on the canine microbiome, a few have reported the impact of types of dietary fiber on the canine microbiome,[16–18] and some have assessed the effect of one type fiber against another fiber(s) when incorporated into a similar food background, limiting the generalizability of the results.[19,20] In contrast, in this study, a novel fiber bundle was added separately to two foods that differed greatly in their composition in order to examine how the food matrix impacts the effect of fiber. This fiber bundle included three types of fibers: at the highest inclusion levels, lignin from pecan fiber as an insoluble non-water-retentive bulking fiber with low fermentability in most mammalian monogastric microbiomes,[21] thus providing low organic matter disappearance during total tract transit and bulking stool without excess water retention; at lower levels, whole flax for fiber of moderate fermentability that retains and swells with water to aid in stool transit; and at the lowest levels, commercially available sources of moderately fermentable hemicellulose and pectin (cranberry, citrus, and beet powders) to aid in curation of a healthy microbiome. These fiber sources also contain plant secondary metabolites that are inaccessible to digestion in the upper intestinal tract but that become substrates for the diverse catabolic capacity of the intestinal microbiome.

In this study, the two foods to which the fiber bundle was added were a high meat/low grain food (hydrolyzed meat [HM]) and a high grain food (grain-rich [GR]) (Table 1, **S1**). The fiber bundle was hypothesized to benefit healthy dogs and improve the condition of those with chronic enteritis/gastroenteritis regardless of the background food matrix. Parameters of digestibility and stool quality, a general marker of gastrointestinal health, were examined, and the fecal microbiomes and metabolomes were evaluated to assess possible mechanisms underpinning the hypothesized beneficial effects of this fiber bundle such as beneficial changes in SCFAs and shifts in saccharolytic and proteolytic bacteria.

Results

Food intake and weight changes

The characteristics of healthy beagles (n = 16) and those with chronic enteritis/gastroenteritis (n = 16) in the study are shown in Table S2. The inclusion of fiber into either the HM or GR foods decreased metabolizable energy respective to the controls without added fiber. As has previously been observed,[22] inclusion of fiber led to differences in energy intake regardless of food

Table 1. Food analyses.

	Type of Food			
	HM	HM Fiber	GR	GR Fiber
Moisture	9.4	10.2	9.4	8.1
Fat	14.6	16.7	12.9	14.9
Protein	21.5	25	23.3	23.2
Ash	4.7	5.5	6.1	6.6
Crude fiber	2.8	3.3	2.1	5.5
Nitrogen-free extract calories	46.9	38	47.1	39
Protein calories	20.3	23.3	23.2	23.7
Fat calories	32.8	38.7	29.7	37.4
Food metabolic energy, kcal/kg	3986	3655	3764	3510
Apparent dry matter digestibility	88.3	77.4	83.9	75.9
Apparent protein digestibility	85.9	77.8	84.8	81.5
Apparent fat digestibility	95.3	90.8	91.6	93.8
Apparent carbohydrate digestibility	96	85	91	79

All parameters are reported as percent except where otherwise indicated.
GR, grain-rich; HM, hydrolyzed meat.

background: mean difference HM Fiber – HM = −15.4 kcal/(kg$^{0.75}$), standard error (SE) = 0.81; $p < 0.001$; mean difference GR Fiber – GR = −10.3 kcal/(kg$^{0.75}$), SE = 1.1; $p < 0.001$. Health status did not influence response of energy intake to fiber inclusion in control foods (HM $p = 0.69$; GR $p = 0.83$). Although all dogs' food offerings were freely adjustable with weight changes according to the study protocol, weight differences resulted after consuming foods with and without added fiber (Table S2). Weight losses in general were minimal across the population of dogs (HM Fiber = 1.8% of body weight, SE = 0.6 and GR Fiber = 1.5% of body weight, SE = 0.6). Inclusion of fiber into HM food slightly decreased weights of healthy dogs and dogs with chronic enteritis/gastroenteritis ($p < 0.01$). When added to the GR food, fiber inclusion did not result in reduced weight of dogs with chronic enteritis/gastroenteritis ($p = 0.66$). However, healthy dogs showed reduced weight when consuming GR Fiber relative to the period in which they consumed GR without added fiber ($p < 0.01$).

Stool quality

Figure 1 shows stool scores in healthy dogs or those with chronic enteritis/gastroenteritis after consuming the HM or GR foods with and without added fiber bundle for 4 weeks. The stool

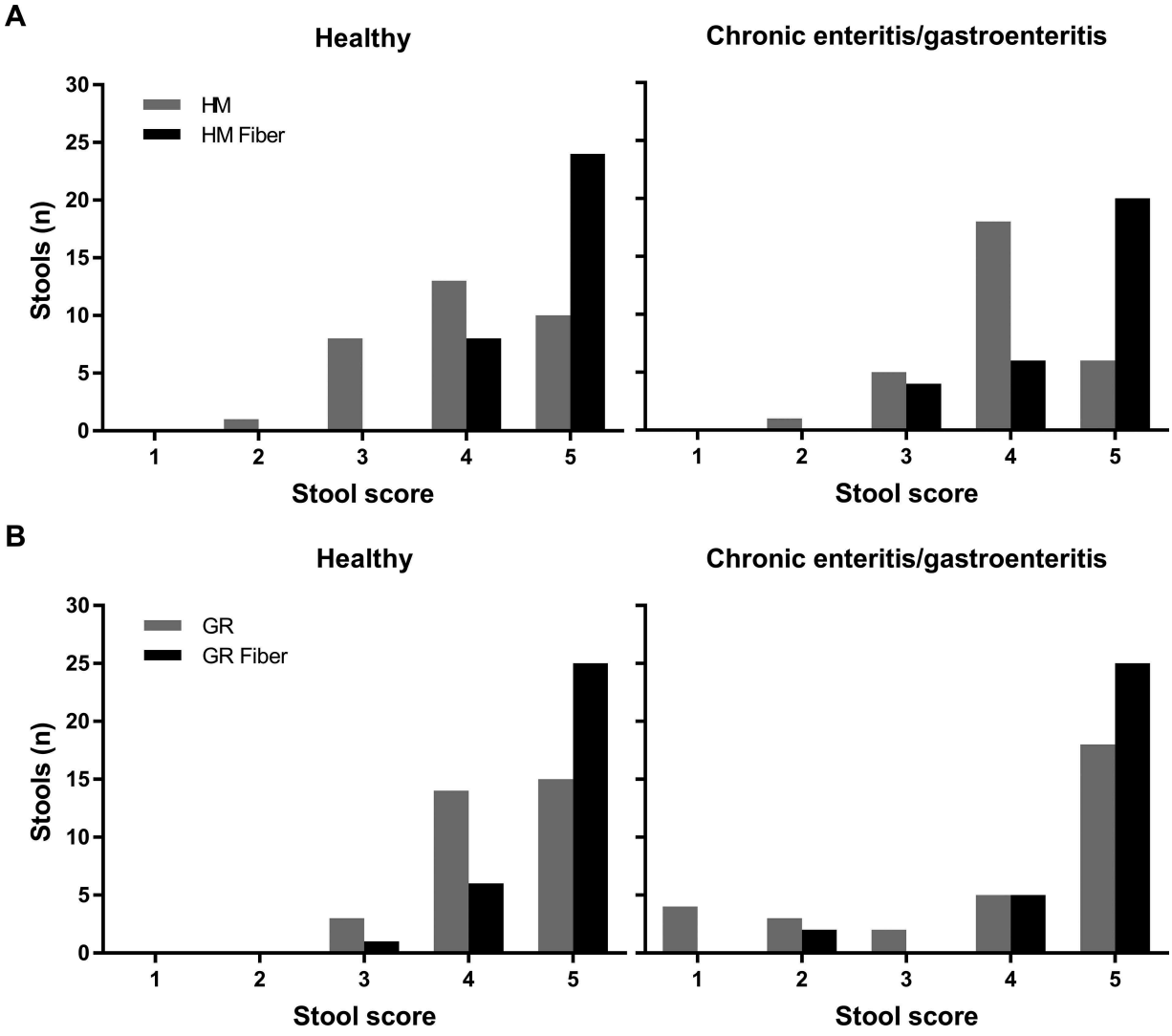


Figure 1. Stool scores in healthy dogs or those with chronic enteritis/gastroenteritis after 4 weeks of consuming the (a) hydrolyzed meat food with or without fiber or the (b) grain-rich food with or without fiber.

GR, grain-rich; HM, hydrolyzed meat. Stool was scored on a 5-point scale in which 1 does not have solid form and 5 is > 80% firm.

quality score was significantly higher across all dogs when fiber was added to either food (HM, $p < 0.01$ X^2 test; GR, $p < 0.01$ X^2 test). Controlling for health did not eliminate the beneficial effect of fiber ($p < 0.02$ Cochran-Mantel-Haenszel test). Although stool scores were significantly lower for dogs with chronic enteritis/gastroenteritis after consuming the GR food relative to their healthy counterparts ($p < 0.02$), there was no significant difference in scores between healthy dogs and those with chronic enteritis/gastroenteritis who ate the GR Fiber food ($p = 0.3$). Similarly, addition of the fiber bundle to the HM food improved stool scores relative to the HM food without the fiber bundle when fed to dogs with chronic enteritis/gastroenteritis ($p = 0.002$).

Stool composition

Macroanalysis of stool characteristics demonstrated that inclusion of the fiber bundle had the consistent effect of significantly increasing fecal organic dry matter when added to either food (Table S3). Moisture levels were not significantly changed upon addition of the fiber bundle to either background food. Levels of total fecal mineral ash were significantly decreased by the addition of fiber to both the HM and GR foods. Predicated on this observation, individual minerals were assessed from feces to ascertain the degree to which they contributed to decreased ash. Analysis of the mineral makeup of feces indicated that all analyzed ions (calcium, phosphorus, copper, iron, magnesium, manganese, and zinc) except the colonic osmolytes sodium and potassium were uniformly decreased in feces by about 27% when fiber was added to the HM food and 22% when fiber was added to the GR food. Fecal potassium was unchanged for all conditions, except it decreased significantly when the GR Fiber food was consumed by dogs with chronic enteritis/gastroenteritis ($p = 0.008$ for response differing by health). Other than the decrease in this fecal osmolyte when added to the GR food, health status did not influence the effects of fiber on stool composition.

Food-based differences in the fecal microbiome

High throughput sequencing was carried out to investigate the effects of the addition of the fiber bundle on the microbiome. The addition of fiber to the HM food resulted in a larger number of significant changes in operational taxonomic units (OTUs; 80 increased, 64 decreased) than did the addition of fiber to the GR food (55 increased, 65 decreased; Table S4). The ratio of increased to decreased taxa was consistent across all taxa levels in the HM food background, whereas in the GR food, higher ratios were seen at the phylum and class levels and lower ratios at the order, family, and genus levels with the addition of fiber.

The background type of food was the strongest driver of microbiome diversity. Consumption of the HM food led to significantly increased overall diversity at the genus level for healthy dogs (expShannon and invSimpson both $p < 0.0001$) compared to consumption of the GR food (Figure 2a); taxa richness did not differ from consumption of HM versus GR. Genus-level relative log evenness (RLE), another component of overall alpha diversity, of the healthy dogs appeared slightly higher with the HM food compared to the GR food, while there was no apparent difference for dogs with chronic enteritis/gastroenteritis (Figure 2b). Overall, there was a significant interaction between food type and health to influence alpha diversity as assessed by a linear mixed model (food type x health status; $p = 0.001$ for expShannon, $p = 0.0003$ for invSimpson; Table S4). When consuming the HM food, the expShannon and invSimpson diversities were significantly lower in dogs with chronic enteritis/gastroenteritis than their healthy counterparts ($p = 0.020$ for expShannon, $p = 0.008$ for invSimpson; Figure 2a), and evenness appeared to be reduced (Figure 2b), although taxa richness did not differ (Figure 2a). Addition of fiber to the HM food did not significantly change the diversity metrics in healthy dogs. However, dogs with chronic enteritis/gastroenteritis had significantly higher taxa richness than healthy dogs when both were consuming HM Fiber ($p = 0.040$), and there were no longer any differences by health status in the expShannon or invSimpson diversities (Figure 2a). Additionally, the RLE curve indicated

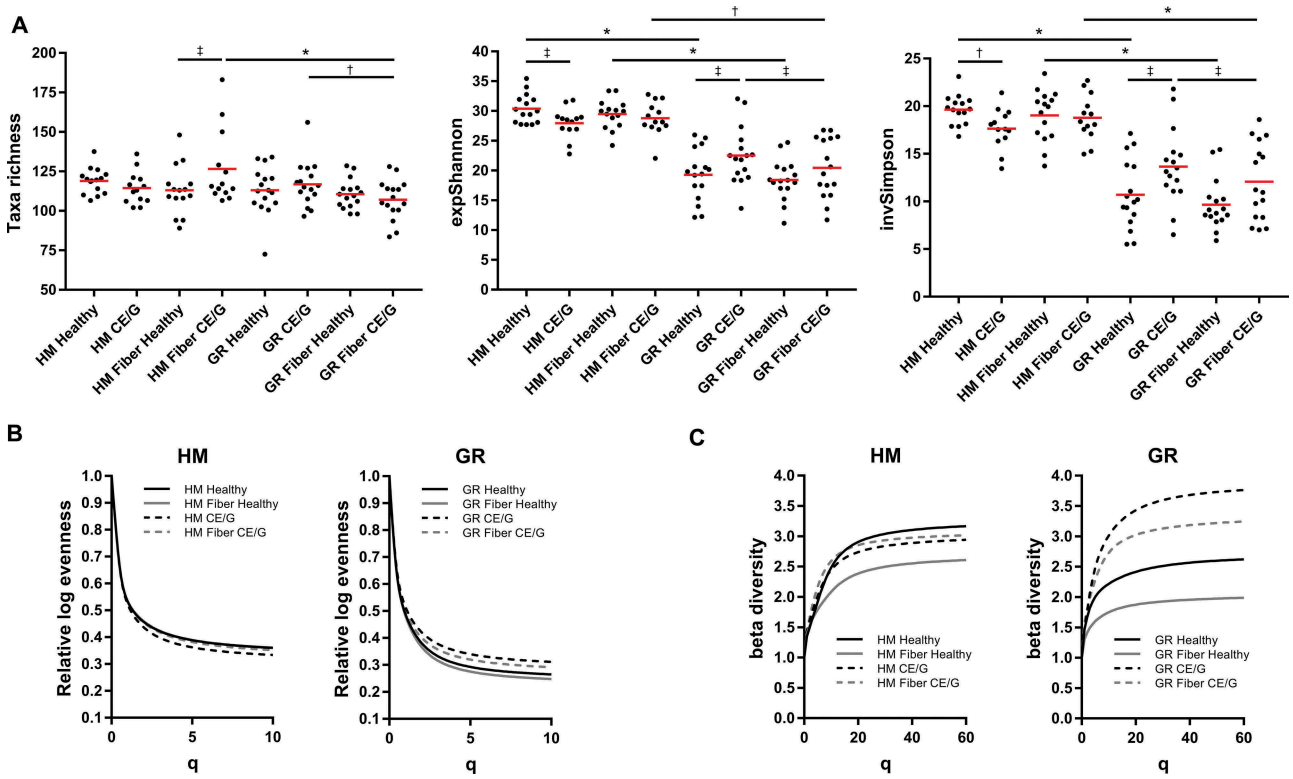


Figure 2. Bacterial diversity measures. (a) Comparisons of alpha diversity measures. Red lines represent the mean for each group. (b) Relative log evenness. (c) Beta diversity as the ratio of gamma/alpha diversity; all in healthy dogs and those with chronic enteritis/gastroenteritis fed the hydrolyzed meat food with or without fiber or the grain-rich food with or without fiber. Significance is derived from a paired t-test for comparisons with health (across food) and an unpaired t-test for comparisons across health (within food). Diversity curves are shown as group means.

CE/G, chronic enteritis/gastroenteritis; GR, grain-rich; HM, hydrolyzed meat.*$p \leq 0.001$; $^{\dagger}p < 0.01$; $^{\ddagger}p \leq 0.05$.

that the addition of fiber to HM essentially eliminated any differences in evenness between healthy and enteritis dogs (Figure 2b).

When dogs consumed the GR food, the dogs with chronic enteritis/gastroenteritis had significantly increased alpha diversity compared to their healthy counterparts ($p = 0.050$ for expShannon, $p = 0.040$ for invSimpson), while taxa richness was not different (Figure 2a). The RLE curves for dogs with chronic enteritis/gastroenteritis consuming the GR food were higher than their healthy counterparts, suggesting that evenness accounted for a portion of the increased overall alpha diversity (Figure 2b). Addition of fiber to the GR food did not significantly change the diversity metrics in healthy dogs, but significantly decreased taxa richness ($p = 0.007$), expShannon ($p = 0.05$), and invSimpson ($p = 0.04$) in dogs with chronic enteritis/gastroenteritis. Suggesting that decreased evenness played a role in the decline in overall alpha diversity with fiber addition, the RLE curves for both healthy and dogs with chronic enteritis/gastroenteritis were lower for GR Fiber than GR. The consequence of adding fiber to GR food was to eliminate the differences in overall alpha diversity between healthy and enteritis; no significant differences remained between health statuses when consuming GR Fiber food. In contrast, the RLE curves for GR Fiber were still higher for dogs with chronic enteritis/gastroenteritis than for healthy dogs.

In the mixed model assessment of interactions among food type, health, and fiber addition, the separate models for each of the three alpha diversity metrics each were significant for food type (HM vs GR; $p = 0.005$ for taxa richness, $p < 0.0001$ for expShannon, $p < 0.0001$ for invSimpson). Additionally, the two models considering the abundance-weighted diversity metrics expShannon and invSimpson had significant terms for food type x health status ($p = 0.001$ for expShannon and $p = 0.0003$ for invSimpson) but had no significant term for food type x fiber added. In contrast, the

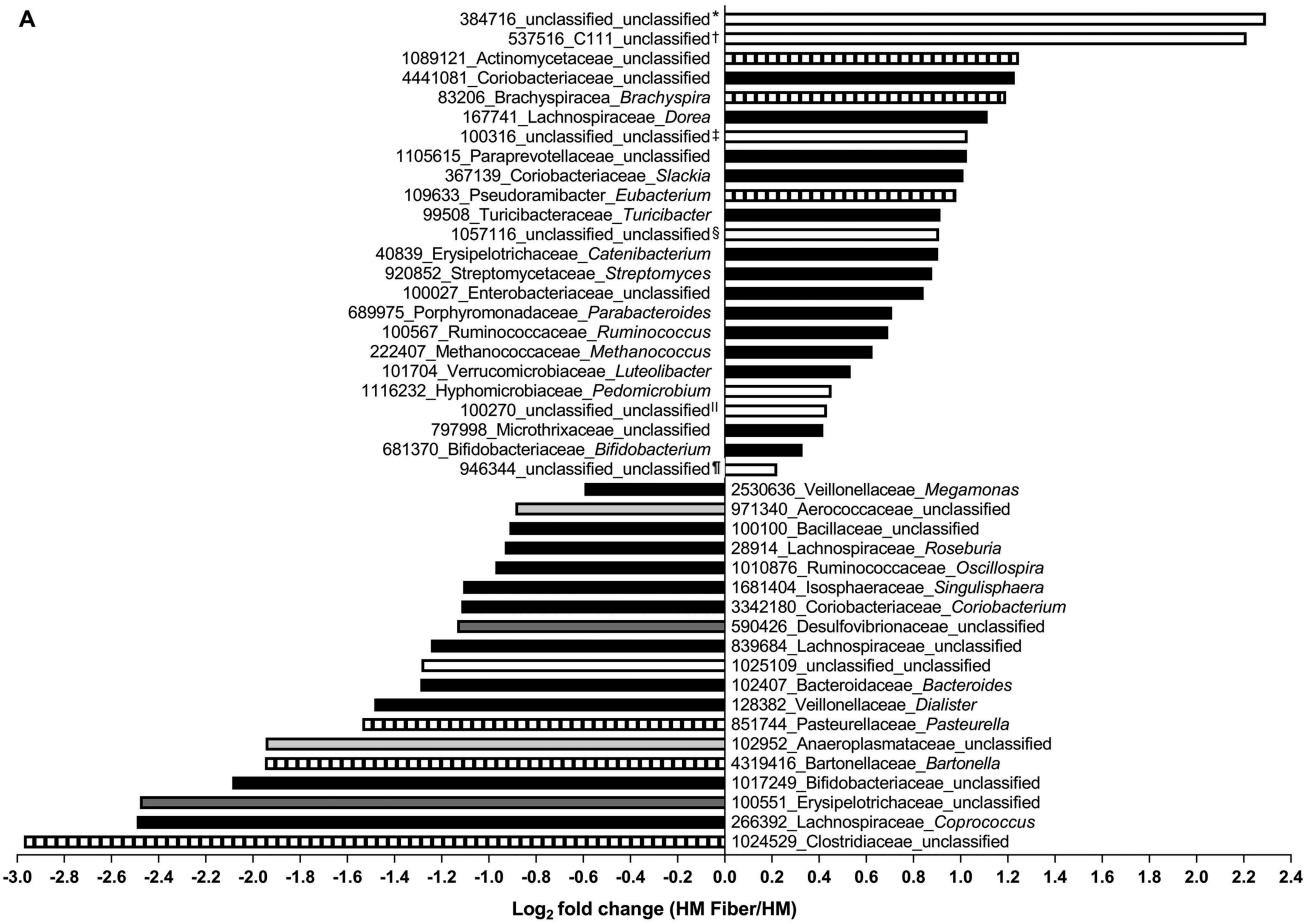
model for taxa richness had no significant term for food type x health status but did have a significant interaction for food type x fiber added (p = 0.028; Table S4) and for the tertiary interaction of food type x health status x fiber added (p = 0.003).

Beta diversity, a measurement of intragroup dissimilarity across separate dog microbiomes within the same health or treatment group, was higher in dogs with chronic enteritis/gastroenteritis than healthy dogs when consuming the GR food (Figure 2c). Further, beta diversity was decreased by fiber addition to the GR food for both healthy and dogs with chronic enteritis/gastroenteritis. In contrast, when consuming the HM food, healthy dogs were marginally higher in beta diversity, and when fiber was added to the HM food, only the healthy dogs showed decreased beta diversity. Assessment of a non-linear transform of the beta diversity to CqN values, which provide a parallel assessment of the degree to which within-group microbiomes are similar, supports the raw beta diversity comparisons (Fig. S1). Principal coordinates analysis (PCoA) plots of the abundance-weighted UniFrac distances did not show any observable group separation by health, food type, or fiber addition (Fig. S1).

Addition of the fiber bundle to either the HM or GR food backgrounds affected a few common OTUs (Figure 3). An unclassified genus in the phylum Bacteroidetes was increased, while the genus *Dialister* and an unclassified genus in the family Desulfovibrionaceae were decreased by fiber regardless of food background.

When added to the HM food, the fiber bundle modulated the abundances of several genera known to participate in saccharolytic fermentation or polyphenol catabolism (Figure 3a). Genus-level OTUs increased by fiber bundle addition to the HM food include the presumed saccharolytic or polyphenol catabolizing genera *Bifidobacterium, Dorea, Parabacteroides, Ruminococcus, Slackia, Turicibacter*; an unknown genus in the family Paraprevotellaceae; and the presumed beneficial commensal Archaeal genus *Methanococcus*. Genus level OTUs decreased by fiber added to HM include the presumed saccharolytic genera *Bacteroides, Coprococcus, Dialister, Megamonas, Oscillospira, Roseburia*; two unknown genera in the families Desulfovibrionaceae and Erysipelotrichaceae with presumed detrimental impact on GI health via proteolytic or inflammatory activity; and the possibly pathogenic genera *Pasteurella* and *Bartonella*. When changes in the microbiome were examined by health status, levels of *Bifidobacterium* and *Slackia* were higher when fiber was added to the HM food in healthy dogs but not those with chronic enteritis/gastroenteritis (p = 0.036 and p = 0.024, respectively, for difference by health status; Table S4).

As when added to the HM food, addition of the fiber bundle to the GR food appeared to modulate the abundances of several bacterial genera known to be involved in saccharolytic fermentation or polyphenol catabolism but also to impact a few that have been documented to have detrimental impact on gastrointestinal health (Figure 3b). Genus level OTUs increased by fiber addition to GR include the presumed saccharolytic or polyphenol catabolizing genera *Blautia, Colinsella, Roseburia, Succinivibrio*, and *Brenneria* as well as unknown genera in the families Veillonellaceae, Enterobacteriaceae, and Lachnospiraceae. Genus level OTUs decreased by fiber added to GR include the presumed saccharolytic genera *Dialister*, two in the family Enterobacteriaceae, and an unknown genus in the family Ruminococcaceae; genera with presumed detrimental impact on GI health via proteolytic or inflammatory activity (*Peptococcus* and *Peptostreptococcus*, as well as an unclassified genera from the family Desulfovibrionaceae and from the order Fusobacteriales); and genera with possible detrimental effects on health through acute pathogenic blooms (*Yersinia, Flexispira, and Plesiomonas*). As noted above for the HM Fiber food, few of the OTU changes were dependent on health status. Only *Colinsella* and *Roseburia* were significantly increased in healthy dogs, with no significant change in dogs with chronic enteritis/gastroenteritis (p = 0.014 and p = 0.022, respectively, for difference by health status; Table S4). An additional OTU (*Ruminococcus*; OTU 110221) showed no overall significant change across all dogs but was significantly increased by the addition of fiber to the GR food in healthy dogs but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100027_Enterobacteriaceae_unclassified
689975_Porphyromonadaceae_Parabacteroides
100567_Ruminococcaceae_Ruminococcus
222407_Methanococcaceae_Methanococcus
101704_Verrucomicrobiaceae_Luteolibacter
1116232_Hyphomicrobiaceae_Pedomicrobium
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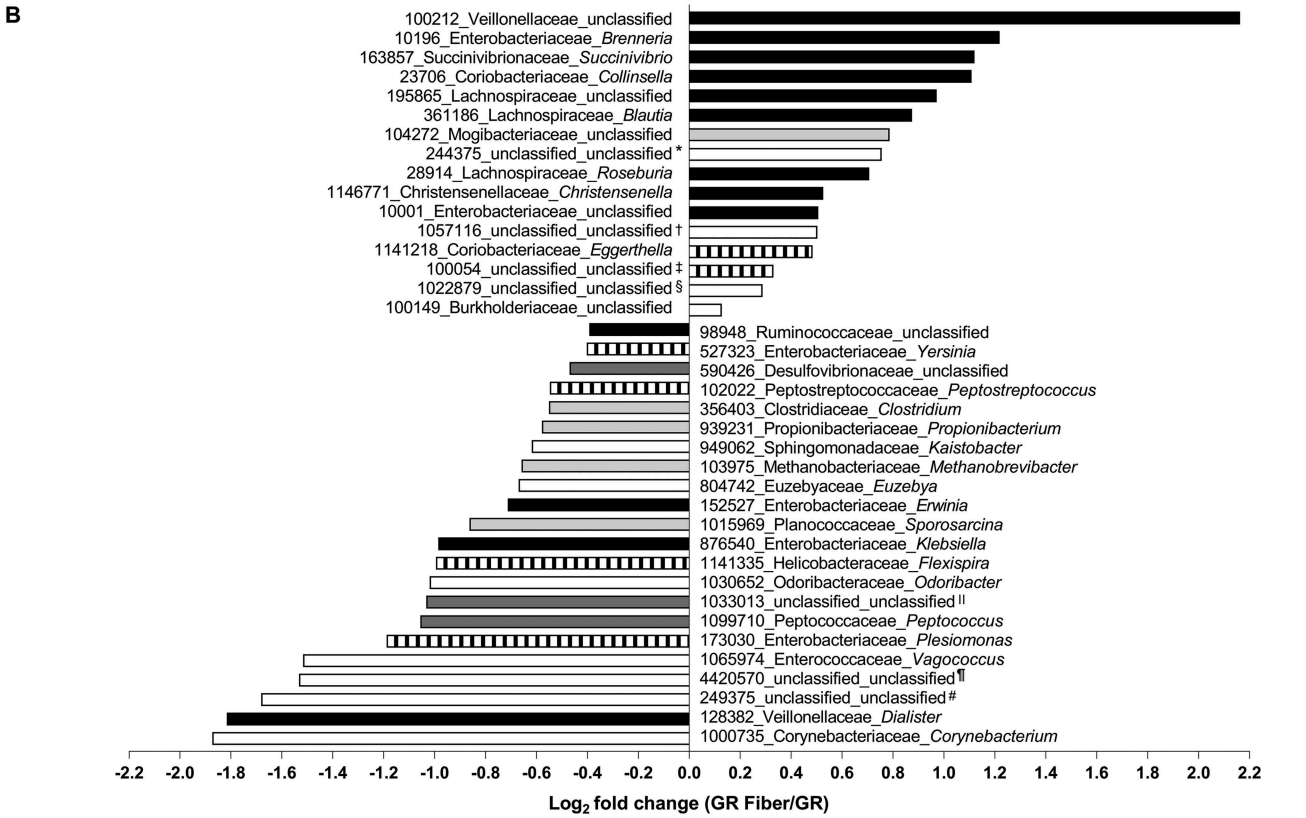
-3.0 -2.8 -2.6 -2.4 -2.2 -2.0 -1.8 -1.6 -1.4 -1.2 -1.0 -0.8 -0.6 -0.4 -0.2 0.0 0.2 0.4 0.6 0.8 1.0 1.2 1.4 1.6 1.8 2.0 2.2 2.4
Log2 fold change (HM Fiber/HM)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23706_Coriobacteriaceae_Collinsella
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104272_Mogibacteriaceae_unclassified
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1146771_Christensenellaceae_Christensenella
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590426_Desulfovibrionaceae_unclassified
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356403_Clostridiaceae_Clostridium
939231_Propionibacteriaceae_Propionibacterium
949062_Sphingomonadaceae_Kaistobacter
103975_Methanobacteriaceae_Methanobrevibacter
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128382_Veillonellaceae_Dialister
1000735_Corynebacteriaceae_Corynebacterium
-2.2 -2.0 -1.8 -1.6 -1.4 -1.2 -1.0 -0.8 -0.6 -0.4 -0.2 0.0 0.2 0.4 0.6 0.8 1.0 1.2 1.4 1.6 1.8 2.0 2.2
Log2 fold change (GR Fiber/GR)

not dogs with chronic enteritis/gastroenteritis ($p = 0.022$ for difference by health status; Table S4).

Assessment of saccharolysis and proteolysis in stool

A global metabolomics screen was employed to assess the molecular mechanisms underpinning the improvements in stool quality and changes in the microbiome due to the addition of the fiber bundle to the HM and GR foods. To gauge the degree of proteolysis, free amino acids in feces were assessed in the metabolomics data (Table S3). When the fiber bundle was added to either food, the fecal excretion of lysine and cysteine significantly decreased ($p < 0.05$). In the HM food background, the addition of the fiber bundle increased excretion of amino acids enriched in the mucus layer of the colon (serine, threonine, and asparagine; $p < 0.005$ for each). Tryptophan levels were also increased in the feces of HM-fed dogs by addition of the fiber bundle ($p < 0.001$), prompting the assessment of tryptophan metabolites detailed further below. In contrast, when the fiber bundle was added to the GR food, amino acid levels in feces were nearly uniformly decreased, with 17/22 amino acids significantly decreased.

Free saccharides in feces were assessed as a measure of saccharolysis (Table S3). The consistent free monosaccharide signature present in feces after fiber bundle addition to either food was an increase in arabinose and ribulose/xylulose ($p < 0.0001$ for all). Fucose levels were higher in response to the addition of the fiber bundle to the HM food ($p < 0.001$). When changes in markers of saccharolysis were examined by health status, fecal glucose was significantly increased on addition of fiber to the HM food in dogs with chronic enteritis/gastroenteritis ($p < 0.001$) but not in healthy dogs, a response to fiber that differed by health ($p = 0.024$ for difference by health status; Table S3). Fecal arabinose was more strongly increased by the addition of fiber to the GR food in dogs with chronic enteritis/gastroenteritis than in healthy dogs ($p = 0.048$ for difference by health status; Table S3).

As a measure of the balance of saccharolysis and proteolysis, fecal pH and SCFAs were assessed in stool. Fecal pH decreased from 6.34 to 6.08 when fiber was added to HM ($p < 0.001$), and from 5.98 to 5.79 when added to GR ($p < 0.001$). Assessment of the changes in fecal SCFAs showed that acetate was significantly increased in feces after fiber bundle addition to either food (each $p < 0.05$; Figure 4, Table S3). Other changes in SCFAs were dependent on the food background. Addition of fiber to the HM food also significantly increased fecal levels of the saccharolytic SCFAs propionate and butyrate (each $p < 0.05$), but there were no significant changes in the levels of fecal branched chain proteolytic SCFAs. In contrast, significant decreases in the levels of fecal branched chain proteolytic SCFAs were seen when fiber was added to the GR food (each $p < 0.01$). The response to the addition of fiber on fecal pH and SCFAs did not differ by health status.

Analysis of postbiotic products in stool

Similar to fecal free amino acids and branched chain SCFAs, polyamines are indicators of putrefaction; putrescine and cadaverine are representative of the putrefaction of arginine and lysine, respectively. The degree to which polyamine levels, as well as their precursor amino acids and catabolic intermediates, were changed when fiber was added to either the HM or GR background foods were evaluated. When assessed in a multivariate fashion by multivariate analysis of variance (MANOVA), the pathway consisting of polyamines, as well as their detected anabolic and

Figure 3. Significant genus-level taxa changes in the fecal microbiome with the addition of the fiber bundle to the (a) hydrolyzed meat or (b) grain-rich food backgrounds. Operational taxonomic unit number, family, and genus are shown. Black bars, saccharolytic; dark gray, proteolytic; light gray, neutral; white, unknown metabolic activity; striped, possibly pathogenic.

(A) *Order Gemellales. †Order Acidimicrobiales. ‡Class Methanomicrobia. §Phylum Bacteroidetes. ‖Class SJA-4. ¶Order Solirubrobacterales. #Order d153.(B) *Order Solirubrobacterales. †Phylum Bacteroidetes. ‡Order Legionellales. §Phylum Acidobacteria. ‖Order Fusobacteriales. ¶Order Streptophyta. #Order Actinomycetales.

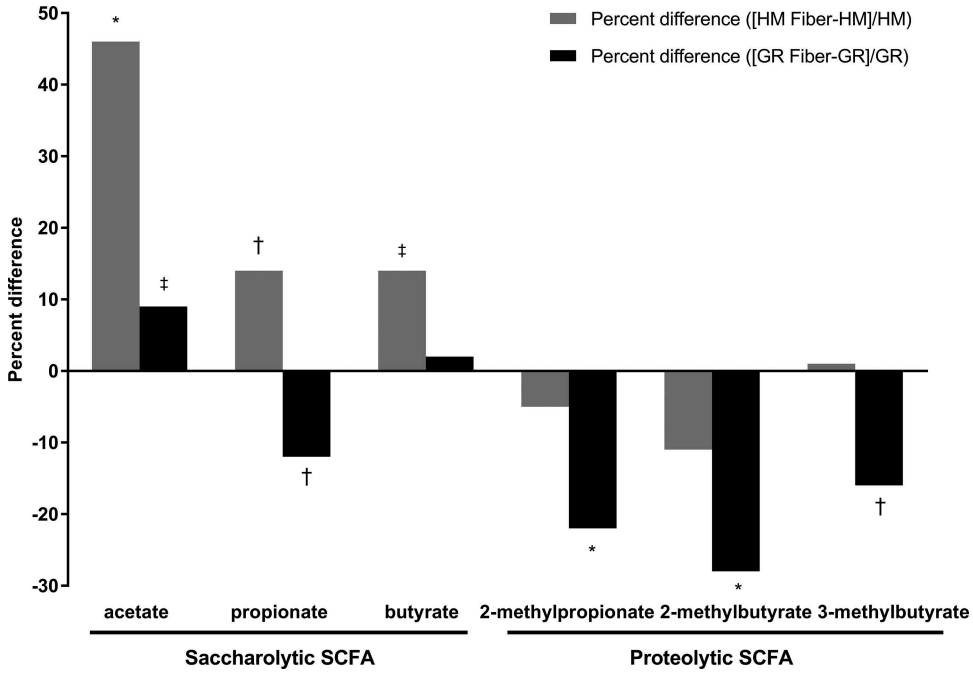

Figure 4. Percent differences in short-chain fatty acids in feces from the fiber-containing and non-fiber-containing versions of the hydrolyzed meat and grain-rich foods.
GR, grain-rich; HM, hydrolyzed meat.*$p \leq 0.001$; †$p < 0.01$; ‡$p < 0.05$.

catabolic intermediates (Table 2) was significantly altered by addition of fiber to the HM ($p < 0.001$) and GR ($p < 0.001$) foods. Substrates for polyamine biosynthesis were surveyed in a univariate manner. Lysine, the precursor to cadaverine via a one-step reaction catalyzed by lysine decarboxylase, was decreased by fiber addition to either food. Arginine, a precursor to putrescine and subsequently spermidine via the intermediacy of agmatine or ornithine, was not significantly changed in either food when fiber was added (Table 2, Table S3). Agmatine, an intermediate of the arginine decarboxylase pathway, was significantly decreased in both healthy dogs and those with chronic enteritis/gastroenteritis only when fiber was added to the HM food. When fiber was added to the GR food, agmatine decreased in the feces of only healthy dogs ($p = 0.013$), a response that differed by health status ($p = 0.002$ for difference by health status). Ornithine, an intermediate of the ornithine decarboxylase pathway, was significantly increased in all dogs when fiber was added to the HM food, but addition of fiber to GR had no significant effect on fecal ornithine levels for dogs when pooled or assessed by health status. Putrescine and spermidine, their mono- and di-acetylated catabolic intermediates, and the near-terminal oxidation product carboxyethyl-GABA were decreased by fiber addition to either food. In support of perturbation of polyamine homeostasis by fiber, although lysine-derived cadaverine and its acetylated derivative were statistically unchanged by fiber addition, they trended to decreased levels with the same magnitude of fiber-induced difference as for other members of the pathway with significant differences, but with greater individual variation of response that may have belied significance (SE of matched pairs assessing fiber influence for individual dogs; Table 2). Fiber-induced changes to polyamines were not dependent on health status.

Because tryptophan was increased in the feces of dogs consuming the HM food when the fiber bundle was added, and due to the importance of tryptophan metabolites to gastrointestinal health, postbiotics in this class were also assessed. The pathway consisting of those compounds listed in Table 3 was significantly altered by addition of fiber to HM ($p < 0.001$) and GR ($p < 0.001$) in the multivariate MANOVA. When individual pathway components were assessed in univariate manner, fecal serotonin was uniformly decreased

Table 2. Fecal polyamine precursors and polyamines in feces from canines fed a hydrolyzed meat or grain-rich food with or without fiber.

	HM				GR			
Metabolite	No fiber (LN fold)	Fiber (LN fold)	Mean Difference (Fiber – No fiber) ± SE	p value	No fiber (LN fold)	Fiber (LN fold)	Mean Difference (Fiber – No fiber) ± SE	p value
Polyamine precursor								
Lysine	−0.04	−0.25	−0.21 ± 0.09	0.021	0.17	−0.19	−0.36 ± 0.06	< 0.0001
Arginine	−0.38	−0.05	0.33 ± 0.17	0.062	0.31	0.03	−0.28 ± 0.21	0.203
Agmatine	−0.26	−0.71	−0.45 ± 0.17	0.011	0.14	0.01	−0.13 ± 0.28*	0.661
Ornithine	−0.17	0.19	0.36 ± 0.13	0.011	0.24	0.07	−0.17 ± 0.12	0.164
Polyamine								
Spermidine	1.34	−1.72	−3.06 ± 0.25	< 0.0001	0.18	−1.99	−2.17 ± 0.18	< 0.0001
Putrescine	0.35	−0.53	−0.88 ± 0.26	0.002	0.13	−0.39	−0.52 ± 0.16	0.003
Cadaverine	−0.17	−0.70	−0.53 ± 0.30	0.087	0.08	−0.37	−0.44 ± 0.28	0.126
Degradation-targeted polyamine								
N(1)-acetylspermine	0.94	−2.27	−3.21 ± 0.27	< 0.0001	−0.17	−3.47	−3.30 ± 0.27	< 0.0001
N-acetylputrescine	0.12	−0.37	−0.49 ± 0.14	0.002	0.08	−0.06	−0.14 ± 0.13	0.275
N1,N12-diacetylspermine	0.00	−1.01	−1.01 ± 0.32	0.004	0.51	−1.51	−2.02 ± 0.25	< 0.0001
N-acetyl-cadaverine	−0.31	−0.87	−0.56 ± 0.30	0.070	−0.04	−0.31	−0.26 ± 0.22	0.243
Degraded polyamine								
Carboxyethyl-GABA	−0.10	−0.31	−0.22 ± 0.12	0.095	0.11	−0.24	−0.35 ± 0.08	< 0.001

*The mean difference was −0.96 (p = 0.013) in healthy dogs and + 0.71 (p = 0.062) in those with chronic enteritis/gastroenteritis. For the differences by health status, p = 0.002.
GR, grain-rich; HM, hydrolyzed meat; LN, natural log; SE, standard error.

Table 3. Components of the tryptophan pathway in feces from canines fed a hydrolyzed meat or grain-rich food with or without fiber.

	HM				GR			
Metabolite	No fiber (LN fold)	Fiber (LN fold)	Mean Difference (Fiber – No fiber) ± SE	p value	No fiber (LN fold)	Fiber (LN fold)	Mean Difference (Fiber – No fiber) ± SE	p value
Tryptophan	−0.48	0.01	0.49 ± 0.11	< 0.001	0.06	0.01	−0.04 ± 0.12	0.713
Serotonin	0.27	−0.10	−0.37 ± 0.08	< 0.0001	0.14	−0.13	−0.27 ± 0.10	0.015
Kynurenine	−0.06	−0.15	−0.10 ± 0.08*	0.255	0.11	0.02	−0.09 ± 0.08	0.296
Kynurenate	0.11	0.08	−0.03 ± 0.28	0.923	0.97	0.25	−0.72 ± 0.26	0.009
N-acetylkynurenine (2)	−0.82	−1.39	−0.57 ± 0.14	< 0.001	−0.10	0.35	0.44 ± 0.23	0.059
3-hydroxykynurenine	ND	ND	ND	ND	−0.48	−0.47	0.01 ± 0.11	0.943
Indole	0.33	−0.23	−0.56 ± 0.22	0.017	−0.03	−0.65	−0.62 ± 0.15	< 0.001
2-oxindole-3-acetate	−0.23	−0.97	−0.75 ± 0.20	< 0.001	0.10	−0.41	−0.50 ± 0.13†	< 0.001
Indolin-2-one	0.57	−0.42	−0.99 ± 0.25	< 0.001	−0.15	−0.81	−0.66 ± 0.28	0.023
Methyl indole-3-acetate	−0.80	−1.17	−0.37 ± 0.14	0.015	0.07	−0.16	−0.23 ± 0.14	0.108
Indoleacrylate	0.30	−0.25	−0.56 ± 0.16	0.002	0.05	−0.22	−0.27 ± 0.12	0.036
3-hydroxyindolin-2-one	ND	ND	ND	ND	−0.17	−0.23	−0.06 ± 0.13	0.650
3-indoxyl sulfate	ND	ND	ND	ND	−1.60	−1.93	−0.32 ± 0.27	0.238
5-hydroxyindoleacetate	ND	ND	ND	ND	−0.48	−0.70	−0.22 ± 0.18	0.227
Indoleacetate	−0.55	−0.32	0.23 ± 0.13	0.090	−0.24	−0.14	0.10 ± 0.15	0.509
Indoleacetylglutamine	−0.28	−0.57	−0.29 ± 0.17	0.103	−0.49	−0.68	−0.19 ± 0.21	0.379
Indoleacetylglycine	ND	ND	ND	ND	−0.33	−0.66	−0.33 ± 0.30	0.269
Indolelactate	−1.03	−0.83	0.20 ± 0.29	0.496	0.40	0.35	−0.05 ± 0.38	0.904
Indolepropionate	−0.29	−0.19	0.11 ± 0.12	0.396	−0.29	0.11	0.40 ± 0.21	0.071

*The mean difference was −0.28 (p = 0.01) in healthy dogs and + 0.09 (p = 0.48) in those with chronic enteritis/gastroenteritis. For the differences by health status, p = 0.026.
†The mean difference was −0.76 (p = 0.002) in healthy dogs and −0.24 (p = 0.15) in those with chronic enteritis/gastroenteritis. For the differences by health status, p = 0.045.
GR, grain-rich; HM, hydrolyzed meat; LN, natural log; ND, not determined; SE, standard error.

across both foods with addition of the fiber bundle. Kynurenate was decreased when fiber was added to the GR food, while acetylkynurenine was decreased by the addition of fiber to the HM food. Further, fecal kynurenine was decreased in only healthy dogs when fiber was added to the

HM, but not the GR, food (p = 0.01), a response that differed by health status (p = 0.026 for difference by health status). Indole, indolin-2-one, and indoleacrylate were decreased by the addition of fiber to both the HM and GR foods, while methyl indole-3-acetate was also decreased by the addition of fiber to the HM food. Fecal 2-oxindole-3-acetate was significantly decreased in both healthy dogs and those with chronic enteritis/gastroenteritis when fiber was added to the HM food, but only in healthy dogs when added to the GR food (p = 0.002), a response that differed by health status (p = 0.045 for difference by health status). Other indole derivatives were not significantly changed by fiber addition to either food.

Levels of bile acids were significantly altered by addition of fiber to HM (p = 0.002) and GR (p < 0.001) as assessed by a multivariate MANOVA analysis. When individual bile acid pathway components were evaluated, the effect of fiber bundle addition to the GR food was more pervasive and consistent than the effect on the HM food (Table 4). With the addition of the fiber bundle to the GR food, levels of 18 out of 28 detected fecal bile acids were significantly decreased but none were increased. In contrast, levels of four bile acids significantly increased and four significantly decreased on addition of the fiber bundle to the HM food. Fiber-induced changes to bile acids were not modified by health status.

The levels of urobilins listed in Table S3 were significantly altered by the addition of fiber to the HM food (p = 0.003) and GR food (p = 0.006) backgrounds when assessed in a multivariate MANOVA model. Similar to the bile acids, there was a more pervasive effect by the addition of the fiber bundle to the GR food than when added to the HM food. Two bilirubins, as well as L-urobilinogen, were decreased by the addition of the fiber bundle to the GR food. Fecal D-urobilin was significantly decreased for only healthy dogs when added to the GR food (p < 0.001), a response

Table 4. Bile acids in feces from canines fed a hydrolyzed meat or grain-rich food with or without fiber.

	HM				GR			
Metabolite	No fiber (LN fold)	Fiber (LN fold)	Mean Difference (Fiber – No fiber) ± SE	p value	No fiber (LN fold)	Fiber (LN fold)	Mean Difference (Fiber – No fiber) ± SE	p value
12-dehydrocholate	ND	ND	ND	ND	0.50	−0.07	−0.56 ± 0.25	0.029
3-dehydrocholate	−1.88	−1.52	0.37 ± 0.18	0.056	−1.05	−1.50	−0.46 ± 0.42	0.280
6-oxolithocholate	0.28	−0.70	−0.99 ± 0.16	< 0.0001	0.62	−0.64	−1.27 ± 0.22	< 0.0001
7,12-diketolithocholate	−0.59	−0.80	−0.21 ± 0.34	0.536	0.09	−0.35	−0.44 ± 0.15	0.005
7-ketodeoxycholate	0.03	0.10	0.08 ± 0.18	0.660	0.55	−0.05	−0.60 ± 0.22	0.012
7-ketolithocholate	−1.45	−1.32	0.12 ± 0.40	0.763	−1.34	−2.35	−1.01 ± 0.24	< 0.001
Chenodeoxycholate	ND	ND	ND	ND	−1.62	−1.70	−0.08 ± 0.08	0.325
Cholate	−0.20	0.23	0.43 ± 0.16	0.012	0.61	0.01	−0.61 ± 0.21	0.007
Dehydrolithocholate	0.41	−0.38	−0.79 ± 0.17	< 0.001	−0.26	−0.23	0.02 ± 0.20	0.904
Deoxycholate	0.29	−0.14	−0.43 ± 0.17	0.017	0.15	−0.23	−0.38 ± 0.13	0.006
Glycochenodeoxycholate	ND	ND	ND	ND	−2.07	−2.98	−0.91 ± 0.36	0.018
Glycocholate	−0.60	0.08	0.68 ± 0.23	0.005	−0.63	−1.06	−0.43 ± 0.24	0.077
Glycodeoxycholate	0.23	0.02	−0.21 ± 0.26	0.434	−0.11	−0.78	−0.67 ± 0.26	0.017
Glycohyocholate	ND	ND	ND	ND	−1.10	−2.30	−1.20 ± 0.29	< 0.001
Glycohyodeoxycholate	ND	ND	ND	ND	−0.87	−1.57	−0.70 ± 0.21	0.002
Glycolithocholate	0.39	−0.02	−0.41 ± 0.23	0.088	ND	ND	ND	ND
Glycoursodeoxycholate	−0.40	−0.51	−0.12 ± 0.17	0.485	−0.50	−0.68	−0.18 ± 0.13	0.177
Hyocholate	−0.04	−0.18	−0.14 ± 0.16	0.365	0.83	−0.28	−1.10 ± 0.15	< 0.0001
Hyodeoxycholate	0.27	−0.01	−0.28 ± 0.14	0.051	0.18	−0.23	−0.40 ± 0.15	0.011
Isoursodeoxycholate	0.22	0.02	−0.20 ± 0.11	0.087	0.39	−0.33	−0.73 ± 0.12	< 0.0001
Lithocholate	0.44	−0.02	−0.46 ± 0.17	0.012	0.07	−0.28	−0.35 ± 0.11	0.004
Tauro-alpha-muricholate	−1.30	−1.19	0.11 ± 0.23	0.628	ND	ND	ND	ND
Tauro-beta-muricholate	−1.92	−1.96	−0.04 ± 0.23	0.858	−0.46	−1.23	−0.77 ± 0.25	0.004
Taurochenodeoxycholate	0.16	0.60	0.44 ± 0.38	0.256	−0.22	−0.73	−0.51 ± 0.40	0.220
Taurocholate	0.23	0.68	0.46 ± 0.22	0.045	0.19	−0.29	−0.48 ± 0.33	0.156
Taurodeoxycholate	0.42	0.46	0.03 ± 0.28	0.908	0.16	−0.66	−0.82 ± 0.33	0.019
Taurolithocholate	0.31	0.25	−0.06 ± 0.35	0.870	−0.05	−0.80	−0.75 ± 0.29	0.013
Tauroursodeoxycholate	−0.66	−0.22	0.45 ± 0.29	0.133	−0.41	−0.64	−0.23 ± 0.20	0.261
Ursocholate	−0.16	0.44	0.59 ± 0.17	0.002	−0.02	0.01	0.03 ± 0.21	0.895
Ursodeoxycholate	−0.49	−0.17	0.32 ± 0.24	0.188	0.08	−0.12	−0.20 ± 0.17	0.266

GR, grain-rich; HM, hydrolyzed meat; LN, natural log; ND, not determined; SE, standard error.

that differed by health status ($p = 0.046$). Fiber addition to HM food significantly reduced D-urobilin and L-urobilin.

Changes in N- and O- linked acylated amino acids and neurotransmitters (NOAN), a broad class of signaling molecules produced by gut bacteria that impact the gastrointestinal function of the host (eg, the endocannabinoid ethanolamides and acylglycerols),[23] were also evaluated. When assessed in multivariate fashion by MANOVA, the pathway consisting of those compounds listed in Table S3 was significantly altered by addition of fiber to HM ($p < 0.001$) and GR ($p < 0.001$). The univariate assessment indicated that fiber addition to the HM food significantly decreased levels of very long chain fatty acid ethanolamides (chain length ≥ 20 carbons) for both healthy dogs and those with chronic enteritis/gastroenteritis alike, but differentially impacted ethanolamides with chain lengths of 16–18 carbons dependent on health status. In general, there were increased C16-C18 ethanolamides in dogs with chronic enteritis/gastroenteritis, reaching significance for linoleoyl, oleoyl, and palmitoyl and trending for palmitoleoyl ethanolamides. Additionally, stearoyl ethanolamide was decreased by fiber addition to the HM food only in healthy dogs ($p < 0.001$). This response differed by health status ($p = 0.014$), although dogs with chronic enteritis/gastroenteritis showed a trend toward the same decrease in stearoyl ethanolamide ($p = 0.31$). Similar to the C16-C18 ethanolamide responses to HM Fiber, fecal N-linoleoylglycine ($p = 0.037$) and oleoyl taurine ($p = 0.004$) increases only reached significance in dogs with chronic enteritis/gastroenteritis. These responses trended toward differing by health status ($p < 0.06$). Intriguingly, fiber addition to the HM food significantly increased the levels of all detected O-linked acylglycerols (oleoyl, linoleoyl, palmitoyl, palmitoleoyl) in a uniform fashion, but these endocannabinoids were not significantly altered by fiber addition to GR food. In contrast, fiber addition to the GR food significantly increased 3 out of 4 detected N-linked acylated cholines (oleoyl, linoleoyl and palmitoyl, but not stearoyl). Overall, while there was some impact of health status on changes in NOAN observed for the HM Fiber food, health status did not modify GR Fiber-induced changes to these postbiotics.

Finally, the fecal metabolome was assessed for the presence of plant bioactives known to positively impact gastrointestinal health and that are bound to polysaccharide matrices in their native form (Table S3). The flax-derived lignan secoisolariciresinol diglucoside was significantly higher in feces from dogs fed the HM or GR foods with added fiber as was its microbial de-glycosylated product, secoisolariciresinol, and the phytoestrogen enterodiol. However, the other known microbial lignan product of secoisolariciresinol, enterolactone, was not enriched in fiber-fed dogs. Citrus and cranberry polyphenols were prominent in the feces in fiber-fed dogs, with many significantly increased, including hesperetin, ponciretin, hesperidin, naringenin, diosmetin, eriodictyol, limonin, chrysoeriol, and pheophorbide A. There were no significant differences in fecal levels of phenolic acids such as vanillate, ferulate, or dihydroferulate when the fiber bundle was added to the GR food, but these phenolic acids were increased in feces in the HM Fiber food. Fiber-induced changes to fecal bioactives were not modified by health status.

Discussion

Here, the effect of the addition of a fiber bundle to both HM and GR foods on canine gastrointestinal health was examined via stool quality as well as characterization of the changes in the fecal microbiome and metabolome. Overall, the results supported the hypothesis that the fiber bundle had a consistent effect regardless of food background or dog health status. Addition of the fiber bundle to the HM and GR foods improved stool quality in both healthy dogs and those with chronic enteritis/gastroenteritis, with no significant difference in stool scores between dogs with or without chronic enteritis/gastroenteritis after consuming the fiber-added foods. Thus, the addition of the fiber bundle appeared to improve stool quality in dogs diagnosed with chronic enteritis/gastroenteritis toward a more healthy state. The improvement in stool quality with the addition of the fiber bundle is likely due to increased organic dry matter content of the feces, which contains carbohydrate and protein components, observed experimentally as decreased carbohydrate and protein digestibility,

respectively. Slightly decreased digestibility of protein[24] and starch[25] with the addition of fermentable fiber has been previously observed. In contrast, addition of the fiber bundle decreased fecal levels of calcium, magnesium, iron, and other minerals, in agreement with reports indicating enhanced mineral bioavailability with increased dietary soluble fiber,[26] while leaving the osmolytes sodium and potassium largely unchanged.

The primary finding regarding alpha diversity was that the HM food generally increased diversity relative to the GR food apart from the addition of fiber, and that there was a significant interaction between food type and health. The background food influenced the number of genera detected (richness) as well as the evenness (relative abundance). Prior studies have found conflicting results regarding food type. Diversity increased in client-owned dogs fed mostly raw meat with added vegetables compared with commercial feed,[27] but another study found a decrease in diversity in dogs changed from a commercial dry food to boiled meat[9]; both studies tested overtly healthy dogs.

The secondary finding of the alpha diversity analysis was that the genus-level alpha diversity of dogs with chronic enteritis/gastroenteritis was different than that of their healthy counterparts, but that this difference was directionally opposite depending on whether dogs were consuming the HM (chronic enteritis/gastroenteritis < healthy) or GR (chronic enteritis/gastroenteritis > healthy) foods. A previous study investigating microbiome diversity in dogs with inflammatory bowel disease observed lower diversity compared with healthy dogs; dogs were fed a variety of commercial foods.[28] In summary, the addition of fiber moved the alpha diversity of the fecal microbiome of dogs with chronic enteritis/gastroenteritis to be more similar to their healthy counterparts by modulating both the number of taxa (richness) and the evenness of the abundances of microbiota.

Whereas alpha diversity expresses the diversity of a microbial community within a single dog, beta diversity instead expresses the total number of distinct microbiomes shared within a group of dogs. Decreased beta diversity indicates that fiber addition resulted in dogs having more similar microbiomes (fewer distinct microbiomes within the group) than when consuming foods without fiber. It is interesting that only the dogs with chronic enteritis/gastroenteritis consuming the HM food did not present with more similar microbiomes after addition of fiber, although they did when fiber was added to GR food. A more heterogeneous set of microbiomes within an enteritis subject population could pose a more challenging target for dietary or medical interventions targeting gut microbes. In this light, the fiber addition to a GR-type food may offer an opportunity to converge heterogeneous microbiomes in enteritis cases toward a more shared state and facilitate greater success when applying a uniform medical intervention. In contrast to observable separation by group when plotting continuous beta diversity curves, the weighted UniFrac PCoA plots showed no group differences. While UniFrac incorporates phylogenetic information in its distances, the beta diversity metric does not consider phylogeny. It may be that the influence of health, food, and added fiber on the degree to which dog microbiomes become more similar to each other does not necessarily depend on specific taxa relationships but is rather a convergence about a similarly structured rank abundance distribution.[29]

Addition of the fiber bundle also resulted in a larger number of observed significant changes to OTUs resulting from addition of the fiber bundle to the HM food than to the GR food. When added to either the HM or GR foods, the fiber bundle modulated the relative abundance of potentially saccharolytic commensal genera and decreased several OTUs known to mediate proteolytic catabolism. Proinflammatory and potentially detrimental genera were decreased by fiber bundle addition to the GR food. With few exceptions, the effects of fiber addition to the HM and GR foods were maintained regardless of health status. Thus, differences in endpoints resulting from fiber addition to a given food largely did not depend on health, consistent with the observations on alpha diversity richness.

While there are a number of published studies on the effect of food on the canine microbiome, relatively few have specifically examined the effect of the addition of fiber to the food. One study that examined the microbiome of dogs with chronic diarrhea found a greater similarity between those dogs and healthy dogs after consuming a fiber-

supplemented food (banana flakes, rice brain, and Yeast SAFpro 190), as measured via denaturing gradient gel electrophoresis.[30] Significantly lower counts of Desulfovibrionales were seen with the addition of that fiber blend, similar to the present study when the fiber bundle was added to either the HM or GR food backgrounds. In contrast to other studies in which addition of beet pulp or potato fiber decreased Fusobacteria and increased Firmicutes,[16,17] the present study found both increases and decreases in Firmicutes and few significant differences in Fusobacteria on addition of the fiber bundle to either the HM or GR foods. These varying results may be explained by differences in the compositions and thus fermentability of the added fibers. Of course, other studies may not be directly comparable to the present one due to differences in design and methodology.

In concert with the fiber-induced changes to the fecal microbiome, metabolomics data were captured for the fecal levels of free saccharides and free amino acids, which are molecular measures of the beneficial microbial process of saccharolysis and the detrimental process of proteolysis. Addition of the fiber bundle to the HM food increased several free saccharides and increased amino acids in proportions that indicated those amino acids were derived from endogenous mucoid sources rather than from the dietary bypass of digestion. The addition of the fiber bundle to the HM food may encourage integration of the microbiome with host processes by supporting liberation of host glycan-derived nitrogen (eg, serine, threonine, asparagine) with subsequent cross-feeding of microbiota.[31] In contrast to the observations with addition of the fiber bundle to the HM food, uniformly decreased levels of free amino acids were seen in the GR Fiber food. Arabinose and ribulose/xylulose were consistently increased in feces from dogs fed foods with fiber (both HM and GR), providing a saccharide signature for ingestion of this fiber bundle. Consistent with the concept of the fiber bundle encouraging cross-feeding from host glycans when added to the HM food,[32] fucose was increased in feces from dogs fed the HM Fiber food.

Effects of the addition of the fiber bundle on saccharolysis and proteolysis in this study were somewhat dependent on the background food. Addition of fiber to the HM food increased saccharolytic SCFAs in feces and decreased the pH, consistent with the observations of other studies that tested the addition of dietary fibers to canine foods (soybean hulls with beet pulp[33] and potato fiber.[34]) Consistent with prior work, fecal pH was higher in the HM group (regardless of the addition of fiber) compared with the GR group.[9,35] While GR Fiber significantly increased the fecal saccharolytic SCFA acetate, the proteolytic SCFAs were decreased, indicating that saccharolytic processes increased while proteolysis decreased. As evidence of this shift to saccharolysis at the expense of proteolysis, the pH of feces was significantly decreased by fiber addition to either food. When the microbiome composition was considered, there was a greater downregulation of proteolytic genera with fiber addition to the GR food. There were statistically significant effects of fiber addition to either HM or GR foods on the levels of bacterial-derived postbiotics and host-microbiome cometabolites. In general, there was a greater impact on the postbiotic classes when fiber was added to the GR food, but significant alterations to each class of postbiotics were also detected for fiber addition to the HM food.

Markers of putrefaction, fecal polyamines derived from arginine (putrescine, spermidine) and lysine (cadaverine), were decreased by fiber addition. Also decreased were their immediate metabolic precursors (agmatine and ornithine, and lysine respectively). Polyamine decrements were largely independent of health status but were influenced by background food in which the fiber was consumed. Although present at low levels overall, polyamines contain multiple basic amine sites and their decrease is consistent with the reduced pH reported above.

Although fecal free tryptophan was not decreased (GR food) or was increased (HM food) by fiber addition, all changes in fecal tryptophan catabolic products resulting from fiber addition to either GR or HM foods that reached significance were decreases; no tryptophan catabolites were significantly increased by fiber addition. Notably, fecal serotonin was decreased for all dogs with addition of fiber to either food. Serotonin is a host-microbial co-metabolite whose production is dependent upon interaction of microbiota with

enterochromaffin cells,[36] recently reviewed by O'Mahony et al.[37] Serotonin influences gastrointestinal motility,[38] and it has been proposed that increased serotonin synthesis in the colon contributes to irritable bowel symptomology in Crohn's disease in humans.[39] Intriguingly, serotonin production has been shown to be driven by increased SCFA production in a mouse model[36]; however, in the current study, the addition of fiber decreased serotonin production while it concurrently increased SCFA production. Decreased colonic serotonin production might be expected to contribute to firming of stools by fiber, as application of the serotonin antagonist alosetron has been used to reduce diarrhea in irritable bowel syndrome.[38] Indole and associated congeners (oxidized and methyl indoleacetates, indolin-2-one, and indoleacrylate) were also decreased by fiber, as were some members of the kynurenine pathway. In summary, two metabolic products of tryptophan, serotonin and indole, were decreased by fiber addition, and there were minor decreases of members of the proinflammatory kynurenine pathway.

Bile acids are canonical examples of host-microbiome co-metabolism. In dogs, cholate, chenodeoxycholate, and their urso-, muri-, and hyocongeners form the primary bile acids; these primary bile acids can be glyco- or tauro- conjugated. Fiber addition to GR provided qualitatively similar effects on levels of microbial-produced secondary bile acids, but stark differences on host-produced primary bile acids. The decrease in secondary bile acids by fiber addition to GR food was more extensive than when added to HM food. Intriguingly, fiber addition to HM food increased fecal levels of cholate as well as its urso- variant and the glyco- and tauro- conjugates. In contrast, fiber addition to GR decreased cholate and had no significant effect on the aforementioned variants. Regarding chronic enteritis, deoxycholate is proinflammatory and contributes to gastrointestinal disease.[40] That deoxycholate levels decreased with fiber addition to either HM or GR foods implies a potentially conserved bile acid-mediated mechanism of fiber to decrease intestinal inflammation. Supporting this interpretation, neither ursodeoxycholate nor its tauro- and glycoconjugates were decreased by fiber addition to either food. Ursodeoxycholate and congeners are proposed to benefit intestinal health by decreasing inflammatory processes.[41] Taken together, it would appear that there are different qualitative effects of fiber and fermentation on canine synthesis of primary bile acids depending on food background, but commonalities are present for secondary bile acids that are independent of the food matrix.

Heme is catabolized to biliverdin before enzymatic reduction to bilirubin and excretion in bile. Urobilins are microbial metabolites of bilirubin, and fecal levels are higher in human subjects with irritable bowel syndrome who respond to low fermentable substrate foods, implying that urobilins are markers for specific microbial activity associated with bowel pathophysiology.[42] Indeed, bile pigment deconjugation and enterohepatic recirculation has been shown to be mediated most strongly by only a few bacterial commensals,[43] and levels of urobilins are decreased in human Crohn's-type inflammatory bowel disease.[44] In this study, levels of urobilins were more strongly impacted when fiber was added to GR food than to HM food. On balance, urobilinoids were decreased by inclusion of fiber into either food; 2 of 3 detected urobilinoids were decreased on either HM or GR foods. Biliverdin was not changed on either HM Fiber or GR Fiber, whereas bilirubins themselves were decreased by inclusion of fiber into GR but not HM food. The decreased bile pigment effects appear to be microbiome mediated, as fiber *per se* would be expected to entrap and promote excretion of luminal bile pigments, increasing fecal levels. There may be instances where modulation of microbiome urobilin postbiotic production has physiological utility, and dietary fiber from natural sources could be a safe dietary way to effect this change.

The impact of fiber addition to the GR food background had a more pervasive impact on ethanolamide levels than addition to the HM food, as 10 out of 11 detected ethanolamides, regardless of chain length, were decreased in feces from dogs fed GR Fiber. Intriguingly, fiber bundle addition to the HM food increased the acylglycerol class of endocannabinoids while fiber addition to the GR food resulted in greater detection of and uniform increases in N-linked acylcholines.

These changes in the metabolome perhaps provide a mechanistic underpinning of the beneficial

effects observed on stool quality. Interestingly, there were but minimal effects of health status on most microbiome and metabolomic endpoints, with a few saccharolytic bacteria and NOAN endocannabinoids as the prominent exceptions. Despite altered responses to fiber for these endpoints in dogs with chronic enteritis/gastroenteritis, these dogs otherwise benefitted from fiber inclusion into either food in terms of stool quality, SCFA production, decreased pH, microbiome composition, and metabolite levels. The indigestible polysaccharides present in the fiber bundle may explain the observed effects. Additionally, plant bioactives, including lignans, phenolic acids, and flavonoids, were enriched in feces from dogs fed HM and GR foods with added fiber, and these could also account for the observed modulation of the canine gut microbiome and shifts in metabolic capacity.

The fiber bundle contains plant-derived secondary metabolites (ie, bioactive molecules) with anti-inflammatory properties. For example, pecan fiber demonstrates antioxidant action in both chemical and cellular models of oxidative stress.[45] The citrus powder used in this formulation contained approximately 2% flavonoids (primarily hesperidin) by independent analysis (data not shown). The flavonoids, phenolics, and flax lignans present in the sources of fiber of this fiber bundle have beneficial effects in gastrointestinal health maintenance and in ameliorating gastrointestinal disease. Most of the ingredients in the fiber bundle contain fiber-bound bioactives that are inaccessible to canine digestion in the upper intestinal tract, but that can be liberated by the hindgut microbiota. Once separated from the fibrous matrix, these bioactive molecules then become substrates for the diverse catabolic capacity of the microbiome. Consistent with this idea, the flax-derived lignan secoisolarisiresinol diglucoside was significantly higher in feces from dogs fed either food with added fiber as were some of its microbial products. However, one lignan product of secoisolariciresinol, enterolactone, was not enriched in fiber-fed dogs. Thus, the canine microbiome may metabolically support the production of some lignan products but not others.

A limitation of this study is that only feces were used to assess metabolites and the microbiome, which may not provide a full picture of the changes resulting from the addition of the fiber bundle along the gastrointestinal tract. However, stool quality is a good indicator of gastrointestinal health. In addition, it appears that the microbiome of the large intestine (as measured through fecal samples) is relatively constant over time, in contrast to that of the small intestine.[46] Thus, studying fecal output likely avoids the fluctuations seen higher in the gastrointestinal tract. A further limitation is the split study design whereby direct comparison of the levels of metabolites cannot be directly compared across the HM versus GR foods, due to the expression of these metabolomics values as relative fold and being analysis-dependent. However, for endpoints for which absolute values were measured, effects consistent with expected results were observed. For example, in the absence of fiber addition, fecal pH was lower with consumption of the GR food than the HM food, in agreement with the expectation that undigested native starch from whole grains imparts a benefit to microbiome fermentation not manifest on foods containing highly digestible starch. As well, total fecal SCFAs (acetate, propionate, butyrate) were higher in feces from GR-fed dogs compared to feces from dogs consuming the HM food. In contrast, the ratio of proteolytic to saccharolytic (branched to unbranched) SCFAs was higher in feces from HM- vs GR-fed dogs.

In conclusion, several beneficial effects were seen upon the addition of the fiber bundle to the HM and GR foods in canines. The improved canine stool quality; microbiome composition and shift in the alpha diversity of dogs with chronic enteritis/gastroenteritis toward that of healthy dogs; and microbiome metabolism of postbiotics such as SCFAs, polyamines, tryptophan metabolites, bile acids, and endocannabinoids indicate that the fiber bundle positively contributed to gastrointestinal health.

Materials and methods

Animals and ethics statement

All study protocols and this study were reviewed and approved by the Institutional Animal Care and Use Committee, Hill's Pet Nutrition, Inc., Topeka, KS, USA, and complied with the National Institutes

of Health guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978). Studies were conducted using dogs from a colony of beagles (Table S2). Inclusion criteria for dogs with chronic enteritis/gastroenteritis were diagnosis following endoscopy and histopathologic analysis of excised tissues confirming plasmacytic/lymphocytic inflammation. In a few cases, the research veterinarian deemed that the endoscopic procedure was not in the best interest of the dog's health, and observation of chronic symptomology sufficed. Healthy dogs were matched to chronic enteritis dogs by sex, reproductive status, and approximate age and weight. Dogs were considered healthy when there was no evidence of chronic systemic disease from physical examination, complete blood count, serum biochemical analyses, urinalysis, or fecal examination for parasites; exclusion criteria were recorded instances of gastrointestinal upset (vomiting, diarrhea) or abnormally low appetite. All dogs were pair-housed in spacious indoor rooms with natural light. Dogs received behavioral enrichment by interacting with each other, as well as through play time with caretakers, daily opportunities to run outside, and access to toys. Dogs were fed once daily and had ad libitum access to water. All dogs were immunized against canine distemper, adenovirus, parvovirus, *Bordetella*, and rabies, were monitored for parasites, and received routine heartworm preventative. Symptoms of dogs with chronic enteritis/gastroenteritis were managed with bismuth subsalicylate, prednisolone, cobalamin, omeprazole, and prednisone as needed to maintain their quality of life (Table S2).

Study foods

Nutrient profiles of the four dry extruded foods utilized in the study are listed in Table 1. Addition of the fiber bundle to the HM and GR food backgrounds slightly increased fat (likely from the inclusion of whole flax seed), fiber, and ash in foods. The two control foods without added fiber were chosen because they contain similar macronutrient composition and micronutrient profiles as the leading brands of available commercial dog foods for adult maintenance and provided different sources of macronutrients, particularly protein and carbohydrate. All foods were adequate for adult maintenance according to the Association of American Feed Control Officials 2015 Official Publication. Foods were produced at the experimental foods pilot plant at Hill's Pet Nutrition®, Topeka, KS.

The hydrolyzed meat food (HM) was formulated primarily from enzymatically hydrolyzed chicken heart and liver, cornstarch, and cellulose for fiber along with vitamins, minerals, and taurine (Table S1). For the HM food containing fiber (HM Fiber), the fiber bundle replaced a portion of cornstarch and all of the cellulose.

The grain-rich food (GR) was formulated primarily from chicken meal, egg, and whole grains along with cellulose and < 1% beet pulp as supplemental fiber sources (Table S1). For the GR food containing fiber (GR Fiber), the fiber bundle replaced a portion of whole yellow corn and all of the cellulose. The < 1% beet pulp was retained in the formulation of the control GR food not containing the fiber bundle, which is about five times less than the beet pulp level in the GR Fiber food.

Design of studies

Each of the four foods was initially screened for digestibility and acceptance in separate digest panels. All foods provided acceptable stool quality and digestibility; fiber inclusion slightly decreased digestibility and improved stool quality (data not shown). Two separate dietary intervention trials were then performed, each individually assessing the impact of adding fiber to either the HM food (study #1) or the GR food (study #2). After a 1-week prefeed period during which dogs consumed a commercial maintenance food (Hill's Pet Nutrition® Prescription Diet® canine i/d®), both healthy dogs and those with chronic enteritis/gastroenteritis were randomized into two groups and provided HM or HM Fiber (study #1) for 4 weeks, at which time feces and blood samples were collected (Fig. S2). Subsequently, the groups of dogs crossed-over and consumed the HM or HM Fiber food that was not consumed during the first treatment feeding phase. Study #2 employed the same design but utilized GR and GR Fiber as experimental foods. Food quantities were calculated to maintain weight and provided to dogs in individual feed units for 30 minutes with pre- and post-weights recorded to assess intakes.

Digestibility tests

The digestibility tests were run according to the Association of American Feed Control Officials digestibility protocols.[47]

Stool scoring

The primary endpoint in this study was to assess changes in stool quality when fiber was added to the HM or GR foods. Fresh feces were assessed for subjective quality parameters according to a 5-point index, where 1 does not have solid form and 5 is > 80% firm.[48]

Serum chemistry and cell counts

Serum chemistry and complete blood count profiles were obtained from fasted dogs (minimum of 12 hours) to assess continued health of dogs throughout the study. These values did not exceed or diminish beyond acceptable clinical ranges.

Fecal sample collection

Fecal samples were collected, homogenized and frozen as aliquots within 1 hour of defecation. Whole feces were collected after defecation and homogenized thoroughly by hand until visually uniform. Homogenous samples were aliquoted into labeled cryovials. The tubes were snap-frozen immediately in liquid nitrogen followed by storing at −80° C until further processing.

Fecal proximate analyses and mineral composition of ash

Proximate, vitamin, amino acid, fatty acid, and mineral analyses were performed using certified official compendial methods where available by ISO accredited commercial laboratories. Moisture of fecal samples was determined by spreading feces in an aluminum pan and drying for approximately 3 hours (modified AOAC 935.29). Ash values were determined by weighing a portion of the fecal sample in a small ceramic crucible and heating to 600°C for approximately 2 hours (AOAC 942.05). Minerals were evaluated by grinding the dried fecal sample in a ball mill followed by acid digestion (Modified EPA 200.2); mineral analysis took place on an Agilent 5100 OES.

Fecal metabolomics and short chain fatty acids analysis

Analysis of fecal metabolomic profiles was performed by a commercial laboratory (Metabolon, Morrisville, NC) as previously described.[49] SCFAs were separated from fecal matter by liquid-liquid extraction under basic conditions with inclusion of an internal standard. Extracts were clarified by centrifugation and then acidified in the presence of methyl-t-butyl ether (MTBE). MTBE layers were separated by centrifugation, and SCFAs were resolved by capillary gas chromatography with flame ionization detection (Agilent 6890 Gas Chromatograph).

DNA extraction, 16S rDNA amplicon sequencing, and processing

Total DNA was extracted from frozen feces samples using the PowerFecal DNA isolation kit (MO BIO, Carlsbad, CA) according to the manufacturer's instructions with the modification of introducing a sonication step before vortexing the bead tubes with fecal samples horizontally for 15 minutes. PCR amplification was performed by using the primer pairs 341F and 806R spanning the V3-V4 hypervariable regions of the 16S rRNA gene along with Illumina adapters.[50] Amplicon sequencing was performed using the Illumina 16S metagenomic sequencing library preparation protocol (15044223 Rev. A). The sequences were de-multiplexed based on the dual index sequences by employing the Miseq built-in metagenomics workflow to obtain FASTQ files. FASTQ sequence files were processed using standard parameters for QIIME.[51] Sequences were deposited in the NCBI Sequence Read Archive under Accession No. SRP159154.

Data transformation and bioinformatics processing

FASTQ files were pre-processed into contigs from pairs of reads, chimeras removed, and bacterial taxonomic classification obtained using Mothur

software.[52] A modified protocol of the MiSeq standard operating procedure[53] published on the Mothur website[54] was used to perform bacterial taxonomic classification per the GreenGenes reference taxonomy.[55] OTUs were identified based on taxonomic hierarchy. OTUs were further processed using the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) protocol[56] to correct for copy numbers of the 16S genes in their respective taxa. Since numerical values obtained in all the steps above are inherently compositional and not normally distributed, they were centered log-ratio (CLR) transformed to enable appropriate statistical analysis in the Real Space, using the natural log.[57] Rows and columns of data that contained only zeros were removed and remaining zeros were imputed using Bayesian-multiplicative treatment of count zeros with simple replacement on the matrix of estimated probabilities[58] before the CLR transformation. Further statistical analyses were performed on the data as described below.

Statistical analysis

The sample size for enrollment was based on a power analysis using data from initial digestibility studies. For stool scores from the HM digest test versus those from the HM Fiber digest test, a sample size of 11 dogs in each group would provide 80% power to detect a between-group difference at a significance level of 0.05. For microbiome analysis, two stool samples were collected on subsequent days as described above, extracted and sequenced separately, and analyzed as an average of the data from both samples. All dogs produced two stools for all foods except subjects #9 (HM Fiber food) and #31 (HM food), each of whom produced only one stool on the indicated foods; thus, microbiome data from the single stool was used. Stool was successfully collected from subject #30 only when consuming the HM food, and data from this sample was excluded from paired t-test analyses.

Alpha diversity is presented as genus level taxa richness and overall diversity and was calculated on genus-level count data using the R vegan package.[59] For overall alpha diversity, the exponential of the Shannon index and the inverse of the Simpson index were utilized. Together with taxa richness, these metrics estimate where 1) taxa abundance is not considered in the diversity calculation (taxa richness), 2) taxa contribute to the estimator equally according to their abundance ($e^{Shannon}$; expShannon), and 3) more highly abundant taxa contribute more to the resulting diversity value (1/Simpson; invSimpson).[60] Formally this is noted as "true diversity" of orders "q" = 0, 1, 2. RLE, which assesses the contribution of community evenness to overall diversity,[61] was calculated with custom R scripts.[62] These scripts are exercised through a browser interface using the R shiny package[63] and are available upon request. Beta diversity was calculated as the ratio of gamma/alpha diversity or as CqN and plotted using the same custom R scripts as with RLE.[64] Statistical comparison of alpha diversity metrics across health status were performed as Student's t-test (unpaired), while comparisons within health status were performed using a within-animal paired t-test. RLE and beta diversity are presented as qualitative curves where increasing values of "q" on the x-axis denote the increasing influence of higher abundance taxa (and increasing negligence of lower abundance taxa) on diversity plotted on the y-axis.[65] Phylogenetically derived abundance UniFrac distances were also calculated and plotted as PCoA using the R package phyloseq.[66]

In order to assess the interactions of food background (HM vs GR), health status, and addition of fiber on alpha diversity, a full factorial mixed model analysis was performed on the diversity metrics that included the 15 healthy and 14 enteritis dogs that had consumed all four diets across both studies (HM ± Fiber, GR ± Fiber). 'Individual dog' was designated as a random effect while fixed effects were 'food type,' 'health status,' and 'fiber added.' The full factorial table of parameters is in Table S4. Commercially available software was used to perform mixed model analysis (JMP®, Version 12.1–13.1. SAS Institute Inc., Cary, NC).

For multivariate analysis of the degree to which biochemical groupings changed with the addition of fiber, MANOVA was performed. For univariate endpoints, a within-individual paired t-test was used to determine group differences resulting from the comparison of HM versus HM Fiber as well as GR versus GR Fiber.

Differences are reported as significant if $p < 0.05$. Stool quality scores were assessed by likelihood ratio and Pearson Chi-Square tests for group differences. All other end points were exploratory and uncorrected P values are reported.[67] A post hoc, paired t-test analysis of the change with fiber addition was performed to assess the effect of health status. JMP® software was used to perform multivariate and paired or unpaired univariate tests.

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Disclosure of interest

M.I.J. and D.E.J. are employees of Hill's Pet Nutrition, Topeka, KS, USA.

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