Importance of gut microbiota for the health and disease of dogs and cats

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Implications

- Molecular tools for bacterial identification and metagenomics, and mass spectroscopy for metabolomics allow a better understanding of the role of intestinal microbiota in health and disease
- Profound alterations in the intestinal microbiota are found in chronic and acute gastrointestinal diseases of dogs and cats, but the microbiota also play roles in extraintestinal diseases such as atopic dermatitis, obesity, and diabetes
- Phylogenetic and functional changes in the microbiota of animals with inflammatory bowel disease appear to be similar across dogs, cats, and humans, suggesting a core microbiome with conserved functions across mammals

Key words: bacteria, immune, inflammatory bowel disease, intestinal probiotics

Introduction

The intestinal microbiota can be defined as the dynamic collection of microorganisms within the gastrointestinal (**GI**) tract and the system of interactions these organisms have with each other and with the host cells. Molecular tools have allowed us to characterize the intestinal microbiota of dogs and cats in more detail. Comparative analyses of the bacterial 16S rRNA gene have provided vast amounts of phylogenetic data from both healthy and diseased animals (Suchodolski et al., 2008a; Handl et al., 2011). More recent functional approaches including metagenomics and metabolomics have begun to relate phylogenetic information to physiologic function (Swanson et al., 2011; Deusch et al., 2014). The intestinal microbiota is a dynamic system and the composition varies within an individual (i.e., different locations of the GI tract, luminal vs. mucosa-adherent, or different time points; Ritchie et al., 2008; Suchodolski et al., 2008a) as well as between individuals (Desai et al., 2009). The composition can also be influenced by diet (Lubbs et al., 2009; Swanson et al., 2011; Deusch et al., 2014), antibiotics (Suchodolski et al., 2009; Igarashi et al., 2014), GI disease (Inness et al., 2007; Guard et al., 2015), age (Deusch et al., 2015), and other genetic and environmental factors. The intestinal microbiota has several roles in the maintenance of host health including defending against non-resident intestinal pathogens, aiding in development of a healthy epithelium and immune system, and providing nutrients for the host via fermentative and metabolic activities (Suchodolski, 2011). It is these complex interactions among the microbiota, immune system, and host genetics that influence the balance between health and disease. This review will characterize the healthy intestinal microbiome, summarize methods of characterization, and discuss changes that occur in dogs and cats with gastrointestinal diseases.

Characterization of the Microbiota

Traditional bacterial culture methods have now been largely replaced by molecular tools for characterization of the complex intestinal microbiota (Tannock, 2005). The first of these molecular methods is next generation (high-throughput) sequencing of the bacterial 16S rRNA gene. This gene contains highly conserved sequences of nucleotide bases that are unique to bacteria as well as a region that contains phylogenetic information about the group and species level (Tannock, 2005). Using this method, we have been able to identify major bacterial groups in feces of healthy dogs and cats. The phylum Firmicutes comprised the majority of bacterial sequences in both dogs and cats in some studies (Ritchie et al., 2010; Handl et al., 2011). Clostridia was the most predominant class of bacteria with more than 65% of sequences belonging to this group containing important bacterial groups such as *Clostridium* clusters XIVa and XI and *Ruminococcus* (Handl et al., 2011). Other studies identified predominant phyla including Bacteroidetes, Proteobacteria, Actinobacteria, and Fusobacteria; however, relative abundances of these bacterial groups varied significantly between studies (Ritchie et al., 2010; Handl et al., 2011; Garcia-Mazcorro et al., 2011; Guard et al., 2015). These discrepancies may be due to differences in genomic DNA extraction methods, DNA amplification protocols, and sample storage conditions. Handl et al. (2011) examined the operational taxonomic units (**OTUs**) at the genus level and found that only 5 of 85 OTUs identified were detected in all 12 dogs, and 14 of 113 OTUs identified were detected in all 12 cats. This suggests that the intestinal microbiota is quite different between individuals. The intestinal microbiota also varies from one collection site in the GI tract to another (i.e., duodenum vs. ileum vs. feces). There is a general increase in diversity and total number of bacteria moving from the duodenum to the colon, and aerobic or facultative anaerobic bacteria predominate in the small intestine while anaerobes thrive in the large intestine (Mentula et al., 2005; Ritchie et al., 2008; Suchodolski et al., 2008a). In dogs, Clostridiales decreased along the GI tract from 40% of 16S rRNA clones in the duodenum to only 26% in the colon (Suchodolski et al., 2008a). In cats, Clostridiales did not exhibit this same decline, however, *Lactobacillus* greatly decreased from the jejunum to the colon (Ritchie et al., 2008). *Clostridium* cluster IV was most abundant in the small intestine and cluster XIVa was most abundant in the large intestine in both dogs and cats (Ritchie et al., 2008; Suchodolski et al., 2008a). *Clostridium* clusters IV and XIVa are important producers of short-chain fatty acids (**SCFAs**) and other metabolites that are

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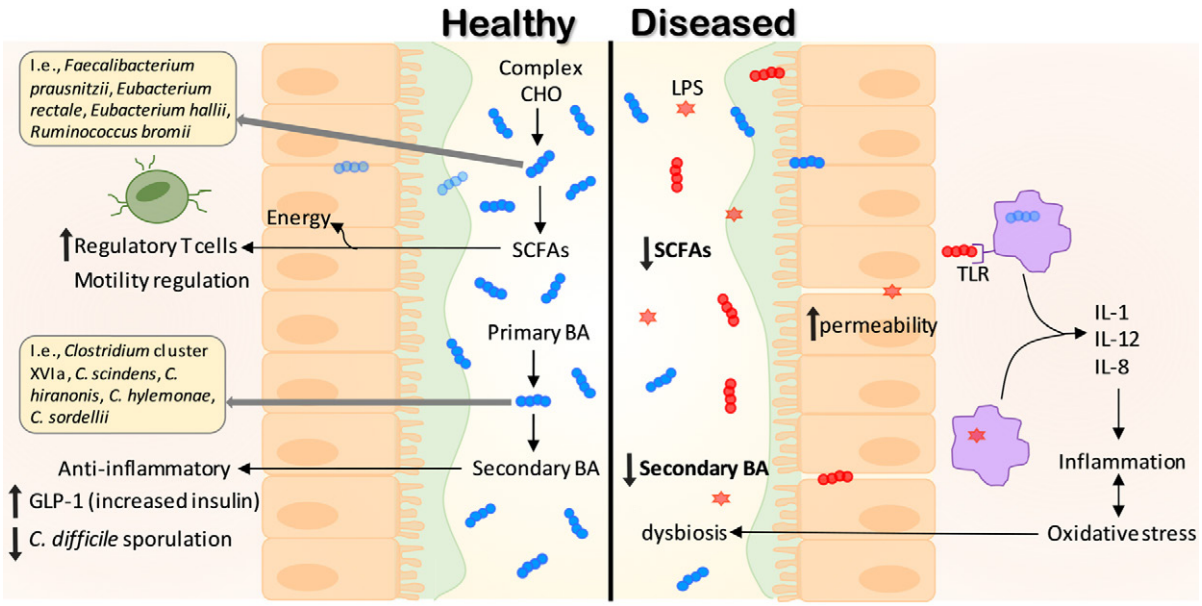

Figure 1. Schematic diagram representing some major microbiota-associated pathways in health and disease. Commensal bacteria in a healthy state convert complex carbohydrates (CHO) into beneficial short-chain fatty acids (SCFAs) that provide energy for endothelial cells, increase anti-inflammatory regulatory T cells, and modulate intestinal motility. Commensal bacteria in the colon also drive the conversion from primary bile to secondary bile acids, and these have anti-inflammatory properties, induce GLP-1 (increases insulin), and decrease, for example, sporulation of *Clostridium difficile*. In a diseased state, the decreased production of antimicrobial peptides and mucus leads to an increase in the permeability of the endothelium and the translocation of bacteria. Toll-like receptors (TLR) on macrophages and other cells recognize specific pathogen-associated molecular patterns, such as lipopolysaccharides in bacterial cell walls (LPS), and trigger inflammatory reactions. Macrophages phagocytize pathogenic microbes, which also triggers an immune response in the host that can lead to oxidative stress. Oxidative stress, in turn, can cause intestinal dysbiosis

beneficial to intestinal epithelium and the host immune system. While next-generation sequencing of the 16S rRNA gene is one of the most powerful methods for characterization of the gut microbial communities, it is important to note that it still has limitation for detecting low abundant bacterial taxa within the total community. Therefore, a combination of next-generation sequencing and quantitative PCR for specific bacterial taxa is often employed to characterize these low abundant bacterial groups.

Shotgun sequencing, also referred to as whole metagenome sequencing, uses short-sequence reads of all of the genes present and therefore provides functional information of the microbiome in addition to phylogenetic information. The reads are referenced against libraries of microbial genomes to predict functional genes and bacterial species present (Qin et al., 2010). However, this method is fairly expensive and computationally demanding when compared with 16S rRNA sequencing, and therefore has been only sparsely applied in veterinary medicine today. To overcome this hurdle, Langille et al. (2013) created a computational approach that can predict the functional gene families based off of the 16S rRNA data with high accuracy. The program is called PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) and is available online as free software. Swanson et al. (2011) demonstrated that GI metagenomes are more closely related between dogs and humans followed by rodent models. Due to many of these primary functional gene categories relating to metabolism, this suggests that humans and dogs also share many metabolic microbial pathways.

An emerging approach to identify bacterial and host metabolites is untargeted metabolomics. Identifying these metabolites allows better correlations between metabolic changes within the host and microbiome alterations. Commonly used platforms are GC–MS, LC-MS, and nuclear magnetic resonance spectroscopy. These analyses are ideally performed on multiple samples from the same individual (e.g., serum, urine, and feces) to better understand the metabolic changes in different organ systems. Once metabolites of interest are identified in an untargeted approach, then assays can be developed to specifically target these compounds (Weckwerth, 2003). Common metabolic end products produced by the intestinal microbiota include lactate, ammonia, and SCFAs. Metabolite profiles and functional metagenomes are more similar between individuals than phylogenetic composition, which supports the hypothesis that there is a core microbiome that exhibits functional redundancy (Dethlefsen et al., 2008; Turnbaugh et al., 2009).

In addition to bacteria, the GI tract is home to various other microorganisms including fungi, viruses, and parasites. The complex interactions these organisms have with the host and bacterial microbiota are not well understood. Recent studies have used next-generation sequencing with panfungal primers to reveal the fungal component of the microbiota in dogs and cats (Suchodolski et al., 2008b; Handl et al., 2011; Foster et al., 2013). *Ascomycota* was the most abundant fungal phylum in dogs (99.62%) and was the only phylum found in cats (Handl et al., 2011). The fungal phyla Basidiomycota, Glomeromycota, and Zygomycota were also found in dogs, with *Nacaseomyces* being the most abundant genus (76.72%) in one study (Handl et al., 2011) and *Candida* being the most abundant genus (5.2%) in another study (Foster et al., 2013). *Saccharomyces* (58.31%) and *Aspergillus* (11%) were the most abundant fungal genera found in cats (Handl et al., 2011). It is also interesting to note that fungal DNA was significantly more prevalent in mucosal brush samples than luminal samples, suggesting that some resident fungi may be mucosa adherent (Suchodolski et al., 2008b). Studies on the viral component in the GI tract of dogs and cats are limited. Swanson et al. (2011) used 454 pyrosequencing methods on canine fecal DNA and found that 99% of the viral sequences identified in the samples could be classified as bacteriophages. Shotgun sequencing evaluation of dsDNA viruses in feline feces revealed only one order of bacteriophages, Caudovirales (Tun et al., 2012). Future

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studies are needed to elucidate the complex interactions between host cells and all microbial populations of the canine and feline GI tract.

Various studies performed in dogs and cats analyzed the effects of dietary compositions on GI microbiota. Using qPCR methods, Lubbs et al. (2009) showed minor effects on the microbiota in cats fed a high-protein diet vs. a moderate protein diet. In contrast, Deusch et al. (2014) found that different protein-to-carbohydrate ratios have an impact on the microbiota of growing kittens. A high-protein/low-carbohydrate diet led to an increase in species richness when compared with a moderate-protein/moderate-carbohydrate diet, and functional differences related to metabolism and amino acid biosynthesis were also identified between groups (Deusch et al., 2014). Another study looked at the effect of dietary fiber in dogs, comparing a low-fiber diet to one that contained 7.5% beet pulp (Swanson et al., 2011). The low fiber led to greater percentage of Bacteroidetes, *Fusobacteria*, and Proteobacteria and a lower percentage of Firmicutes and the Chlorobi group of *Bacteroidetes* when compared with the diet containing 7.5% beet pulp. However, functional groups in the metagenome were not significantly altered (Swanson et al., 2011).

Also, there are many other factors that influence the composition of the microbiota, for example, antibiotic and probiotic administration. A variety of antibiotics have been shown to alter the GI microbiota (Suchodolski et al., 2009; Igarashi et al., 2014), and while the majority of bacterial taxa return to pre-treatment state within several weeks, some remain altered for extended periods of time. For example, one study in healthy humans revealed that some bacterial groups failed to recover within 6 mo after treatment with ciprofloxacin (Dethlefsen et al., 2008). Probiotic strains are detectable in feces of dogs and cats during administration and can alter the intestinal microbiota to a limited extent, but these alterations generally revert after administration is stopped (Garcia-Mazcorro et al., 2011). The effect of probiotics on the canine and feline microbiota are discussed in further detail elsewhere (Schmitz and Suchodolski, 2016). While these initial studies have shown that diet, probiotics, and antibiotics may impact the intestinal microbiota, further in-depth experiments are needed to evaluate how these modifications influence the microbial transcriptome and metabolome as well as the immunological responses of the host. Furthermore, while most studies analyzed only fecal samples, as they are easier to collect in a practical setting, it will be useful to also evaluate the microbiome, metabolome, and transcriptome in proximal parts of the GI tract to more comprehensively understand the effects of diet, probiotics, and antibiotics.

Roles of the Microbiota in Health

The intestinal microbiota plays a large role in maintaining the overall health of the host's gastrointestinal tract. Its functions include defending against non-resident intestinal pathogens, aiding in development of a healthy epithelium and immune system, and providing nutrients for the host via fermentative and metabolic activities. After initial colonization of the intestinal tract in utero or shortly after birth, the microbial community evolves into the typical adult population over several months (Buddington, 2003). The commensal bacterial population provides the host with colonization resistance where the resident microbes outcompete non-resident microbes for vital resources. This system can fail if pathogens outcompete the resident microbes or if certain opportunistic pathogens in the resident microbiota overgrow and cause an imbalance. Some examples of enteric pathogens associated with diarrhea in dogs and cats are *Salmonella*, *Campylobacter jejuni*, toxigenic *Clostridium perfringens*, and *Escherichia coli*; however, these groups are often isolated from healthy animals as well (Marks et al., 2011). One type of *E. coli* has recently been associated with granulomatous colitis in boxer dogs, and it is mucosa adherent and invasive (Craven et al., 2011). This finding shows that resident bacteria interact closely with the host immune system and suggests that colonization resistance and innate immunity susceptibilities may have an underlying genetic breed component.

The intestinal microbiota has a significant impact on the intestinal epithelial structure, as demonstrated by several studies in animal models (Al-Asmakh and Zadjali, 2015). Germ-free mice have changes in the intestinal morphology, motility, physiology, and function when compared with specific-pathogen-free and wild-type mice. Germ-free mice show a decrease in the small intestinal surface area, shorter ileal villi and crypts, thinner lamina propria, longer transit time, lower intestinal fatty acid concentrations, and reduced osmolarity (Al-Asmakh and Zadjali, 2015). These findings re-emphasize the importance of the intestinal microbiota in the proper development of the epithelial tissue structure. The microbiota also helps the immune system develop and function properly. Studies have shown that probiotic administration to dogs and cats can have immunomodulatory effects (Benyacoub et al., 2003; Marshall-Jones et al., 2006; Rossi et al., 2014). When healthy adult cats were given the probiotic *Lactobacillus acidophilus* DSM13241 (2×10^8 CFU/d) for 4.5 wk, phagocytic capacity was increased in the peripheral granulocytes and plasma endotoxin concentrations decreased (Marshall-Jones et al., 2006). Administration of probiotic lactic acid bacteria *Enterococcus faecium* SF68 (5×10^8 CFU/d) to puppies for 20 wk resulted in increased fecal IgA concentration, improved immune response to canine distemper virus vaccine, and increased proportion of mature B cells (Benyacoub et al., 2003). A recent in vitro study revealed that the probiotic *Lactobacillus acidophilus* LAB20 attenuated lipopolysaccharide-induced inflammatory cytokine IL-8 secretion from enterocytes and strengthened the intestinal barrier (Kainulainen et al., 2015). The probiotic mixture VSL#3 (112 to 225×10^9 lyophilized bacteria per 10 kg per day) also induced immune responses in dogs with inflammatory bowel disease, decreasing CD3+ T cell infiltration and increasing regulatory T cell markers (Rossi et al., 2014). Therefore, changing the microbiota composition can have direct effects on animal health by influencing immune function. Future studies should investigate the use of probiotics as therapeutic agents for the management of gastrointestinal diseases in dogs and cats.

The intestinal microbiota provides nutrients for the host via fermentative and metabolic activities. Complex carbohydrates generally pass through the small intestine undigested by the host and reach the large intestine. Here, microbial fermentation creates beneficial SCFAs, which provide energy for endothelial cells and regulate intestinal motility, an important defense mechanism against adherent bacteria (Figure 1). Furthermore, SCFAs induce regulatory T cells, which are anti-inflammatory (Suchodolski, 2011). Some examples of SCFA-producing bacteria are *Faecalibacterium prausnitzii*, *Eubacterium rectale*, *Eubacterium hallii*, and *Ruminococcus bromii* (Louis et al., 2010; Ze et al., 2012). Other bacterial metabolic end products may have direct or indirect effects on host health, but more studies are needed to elucidate these effects in dogs and cats.

Microbiota Alterations in Disease

Although the intestinal microbiota is generally associated with influencing gastrointestinal diseases, it has recently been discovered that the microbiota has a role in many extraintestinal disorders as well. Studies in humans have shown that the intestinal microbiota impacts several disease

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processes such as atopic disorders (Rutten et al., 2015), central nervous system disorders (Catanzaro et al., 2015), autoimmune diabetes (Dolpady et al., 2016), and multiple sclerosis (Wekerle, 2015). Future research in canine and feline models of disease will help us to understand the multi-system role that the intestinal microbiota has in health and disease.

Short-term changes in the intestinal environment, such as in cases of acute diarrhea, affect the microbial composition. Dogs with acute diarrhea show decreased microbial diversity, with lower numbers of Bacteroidetes and *Faecalibacterium* and higher numbers of *Clostridium* (Guard et al., 2015). In this same study, fecal concentrations of propionic acid significantly decreased in dogs with acute diarrhea, and functional genes for transposase enzymes and methyl-accepting chemotaxis proteins were also underrepresented in these dogs compared with healthy controls (Guard et al., 2015). Chronic enteropathy in dogs and cats is characterized by persistent signs of GI disease (i.e., vomiting or diarrhea) and can be clinically classified as food-responsive, antibiotic-responsive, or steroid-responsive, depending on the response to treatment. Idiopathic inflammatory bowel disease (**IBD**) is a subset of chronic enteropathies that exhibit inflammation in the GI tract, GI symptoms with no known cause, and poor response to the above treatments (Simpson and Jergens, 2011). The pathogenesis of IBD is not well understood, but there are several hypotheses that link microbial dysbiosis, intestinal inflammatory immune response, and genetic predisposition as factors that influence the development of IBD (Packey and Sartor, 2009). Interestingly, studies performed on multiple species concluded that IBD is associated with decreases in, for example, Firmicutes, and increased gram-negative bacteria (i.e., Enterobacteriaceae; Simpson and Jergens, 2011). In duodenal biopsies from dogs with IBD, 454-pyrosequencing revealed decreased proportions of Prevotellaceae, Clostridiales, Bacteroidaceae, and Fusobacteria when compared with healthy dogs (Suchodolski et al., 2012). In another study, duodenal brush samples indicated that dogs with IBD have a distinct microbiota with increased mucosal Enterobacteriaceae and Firmicutes and decreased Bacteroidetes and Spirochaetes (Xenoulis et al., 2008). Fluorescence in situ hybridization on duodenal biopsies from cats with IBD revealed increased mucosal Enterobacteriaceae that correlated with changes in the intestinal epithelial structure and clinical signs of disease (Janeczko et al., 2008). Furthermore, there was an increase in pro-inflammatory cytokines IL-1, IL-8, and IL-12 in cats with IBD (Janeczko et al., 2008). Anti-inflammatory cytokines and regulatory T cells have also been shown to decrease in dogs with IBD (Maeda et al., 2016). Toll-like receptors (**TLR**) are pro-inflammatory receptors in the innate immune system that recognize molecular patterns associated with microbes (Figure 1). Expression of TLR-2, 4, and 9 have been shown to increase in duodenum and colon mucosa of dogs with IBD (Burgener et al., 2008). German shepherd dogs are anecdotally more susceptible to GI diseases. Kathrani et al. (2010) found single-nucleotide polymorphisms (**SNPs**) in TLR-4 and TLR-5 that were significantly associated with IBD. Furthermore, SNPs in TLR-5 were associated with hyper-responsiveness to flagellin (Kathrani et al., 2012), the major protein component of bacterial flagella. More studies are needed to determine other potential genetic factors and their functional importance in the pathogenesis of canine and feline IBD.

While there are abundant studies looking at the metagenome and metabolome in humans with GI disease, studies focused on dogs and cats are limited. It has been demonstrated in humans that functional gene changes, including alterations in oxidative stress pathways and decreased amino acid biosynthesis, are more consistent than phylogenetic changes in patients with IBD (Morgan et al., 2012). Similar functional changes were recently found in cats and dogs with chronic GI diseases (Minamoto et al., 2015; Suchodolski et al., 2015). Cats with diarrhea had increased functional genes for transcription factors, tryptophan metabolism, epithelial cell signaling, and glycerolipid metabolism and decreased functional capacity for biosynthesis of secondary metabolites and biotin metabolism (Suchodolski et al., 2015). Dogs with IBD also had increased functional genes for transcription factors and decreased genes for amino acid metabolism (Minamoto et al., 2015). Minamoto et al. (2015) performed untargeted metabolomics analysis on the serum of dogs with IBD and found that several metabolites increased, including 3-hydroxybutyrate, hexuronic acid, ribose, and gluconic acid lactone. Ribose and gluconic acid lactone are both involved in the pentose phosphate pathway, which protects the cells from oxidative stress, suggesting that dogs with IBD also have increased oxidative stress (Minamoto et al., 2015). A preliminary study using untargeted metabolomics on feces of dogs with IBD showed decreased tryptophan metabolites, secondary bile acids, and phytosterols and increased primary bile acids when compared with healthy dogs (Honneffer et al., 2015). Secondary bile acids are anti-inflammatory, increase secretion of GLP-1, which is involved in insulin up-regulation, and decrease *C. difficile* sporulation, making them an important part of the healthy intestinal ecosystem (Figure 1) (Katsuma et al., 2005; Koenigsknecht et al., 2015). Conversion of primary to secondary bile acids involves generally two steps: deconjugation of glycine or taurine by bile salt hydrolase (**BSH**) and dehydroxylation. Commensal bacteria in the colon include dehydroxylating bacteria such as *Clostridium scindens*, *C. hiranonis*, *C. hylemonae*, and *C. sordellii*, as well as *Lactobacilli*, a major source of BSH (Ridlon et al., 2014). The overall objective of investigating the microbial and metabolic alterations would be to identify biomarkers for chronic GI diseases and possible therapeutic treatments.

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Summary and Conclusions

There have been many recent advances in the characterization of canine and feline intestinal microbiota in health and disease. Profound alterations in the intestinal microbiota have been found in chronic and acute gastrointestinal diseases, but it is recently being discovered that the microbiota may also have roles in extraintestinal diseases. We are in the beginning stages of relating phylogenetic changes to functional changes and broadening our search for biomarkers and therapeutic agents for disease. Studies suggest that much of the research on the mammalian intestinal microbiota can be translational to other species due to a core microbiome that has conserved functional genes across mammals. Some limitations of the current microbiome studies on dogs and cats are small sample sizes and difficulties distinguishing bacterial groups that have only a small amount of DNA present in the biological sample. Further studies are needed to elucidate the complex interactions of the intestinal microbiome with the host immune system and host genetics so that we may examine the full breadth of effects on health and disease.

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