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Review

# Diagnosis and interpretation of intestinal dysbiosis in dogs and cats

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ABSTRACT

The intestinal tracts of dogs and cats harbor a highly complex microbiota, which consists of bacteria, fungi, viruses and protozoa. Until recently, traditional bacterial culture was commonly used to identify bacteria present in the gastrointestinal tract, but it is now well recognized that standard plating techniques do not have enough resolution for identification of the mostly anaerobic bacteria that reside within the gut. Molecular methods are now established for assessing intestinal dysbiosis in dogs and cats with gastrointestinal disease, but these approaches are not yet widely available for routine diagnosis. The loss of normal commensal bacterial microbiota (i.e. Lachnospiraceae, Ruminococcaceae, and *Faecalibacterium* spp.) in acute and chronic intestinal diseases has been linked to metabolic changes, for example alterations in immunomodulatory bacterial metabolites, such as short chain fatty acids and secondary bile acids. This highlights the importance of dysbiosis in the pathophysiology of gastrointestinal diseases. Development of molecular based assays for specific bacterial groups, calculations of microbial dysbiosis indices and assays for microbial functional metabolites are currently underway to help assess dysbiosis. These will yield a better understanding of the pathophysiology of gastrointestinal diseases and may also lead to new diagnostic and therapeutic approaches to dysbiosis.



## Introduction

The intestinal microbiota is the consortium of all living microorganisms (bacteria, fungi, protozoa and viruses) that inhabit the gastrointestinal (GI) tract. Whilst the term 'microflora' is often used in the older literature, 'microbiota' (from 'bios', Greek: 'life') is the proper term. Bacteria are the most abundant microbes in the intestine (Swanson et al., 2011). Until recently, traditional bacterial culture was commonly used to identify bacteria present in the GI tract. It is now recognized that the vast majority of intestinal bacteria cannot be cultured using standard plating techniques.

There is no correlation between bacterial counts in the small intestine and disease status (German et al., 2003). Qualitative fecal culture does not yield enough resolution to characterize the complex large intestinal microbiota comprehensively. Molecular methods, mostly targeting the 16S rRNA gene, are now the recognised standard for identification of bacterial microbiota. Such approaches have demonstrated that the canine and feline GI tracts harbor a highly complex microbial ecosystem, consisting of several hundred different bacterial phylotypes (Handl et al., 2011).

An estimated trillion ($10^{12}$–$10^{14}$) microbial cells are present in the GI tract, which is approximately 10 times more than the number of all host cells. Furthermore, the combined genetic pool of all intestinal bacteria outnumbers the host gene content by a factor of 100. This new insight into the complexity of the intestinal microbiota and its intimate relationship with the host has spurred research to better understand the importance of a balanced microbial ecosystem for regulation of host health and immunity. Intestinal dysbiosis can be defined as an alteration in the composition and/or richness (i.e. the number of unique bacterial species) of the intestinal microbiota. Studies in human beings and veterinary species have associated intestinal dysbiosis with various GI disorders, such as inflammatory bowel disease (IBD), granulomatous colitis and irritable bowel syndrome (IBS) (Suchodolski et al., 2012a and b; Honneffer et al., 2014; Minamoto et al., 2014).

Although it is not always clear whether dysbiosis is a cause or an effect of GI disease, there is likely to be an overlap, since inflammation will cause dysbiosis, and recent functional studies have demonstrated that dysbiosis, when present, is a risk factor that may exacerbate inflammation in genetically susceptible individuals. Therefore, reestablishment of normobiosis should be a desired treatment outcome. However, research to better define signatures of dysbiosis associated with different diseases is still at an early stage.

It is also important to note that there is an overlap in the dysbiosis patterns of many GI diseases. At this time, no specific dysbiosis signatures for GI diseases have been described that can be used diagnostically to distinguish subsets of chronic enteropathies (CE). However, various dysbiosis indices and metabolic alterations currently are being evaluated, and these may have diagnostic and therapeutic utility in the future. This review will provide a brief

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overview of methods to assess the GI microbiota and dysbiosis, the major bacterial groups in the canine and feline GI tracts, and the role of dysbiosis in the pathophysiology of GI diseases.

## Assessment of microbiota and dysbiosis

There is no single gold standard for assessing the GI microbiota and dysbiosis. Most current research is focused on evaluating the bacterial microbiota and methods have been optimized for characterization of bacteria. Since the gut microbiota is a complex and dynamic ecosystem, the best diagnostic approach would be a combination of molecular tools that include PCR amplification of 16S rRNA genes using broad universal bacterial primers, followed by analysis of amplicons by next generation sequencing (NGS), direct quantification of specific bacterial taxa by quantitative PCR (qPCR) and the use of fluorescent in situ hybridization (FISH) to visualize the translocation of bacteria into the mucosal epithelium. Future studies will also need to incorporate measurements of bacterial metabolites, such as fecal bile acids and short chain fatty acids (SCFA), to assess microbiota function and to evaluate changes in the host immune system.

Bacterial culture can be a useful technique for detection of specific enteropathogens (e.g. *Salmonella* spp., *Campylobacter jejuni*, *Yersinia* spp.). Cultivation allows the determination of an active infection (i.e. viability of isolated organisms), antibiotic sensitivity testing of clinical specimens and genotyping of cultured isolates for epidemiological studies. However, it is now recognized that bacterial culture is not well suited for in-depth characterization of complex environments, such as the mammalian GI tract. Since the majority of intestinal bacteria cannot be cultured, this method underestimates total bacterial numbers and also does not allow identification of the majority of bacterial groups in the GI tract. Reasons for the inability to culture most bacteria include lack of knowledge regarding their optimal growth requirements and because the canine and feline GI tracts harbor predominantly anaerobic bacteria, which are fragile and prone to handling damage. It is currently estimated that less than 20% of intestinal bacteria are cultivable with standard laboratory techniques.

Molecular tools allow the identification of previously uncharacterized intestinal microbes and these techniques are also able to provide information about the functionality of the microbiome by means of metagenomics. Several methods are available and all of these approaches ideally would be used in a complementary fashion (Table 1). However, most of these methods are currently available for research only. The use of NGS of 16S rRNA genes is a useful tool to assess the intestinal microbiota, since this approach provides an overview of the proportions of all bacterial groups within the entire microbiota. Due to costs and turnaround time of results, NGS is not currently widely available for routine diagnostic use. However, it is important to note that bacterial groups with low abundance (especially pathogens) are typically present at such a low proportion of the total bacteria that they may escape identification even when high throughput techniques are employed. Therefore, for the detection or quantification of bacterial groups in low abundance, the additional use of group specific PCR primers is recommended.

It is important to note that bacterial phylotypes can possess multiple copies of the 16S rRNA gene, which can vary vastly in number amongst individual bacterial species (1–15 copies of the 16S rRNA gene can be present). Therefore, qPCR results cannot be related directly to absolute bacterial cell counts. The use of FISH is currently considered to be the most accurate method for quantification of bacterial groups because it is based on microscopic counts rather than amplification of DNA. However, this approach does not allow high throughput analysis of samples. A further advantage of FISH is to visualize the location of bacteria with regard to the epithelium (i.e. intracellular, adherent or invasive).

It should also be noted that molecular tools, such as 16S rRNA gene based techniques, have inherent limitations. Bias is introduced during DNA extraction, primer selection, PCR amplification and sequence analysis. As examples, insufficient cell lysis during DNA extraction can underestimate the population of Gram positive bacteria, whilst a lysis protocol that is too harsh can diminish DNA recovery from Gram negative bacteria. Some commonly used primers and PCR protocols underestimate the presence of specific bacterial groups, for example *Bifidobacterium* spp. In view of these potential biases, caution should be applied when comparing quantitative results across studies that have used different DNA extraction methods and PCR protocols.

In addition to identification of bacterial groups, a key to understanding the impact of the microbiota on GI health is to explore the functionality of the microbial community. In metagenomics, DNA is extracted from a biological sample and is then directly sequenced without prior amplification of specific genes. The results yield a snapshot of the gene pool and functional potential of the microbiome, and have been applied in dogs and cats (Swanson et al., 2011; Barry et al., 2012). An emerging area is investigation of the role of host and bacterial metabolites in various GI disorders. This approach can improve our understanding of complex metabolic pathways, with the goal to find novel biomarkers for the etiology, progression and treatment of GI diseases. Targeted measurement of specific metabolites has already been performed in veterinary

**Table 1**
Methods commonly used for characterization of the intestinal microbiota.

| Method | Description | Advantages | Disadvantages |
|---|---|---|---|
| Fluorescence in situ hybridization (FISH) | Fluorescent dye-labeled oligonucleotide probes are hybridized to ribosomal RNA sequence in bacterial cells | Identification, quantification, visualization of bacterial cells in tissues (luminal versus cell-adherent versus mucosa-invasive) | Labor intense, fluorescent in situ hybridisation (FISH) probes need to be developed for each bacterial group of interest |
| Quantitative real-time PCR (qPCR) | Target organisms are detected in real-time using fluorescent dye-labeled primers and/or probes | Detection and relative quantification of bacterial groups in a sample; rapid turnaround (few hours), inexpensive | Assays need to be designed for each bacterial group of interest |
| Next-generation sequencing (e.g. 454-pyroseqeucning, Illumina) | Bacteria in a sample are amplified using universal primers; PCR amplicons are then sequenced using a high-throughput sequencer | Identification of (theoretically) all bacteria present in a sample; semi-quantitative; indicates relative changes in bacterial groups within a community | Requires advanced bioinformatics; long turnaround; potential for false positive signals from reagents |
| Metagenomics (shotgun sequencing of genomic DNA) | Genomic DNA is fragmented and then randomly sequenced (without PCR amplification) on a high-throughput sequencer | Provides not only identification of bacteria, but also which functional genes are present in sample | Very expensive, requires advanced bioinformatics |

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medicine, for example measurements of serum concentrations of cobalamin and folate, and fecal concentrations of SCFA.

Untargeted metabolomics is a technique that provides an unbiased profile of metabolites using mass spectrometry. Several hundred metabolites can be measured in a single analysis and can be used to better understand the alterations in biochemical pathways that occur as a consequence of GI inflammation and dysbiosis (e.g. alterations in various amino acids, tryptophan pathways and bile acid dysmetabolism).

## Gastrointestinal microbiota of healthy dogs and cats

Using traditional bacterial culture, initial studies reported that the bacterial load in the small intestine of healthy dogs ranges from $10^2$ to $10^5$ colony forming units (CFU)/g, with some studies observing numbers as high as $10^9$ CFU/g (German et al., 2003). In the colon, the number of cultivable bacteria is much higher than the small intestine, ranging from $10^8$ to $10^{11}$ CFU/g (Mentula et al., 2005). Cats consistently have higher numbers of bacteria in their duodenum than dogs (Johnston et al., 1999).

Molecular methods have enabled more detailed identification of bacteria present within the canine and feline GI tracts (Suchodolski, 2011). The small intestine harbors a mixture of aerobic and facultatively anaerobic bacteria, while the large intestine is home almost exclusively to anaerobes (Suchodolski et al., 2008a). Firmicutes, Bacteroidetes, Proteobacteria and Fusobacteria are the major bacterial phyla, constituting approximately 99% of all gut microbiota in dogs and cats (Handl et al., 2011; Chaban et al., 2012). *Helicobacter* spp. predominate in the canine stomach, but *Actinobacillus* and *Streptococcus* spp. also can be found routinely (Garcia-Mazcorro et al., 2012). The small intestine harbors predominantly *Clostridium* spp., Lactobacillales and Proteobacteria, while Clostridiales, *Bacteroides* spp., *Prevotella* spp. and Fusobacteria predominate in the large intestine. In addition to differences in the composition of the microbiota along the GI tract, each animal harbors a unique microbial profile (Fig. 1) (Suchodolski et al., 2005). However, the metagenomes (i.e. functional gene content) are conserved, suggesting that functional aspects of the microbiomes are similar across individual animals (Guard and Suchodolski, 2016). Mathematical algorithms can be used to combine levels of various bacterial groups

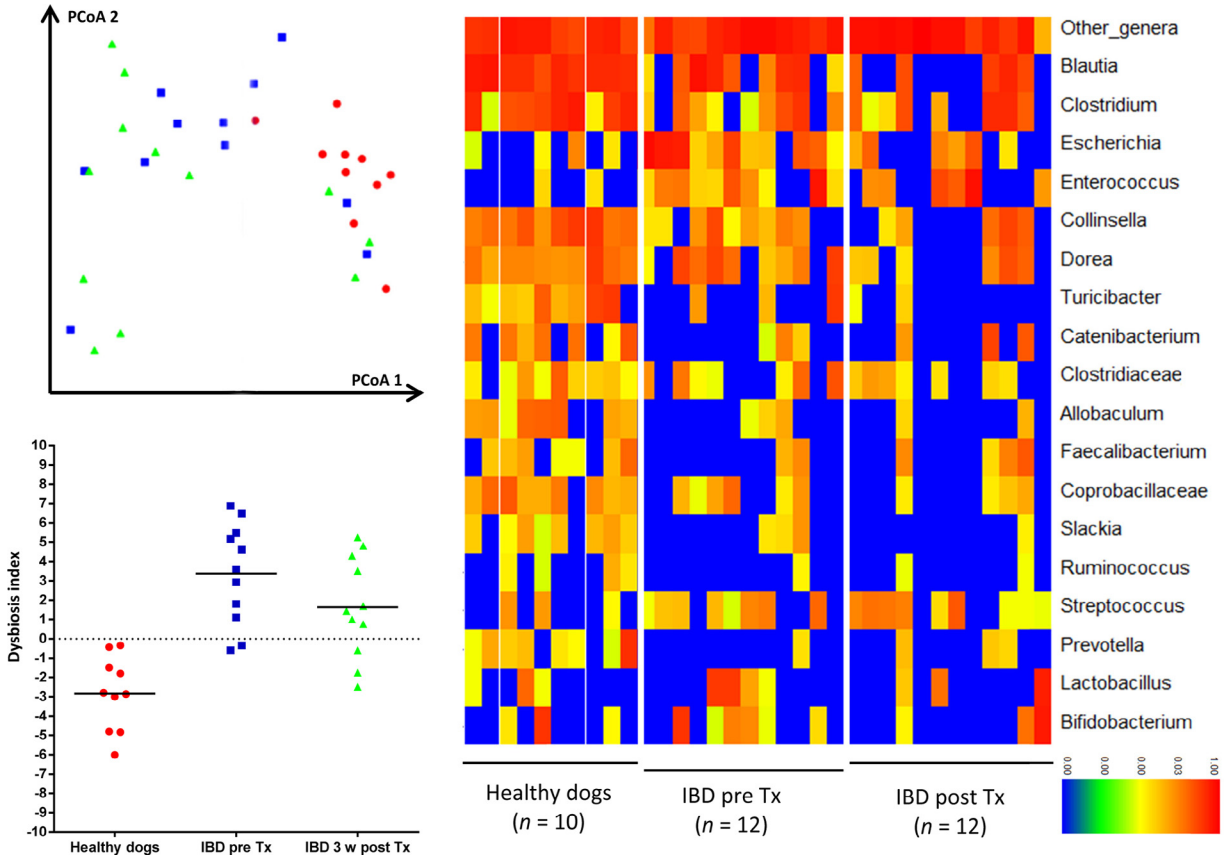


**Fig. 1.** Dysbiosis in dogs with idiopathic inflammatory bowel disease (IBD) before and after 3 weeks of therapy. This figure demonstrates how the same data can be illustrated by a heat map and various mathematical algorithms that take into account the bacterial composition within each fecal sample. Fecal DNA is extracted and then the bacterial composition is identified by amplification of 16S rRNA genes, followed by sequencing. Data based on Minamoto et al. (2015). Left top: The data can be displayed using principal coordinate analysis (PCoA) plot, where the data are displayed across the two main principal coordinates (PCoA 1 and 2). Each dot represents the total bacterial community within one sample. The closer the two dots, the more similar is their bacterial community (i.e. they share many bacterial taxa). The more distance between two dots, the more difference there is between the two bacterial communities. Each dot (i.e. sample) is then colored by phenotype, in this case healthy dogs (red), dogs with IBD before therapy (blue) and dogs with IBD 3 weeks after medical therapy (green). The figure on the left illustrates that the healthy dogs cluster more closely together and away from the IBD dogs, indicating differences in the fecal microbiota between healthy dogs and dogs with IBD. Three weeks after therapy and despite improvement in clinical disease activity (CIBDAI score), the microbiota changed only slightly and did not yet cluster closer to the microbiota of healthy dogs. Left bottom: The same data are displayed as an index that summarizes the abundances of major bacterial taxa in each fecal sample as one single numerical value (a positive number indicates dysbiosis). This index can then be used to monitor changes in gut microbiota over time. As in the left figure, the data indicate that, after 3 weeks of therapy, the microbiota has not returned to a state similar to that of healthy dogs. Right: The most abundant bacterial genera of these dogs are displayed as a heat map. Each column represents the bacterial genera in each sample; each row summarizes the abundance of these genera (the more red the higher the abundance). The heat map shows the major abundant genera in healthy dogs are decreased in dogs with IBD.

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that are different for each individual into one number (Fig. 1), which can be used to track the direction of changes in the microbiota in disease and in response to therapy.

Fungi and viruses also are important members of the microbiota, but their role in health and disease is still being evaluated. Based on metagenomic sequence counts, fungi make up approximately 2% of microbial cells in fecal samples (Swanson et al., 2011). Dogs and cats harbor multiple fungal species in their gut; up to 40 different phylotypes were reported in fecal samples of individual dogs (Foster et al., 2013). Therefore, we expect to find fungi occasionally on routine fecal examinations. The role of fungi in GI disease is uncertain, since no clear differences in specific phylotypes were observed when fecal samples of healthy dogs were compared with fecal samples of dogs with acute diarrhea (Foster et al., 2013) or duodenal samples of dogs with CE (Suchodolski et al., 2008b).

## Intestinal microbiota – Contribution to health

A balanced microbial ecosystem is of crucial importance for host health, since it provides stimuli for the immune system, helps in the defense against enteropathogens and provides nutritional benefits. The presence of bacteria is also important for proper development of gut structure, since germ-free (gnotobiotic) mice have an altered epithelial architecture. An emerging research area is understanding how microbiota modulates host health and disease. Several studies have described the intestinal microbiota and its functional gene pool (metagenome) in dogs and cats (Swanson et al., 2011; Barry et al., 2012; Guard et al., 2015; Minamoto et al., 2015). The interactions between intestinal bacteria and the host immune system are mediated either via direct contact between bacteria and the innate immune system (e.g. toll-like receptors, NOD2 receptors) or through microbial metabolites. These metabolites can be produced directly by bacteria (e.g. vitamins, SCFA) or are primary host metabolites that are converted through bacterial enzymes into secondary metabolites (e.g. conversion of primary to secondary bile acids).

Bile acids are an excellent example of the close interactions between the gut microbiota and the host. Only gut bacteria can convert primary bile acids that enter the colon into secondary bile acids. The optimal ratio of primary to secondary bile acids is considered to be an important regulator of gut homeostasis, since they downregulate inflammation, inhibit germination of *C. difficile* spores, and modulate insulin and glucose metabolism through activation of glucagon-like peptide 1 (GLP-1) (Pavlidis et al., 2015). Gut dysbiosis leads to bile acid alterations, with potential negative metabolic consequences for the host (Duboc et al., 2013).

The bacterial phylum Firmicutes, a major constituent of intestinal microbiota, comprises many phylogenetically distinct bacterial groups, the so-called *Clostridium* clusters. These groups (e.g. *Ruminococcus*, *Faecalibacterium* and *Dorea* spp.), together with Bacteroidetes and Actinobacteria (i.e. *Bifidobacterium* spp.), are believed to be important producers of metabolites that have a direct beneficial impact on host health (Table 2). As examples, nutrient sources for bacteria are complex carbohydrates (e.g. starch, cellulose, pectin and inulin) and fermentation of these substrates results mainly in the production of SCFA (e.g. acetate, propionate and butyrate). These act as energy sources for the host, regulate intestinal motility and are important growth factors for epithelial cells. SCFA also have anti-inflammatory properties, since they induce immunoregulatory T cells ($T_{reg}$) (Arpaia et al., 2013). Other bacterial metabolites, such as secondary bile acids and indole (a byproduct of tryptophan degradation), are also anti-inflammatory, thereby maintaining immune homeostasis and strengthening intestinal barrier function (Bansal et al., 2010; Duboc et al., 2013).

## The role of intestinal dysbiosis in the pathophysiology of gastrointestinal diseases

Some reasons for the development of dysbiosis are summarized in Table 3. Disease processes may be associated with changes in microbiota function (e.g. reduced production of SCFA and other metabolites, and an altered bacterial enzyme pool) rather than shifts in microbiota composition. These functional or immunological alterations are not readily detected, since we are still not able to properly assess the entire microbiota and its functions. The microbiota varies along the GI tract, and there are also clear differences between mucosal and luminal microbiota (Suchodolski et al., 2004, 2005; Manchester et al., 2013; White et al., 2015). Furthermore, it is almost impossible to properly assess the entire interactions between the microbiota and the host immune system (Kathrani et al., 2012). Since these inaccessible factors are likely to play a crucial role in the intricate communication between bacteria and the host immune system, crude assessment of bacterial changes in intestinal samples most often does not reveal the entire disease process. Nevertheless, much progress has been made in characterizing intestinal dysbiosis in GI diseases, and metabolomics have also provided insights into the functional consequences of dysbiosis and its role in the pathophysiology of some GI disorders in human beings (Duboc et al., 2013) and dogs (Honneffer et al., 2015).

### *Intestinal microbiota in acute and chronic enteropathies*

Dysbiosis has been described in dogs with GI diseases (e.g. IBD and acute diarrhea), in cats with CE, and in dogs and cats infected with *Giardia duodenalis* (Suchodolski et al., 2012b, 2015; Guard et al., 2015; Minamoto et al., 2015; Slapeta et al., 2015). In human and

**Table 2**
Importance of microbial derived metabolites in the gastrointestinal tract.

| Metabolic activities of intestinal microbiota | Metabolic end products | Effect on host health and changes in disease |
|---|---|---|
| Fermentation of carbohydrates | SCFA (e.g. propionate, acetate, butyrate) | Anti-inflammatory, energy source of enterocytes, regulation of intestinal motility, regulatory T cells, amelioration of leaky gut barrier; SCFA are reduced due to dysbiosis |
| Vitamin synthesis | Vitamin A, K2, B12, biotin, folate | Important co-factors for various metabolic pathways and generation of regulatory T cells; dysbiosis leads to alterations in vitamin B12 and folate metabolism |
| Induces degradation of sphingomyelin via alkaline sphingomyelinase | Ceramide | Promotes normal apoptotic mechanisms to limit dysplasia and neoplastic transformation |
| Degradation of the amino acid tryptophan | Indole | Increases epithelial-cell tight-junction resistance and attenuates indicators of inflammation; indole pathways are disrupted in dysbiosis associated with IBD |
| Deconjugation/dehydroxylation of bile acids | Secondary bile acids (cholate/deoxycholate) | Intestinal fat absorption; regulation of insulin via GLP-1 activation; provides anti-inflammatory signals; secondary bile acids are reduced in dysbiosis associated with IBD and antibiotic administration |
| Carbohydrate fermentation | D-lactate | Increases in D-lactate due to dysbiosis in EPI and chronic enteropathies; increases associated with encephalopathy |

GI, gastrointestinal; SCFA, short chain fatty acids; IBD, inflammatory bowel diseases; GLP-1, glucagon-like peptide-1; EPI, exocrine pancreatic insufficiency.

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**Table 3**
Disorders associated with intestinal dysbiosis.

| |
|---|
| **Inflammatory diseases of the intestine** |
| Chronic enteropathies (food responsive, antibiotic responsive) |
| Idiopathic inflammatory bowel disease |
| **Acute diarrhea** |
| Acute hemorrhagic diarrhea syndrome |
| Acute diarrhea due to various origins (infectious and non-infectious) |
| **Intestinal stasis** |
| Anatomic abnormalities |
| Congenital blind loops |
| Small bowel diverticula, strictures or adhesions |
| Surgical resection of the ileocolic valve |
| Surgical blind loops (end-to-side anastomosis) |
| Partial obstructions of the small intestine |
| Neoplasia |
| Foreign bodies |
| Chronic intussusception |
| **Motility disorders** |
| Hypothyroidism |
| Diabetic autonomic neuropathy |
| Scleroderma |
| Abnormal migrating motor complexes |
| **Decreased gastric acid output** |
| Atrophic gastritis |
| Administration of acid suppressing drugs ($H_2$-blockers, omeprazole) |
| **Exocrine pancreatic insufficiency** |
| Decreased output of pancreatic antimicrobial factors |
| **Environmental factors** |
| Antibiotics |

canine IBD, there are increases in the proportions of bacterial genera belonging to Proteobacteria (e.g. *Escherichia coli*, *Diaphorobacter* spp.) and decreases in Fusobacteria, Bacteroidetes, and members of the Firmicutes (e.g. *Faecalibacterium* spp., Ruminococcaceae, *Turicibacter* spp., *Blautia* spp.). These changes have been observed in the duodenum (Xenoulis et al., 2008; Suchodolski et al., 2010, 2012a) and in fecal samples (Suchodolski et al., 2012b; Honneffer et al., 2014; Minamoto et al., 2014, 2015) of dogs with IBD. This indicates that, despite differences in microbial composition along the GI tract, dysbiosis due to a disease process in the small intestine can be identified in fecal samples. The dysbiosis in the duodenum was correlated with the severity of histopathological scores, but not with clinical disease severity, i.e. clinical IBD activity index (CIBDAI) (Suchodolski et al., 2012a). Generally, there is similarity in the patterns of dysbiosis observed in chronic versus acute diarrhea, but some notable differences have been described. As an example, in fecal samples of dogs with acute hemorrhagic diarrhea, substantial increases in the populations of *Clostridium perfringens* and Fusobacteria have been reported (Suchodolski et al., 2012b). In contrast, the latter taxon is typically decreased in feces of dogs with IBD. An increase in *C. perfringens* or its detection in fecal samples of dogs with diarrhea is commonly believed to be causative. However, a recent study would suggest that *C. perfringens* overgrowth occurs as an effect of intestinal dysbiosis and the loss of normal microbiota in chronic diarrhea (Minamoto et al., 2014). The role of *C. perfringens* enterotoxin has also been questioned in acute diarrhea (Busch et al., 2015). A novel pore forming toxin (*netF)* has been recently identified in a subset of dogs with canine hemorrhagic diarrhea (Mehdizadeh Gohari et al., 2015); this may be a focus of future diagnostic testing.

Cats with IBD had increased duodenal counts of Enterobacteriaceae, as assessed by fluorescence in situ hybridization (FISH); these counts were positively correlated with changes in mucosal architecture and the density of cellular infiltrates (Janeczko et al., 2008). Less is known about fecal dysbiosis in cats with IBD, since only two studies have evaluated the fecal microbiota of cats with confirmed IBD, using FISH to identify individual bacterial groups rather than sequencing. One study identified an increase in *Desulfovibrio* spp. in cats with IBD (Inness et al., 2007), while the second study did not find any differences between healthy cats and cats with IBD (Abecia et al., 2010). In a study utilizing 16S rRNA sequencing in cats with acute and CE, but with no clear diagnosis, cats with chronic diarrhea had decreased proportions of Bacteroidetes, *Faecalibacterium* spp. and *Turicibacter* spp., and increased proportions of Enterobacteriaceae, similar to dogs with IBD (Suchodolski et al., 2015).

The above studies have clearly identified duodenal and fecal dysbiosis in dogs with IBD. At the present time, no studies have evaluated whether dysbiosis patterns differ between the various forms of CE, food responsive enteropathy (FRE), antibiotic responsive enteropathy (ARE) and IBD. Long term follow-up studies are needed to examine whether the changes in microbiota revert with clinical remission. Initial studies have reported that the GI microbiota and serum metabolome undergo only minor normalization after 3 weeks (Minamoto et al., 2015; Fig. 1) or 8 weeks of therapy (Rossi et al., 2014), even if dogs show improvement in CIBDAI scores. This suggests that the microbiota remains altered due to the underlying disease or the residual histological inflammation present in the intestine (Rossi et al., 2014), which typically does not fully resolve in this time frame. Further studies are needed to correlate the long term outcome of affected animals (i.e. rate of clinical relapse) with the dynamics of dysbiosis.

Dysbiosis, including clinical signs associated with changes in the microbiota, can also be induced by administration of antimicrobial agents. Some broad spectrum antimicrobial agents, such as metronidazole, induce major changes in bacterial taxa; these changes resemble the dysbiosis patterns that are observed in CE (Minamoto et al., 2014, 2015; Gevers et al., 2014). Therefore, administration of antibiotics to healthy dogs may cause changes that mimic the dysbiosis seen in chronic GI disease. Continued administration of antibiotics during therapy may lead to the false impression on follow-up samples that the dysbiosis is persistent due to GI disease, whereas the changes may be attributable to antimicrobial treatment. In cases where antibiotics are administered chronically, serial evaluation of dysbiosis should be interpreted with care.

### *Functional consequences of dysbiosis in chronic enteropathies*

A dysbiotic microbiome may be directly deleterious to the host or the depletion of resident microbiota may lead to reductions in anti-inflammatory metabolites. Therefore, proper characterization of dysbiosis is desirable to enable a better understanding of the disease process in animals with GI disease and to guide treatment decisions. Changes in microbiota result in functional and immunological consequences for the host, but the extent of these changes will depend on the magnitude and the pattern of dysbiosis (i.e. which bacterial groups are altered) and the location of dysbiosis (small intestine versus large intestine). A better understanding of these phylogenetic and functional consequences may result in a better understanding of disease pathogenesis.

Bacteria in the small intestine reside in a very delicate relationship with the host, since many are adherent to the mucosa and, therefore, are important stimulants of mucosal immunity. Sudden dietary changes, including dietary indiscretion, and changes in the architecture of the intestine, with subsequent changes in intestinal motility (e.g. surgical creation of intestinal loops, short bowel syndrome and resection of the ileocolic valve) may lead to changes in bacterial populations. Exocrine pancreatic insufficiency (EPI) has been associated with an increase in total bacterial counts in the duodenum of dogs (Simpson et al., 1989). Subtle changes in microbiota composition may have significant effects on the immune response of the host. The microbiota may also compete with the host for nutrients and may produce deleterious metabolites. Small intestinal microbiota, especially *Lactobacillus* spp. and *Clostridium* spp. (*C. hiranonis* and *C. scindens*), deconjugate bile acids; an abnormal microbial composition may impair fat absorption. Other abnormal

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functions may be the dehydroxylation of fatty acids, destruction of brush border enzymes, damage of carrier proteins and competition for nutrients (e.g. vitamin B12). Enterotoxins produced by pathogenic bacteria can stimulate mucosal fluid secretions, while villous effacement and loss of surface area will diminish mucosal absorptive capacity, resulting in diarrhea. A dysfunction of the mucosal barrier can lead to an increase in intestinal permeability and clinically significant bacterial translocation.

Dysbiosis that occurs in the large intestine is typically associated with decreases in the major abundant bacterial taxa (e.g. *Blautia* spp., *Faecalibacterium* spp., Ruminococcaceae and *Turicibacter* spp.; Fig. 1), which produce SCFA, indoles and other immunomodulatory metabolites. Therefore, major effects on host metabolism are expected. Consequently, decreased abundances of Ruminococcaceae and *Faecalibacterium* spp. were correlated with decreased fecal propionate and increased fecal butyrate concentrations in dogs with acute diarrhea (Guard et al., 2015) and IBD (Minamoto et al., 2015). Dogs with acute diarrhea also had changes in the tryptophan pathway, as indicated by decreased urine concentrations of 2-methyl indole and 5-methoxy-1H-indole-3-carbaldehyde, and decreased serum kynurenic acid (a catabolite of kynurenine and tryptophan), as well as a decreased ratio of tryptophan to kynurenic acid (Guard et al., 2015).

Dogs with IBD had an altered global profile in serum metabolites compared to healthy dogs, with significant increases in oxidative stress pathways (Minamoto et al., 2015). Furthermore, the predicted fecal metagenome was consistent with decreased amino acid metabolism, suggesting that the microbiota of dogs with IBD is involved in dysfunctional protein metabolism. Studies employing untargeted metabolomics have associated fecal dysbiosis with reductions in immunomodulatory secondary bile acids in human beings (Duboc et al., 2013) and dogs (Honneffer et al., 2015), and tryptophan–indole pathways in dogs (Guard et al., 2015). Depletion of commensal groups (heat map in Fig. 1) and their respective immunoregulatory metabolites may impair the ability of the host to down-regulate the aberrant intestinal immune response; thus, dysbiosis becomes a component of the pathophysiology of the chronic disease process.

Recent epidemiological studies in human beings have revealed that dysbiosis caused by administration of drugs (e.g. antibiotics, non-steroidal anti-inflammatory drugs, gastric acid suppressants) is an important risk factor for some chronic diseases. Early exposure to antibiotics in childhood is associated with development of allergies (Metsala et al., 2015) and obesity (Saari et al., 2015), presumably due to antibiotic induced dysbiosis. Reduced diversity of the gut microbiota at the time of allogeneic hematopoietic stem cell transplantation is a risk factor for higher mortality outcomes (Taur et al., 2015). These initial data in human beings, combined with our better understanding of the immunomodulatory properties of the gut microbiota, suggest that proper diagnosis and correction of dysbiosis will become an important therapeutic goal. This could include the use of highly digestible diets and/or prebiotics, probiotic therapy and antimicrobial agents. However, not enough clinical data are currently available to make recommendations as to which dysbiosis patterns will respond best to which therapy.

### *Chronic enteropathies associated with mucosally invasive bacteria*

Granulomatous colitis, sometimes designated histiocytic ulcerative colitis, is a specific form of CE, which responds to antibiotics and has been associated with mucosal infiltration of invasive and adherent *E. coli* in the colon (Craven et al., 2011). Young Boxer dogs (typically <4 years of age) are affected most frequently and a genetic susceptibility has been proposed for this breed. However, other dog breeds, especially young French Bulldogs, may be affected. The predisposition of these dogs to *E. coli* associated granulomatous colitis suggests they harbor a genetic defect that impairs their ability to eliminate invasive *E. coli*. In situ analysis of mucosal biopsies from dogs with granulomatous colitis using FISH probes against *E. coli* demonstrates multifocal clusters of invasive bacteria within macrophages in the intestinal mucosa. Therapy with antibiotics (i.e. enrofloxacin) for 8 weeks correlates with remission from disease (Manchester et al., 2013).

### *Small intestinal dysbiosis: Antibiotic responsive diarrhea*

In human beings, small intestinal bacterial overgrowth (SIBO) is defined as an increased bacterial count in the small intestine (Johnston, 1999). In dogs, the existence of a similar syndrome is currently under debate. Early studies found increased bacterial counts in dogs with diarrhea compared to healthy dogs and the authors of this study defined SIBO as $>10^4$ anaerobic bacteria or $>10^5$ total CFU/mL in fasting duodenal juice (Batt et al., 1983; Rutgers et al., 1995, 1996). However, substantially higher bacterial counts in duodenal aspirates of healthy dogs have been found in subsequent studies (German et al., 2003). Furthermore, there was no correlation between the number of bacteria in the duodenum and clinical signs in dogs with CE (German et al., 2003).

Since this syndrome responds to antibiotic treatment, some authors are using the term antibiotic responsive diarrhea (ARD) (Hall, 2011). Also, a subgroup of dogs with antibiotic responsive diarrhea has been reported that is responsive specifically to tylosin; the term tylosin responsive diarrhea (TRD) has been proposed for this subgroup (Westermarck et al., 2005). Currently, no diagnostic workup is available that would allow a better definition of these subgroups. It is not clear whether these dogs have the same syndrome or whether subgroups exist that could be classified as small intestinal bacterial overgrowth, small intestinal dysbiosis (SID), tylosin responsive diarrhea or generally as antibiotic responsive diarrhea (ARD). It is important to note that disorders caused by potentially pathogenic bacteria, such as *Salmonella* spp., *Campylobacter* spp., enterotoxigenic *Clostridium perfringens* and *C. difficile*, are not included in this syndrome. Whilst healthy cats appear to have much higher duodenal bacterial counts than healthy dogs, and these numbers do not differ from cats with enteropathies (Johnston et al., 1993), there appears to be a subset of cats with CE that favorably responds to antibiotic administration.

Several physiological mechanisms regulate bacterial colonization in the small intestine. These include secretion of gastric acid and antibacterial factors (i.e. pancreatic and biliary secretions), and intestinal motility. Abnormalities in one or more of these control mechanisms may lead to small intestinal dysbiosis, resulting in clinical signs. As an example, dogs that received gastric acid suppressant therapy with a proton pump inhibitor exhibited alterations in the gastric and small intestinal microbiota (Garcia-Mazcorro et al., 2012). Pancreatic juice contains antimicrobial substances and dogs with EPI have significantly increased bacterial counts in the small intestine (Westermarck et al., 1993). This is associated with a poor response to pancreatic enzyme replacement therapy. The formation of blind and stagnant small intestinal loops is a common reason for bacterial overgrowth in humans, which may lead to signs of chronic GI disease.

Certain canine breeds, such as the German shepherd and Chinese Shar-Pei, appear to be predisposed to ARE. A genetic susceptibility for a dysregulation in the cell mediated immune response to normal luminal microorganisms is suspected in these dogs. IgA deficiency as an underlying risk factor has not been confirmed. The histopathology in German shepherds and other dogs with antibiotic responsive diarrhea typically is reported as normal to mild lymphocytic-plasmacytic IBD. However, recent studies have reported an abnormal response in innate immunity (altered toll-like receptor expression) in German shepherds, which may lead to

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hyper-responsiveness to bacterial flagellin in the small intestine (Kathrani et al., 2012).

It is difficult to diagnose SID/ARD definitively. Duodenal culture is not useful and molecular studies have not been reported. Therefore, it is unclear which bacterial groups are altered. A tentative diagnosis can be made on the basis of clinical signs, altered serum cobalamin and folate concentrations, and by an antibiotic therapeutic trial. However, since diseases due to undetected intestinal pathogens may respond to antibiotic therapy, a positive response to therapy does not necessarily confirm the presence of small intestinal dysbiosis. Differential diagnoses, such as parasites, bacterial pathogens, maldigestion due to EPI, IBD, intestinal lymphoma, lymphangiectasia and food intolerance should be ruled out. Histopathological assessment of small intestinal biopsies is often unremarkable. However, occasionally villous atrophy or fusion has been reported. Affected animals also should be evaluated for underlying factors, such as anatomical abnormalities.

### *Serum cobalamin and folate concentrations*

Serum cobalamin concentrations may be decreased and serum folate concentrations may be increased in dogs with SID/ARD. Changes in the small intestinal microbiota may lead to increased bacterial utilization of cobalamin, resulting in decreased absorption of cobalamin by the ileum. Bacteria in the distal small and large intestines produce folic acid, but folate absorption via carriers takes place in the proximal small intestine. When folate producing bacteria accumulate in the proximal small intestine, an increased amount of bacterial folate will be absorbed by the host, resulting in an increased serum folate concentration. However, cobalamin and folate uptake from the small intestine is highly complex and can be affected by several mechanisms; therefore, alterations are not highly specific for SID/ARD. A diet high in folate may lead to increased serum folate concentrations independent of disease. Inflammation of the ileum may damage cobalamin receptors and thus may lead to cobalamin malabsorption. Dogs with EPI have decreased secretion of antibacterial products, with subsequent small intestinal bacterial overgrowth (Simpson et al., 1989). As a consequence, dogs with EPI often have increased serum folate concentrations. Thus, in dogs with an abnormal serum concentration of cobalamin and/or folate, serum trypsin-like immunoreactivity should be evaluated to rule out EPI.

Contrary to expectations, administration of tylosin does not lead to a decrease in serum folate or an increase in serum cobalamin concentration (Ruaux et al., 2005). Therefore, serum folate concentrations may not reflect therapeutic success and serum folate concentrations should always be evaluated together with the clinical findings. When both serum cobalamin and folate concentrations are altered, this is suggestive of SID, but both have a relatively low sensitivity and specificity for the diagnosis of SID; the reported sensitivity of serum cobalamin concentration for a diagnosis is 25–55% and for serum folate concentration is 50–66% (German et al., 2003).

## Conclusions

We are still at an early stage in understanding the complexity of the intestinal microbiota and the metabolic consequences of dysbiosis in GI disease. Recent functional studies have clearly linked dysbiosis with a range of diseases in dogs and cats. However, at this time, not enough clinical data are available to be able to make recommendations as to which dysbiosis patterns will respond best to a specific therapy and affected animals need to be treated based on the entire clinical picture. While in some animals the use of antimicrobial agents is useful (e.g. animals with ARD), their use may exacerbate dysbiosis in other GI diseases that do not respond to antibiotics. Therefore, more clinical studies combining results from phylogenetic and functional microbiota analysis are required to better define the various signatures and therapeutic approaches to dysbiosis.

## Conflict of interest statement

The author is employed by the Gastrointestinal Laboratory at Texas A&M University, which offers gastrointestinal function testing on a fee-for-service base.

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