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CHAPTER 10

The Role of the Microbiota in Feline Inflammatory Bowel Disease

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Inflammatory bowel disease (IBD) is the collective term applied to a group of chronic enteropathies characterized by persistent or recurrent gastrointestinal (GI) signs and inflammation of the GI tract. It is widely accepted that IBD involves a complex interplay among host genetics, the intestinal microenvironment (principally bacteria and dietary constituents), the immune system, and environmental "triggers" of intestinal inflammation.[1] However, the specific steps that lead to IBD and the basis for phenotypic variation and unpredictable responses to treatment are not known. Recent advances in microbiology have enabled unforeseen insights into the composition and spatial distribution of intestinal bacteria, fungi, viruses, and protozoa (collectively the microbiota) in health and disease across species.[2-4] This chapter summarizes current knowledge of the role of the microbiota in feline IBD with a focus on bacteria.

WHAT BACTERIA COLONIZE THE HEALTHY FELINE GASTROINTESTINAL TRACT?

Culture-Based Analyses

Until recently, our knowledge of the bacterial composition of the feline GI tract was based on the culture of duodenal/jejunal juice or mucosa, colonic contents, and feces.[5-8] These studies found that *Bacteroide*s spp., *Clostridium* spp., *Enterococcus* spp., *Streptococcus* spp., *Fusobacteria* spp., and *Eubacteria* spp. are the most common bacteria cultured from the feline GI tract. In general, the number and type of bacteria vary according to the intestinal region, with the number and proportion of strictly anaerobic bacteria increasing from the duodenum to the colon, peaking at 10^{11} colony-forming unit (CFU)/g of feces. Although the small intestine contains fewer bacteria than the colon or feces, bacterial numbers in the small intestines of healthy cats vary widely, from less than 10^2 to greater than 10^8 CFU/mL, and frequently exceed the 10^5 CFU/mL reported as the upper limit of normal in healthy people.[7] This has important implications for clinicians seeking to make diagnosis of small intestinal bacterial overgrowth (SIBO).[7]

Culture-Independent Analyses

The advent of molecular microbiology has enabled the identification of bacteria using their genetic signatures without the need to grow them. Culture-independent analyses have revealed that culture-based methods dramatically underestimate the diversity of the enteric flora, typically identifying only 20% of the bacteria recognized by their 16S RNA signatures.[9] In other words, culture-based methods only identify bacteria that you know how to grow. The principal culture-independent methods used to identify bacteria are polymerase chain reaction (PCR), 16S ribosomal ribonucleic acid (rRNA) sequencing, and fluorescence *in situ* hybridization (FISH; Box 10-1). The 16S sequencing is frequently employed as a first step to create an inventory of bacteria that are present and their relative proportions.[10-12] Bacteria of interest can then be targeted for precise enumeration by PCR (primers with or without probes against a bacterial target) or FISH with oligonucleotide probes directed against bacterial 16 or 23S rRNA.[13] FISH can also be used to gain insight into the spatial distribution of bacteria in formalin fixed biopsies. For example, are they luminal, adherent, crypt associated, or invasive?[13]

16S-based studies in cats indicate that Firmicutes (most are gram-positive bacteria) is the dominant bacterial phylum throughout the GI tract, with Clostridiales (predominately *Clostridium* cluster XIVA) and Lactobacillales (includes the Enterococcaceae, Lactobacillaceae, and Streptococcaceae families) being the predominant orders.[12,14,15] These findings broadly parallel the results of culture-based studies where *Clostridium* spp. were identified in duodenal aspirates of more than 90% of cats.[6-8] *Enterococcus* spp., *Streptococcus* spp., and *Lactobacillus* spp. were the dominant species from the jejunum,[5] and *Enterococcus* spp. and *Lactobacillus* were the dominant species from the colon and feces.[5] The 16S-based analyses to date have indicated that the microbiota tends to be more similar within an individual than when comparing the same intestinal region among different cats.[15] FISH-based studies of duodenal mucosa-associated bacteria in healthy cats have shown that the majority of bacteria are present in free and adherent mucus, with a median of 48

BOX 10-1 Uncultured Approaches for Detecting Bacteria

Are bacteria present?
- 16S rRNA eubacterial PCR/qPCR
- Eubacterial FISH

What bacteria are present?
- Global: 16S rRNA sequencing
- Restricted subset: PCR, array, FISH

Where are bacteria localized?
- Eubacterial or restricted FISH

How many bacteria are present?
- qPCR, FISH

FISH, Fluorescence *in situ* hybridization; *PCR,* polymerase chain reaction; *qPCR,* quantitative real-time polymerase chain reaction; *rRNA,* ribosomal ribonucleic acid.

(0-399) bacteria/mm^2 of mucosa.[13] In healthy cats, the total numbers of bacteria hybridizing to probes against *Clostridium* spp., *Bacteroides* spp., *Streptococcus* spp., and Enterobacteriaceae represented only 6% of bacteria hybridizing to the EUB-338 probe.[13]

BACTERIA AND INFLAMMATORY BOWEL DISEASE/CHRONIC ENTEROPATHIES

When considering the role of bacteria in IBD, it is important to understand that IBD is a term applied to a diverse group of chronic enteropathies that are characterized by persistent or recurrent GI signs and inflammation of the GI tract.[16] IBD is often subcategorized on the basis of clinical signs (e.g., vomiting, diarrhea, and/or weight loss), clinicopathologic abnormalities (e.g., low cobalamin and/or low albumin), ultrasonographic findings (e.g., muscularis hypertrophy and/or regional lymphadenopathy), histopathology (cellular infiltrates and mucosal architecture), the anatomic region(s) involved (e.g., proximal small intestine, ileum, and/or colon), and response to therapy (e.g., diet-responsive, antibiotic-responsive, steroid-responsive, or unresponsive).[13,16-20] Feline IBD is also considered in the context of concurrent inflammation of other organs, such as the liver and pancreas ("triaditis") and kidneys.[21]

The most commonly diagnosed form of feline IBD is lymphocytic-plasmacytic inflammation of the proximal small intestine.[21] Unfortunately, this nomenclature is somewhat misleading as the numbers of lymphocytes and plasma cells in the small intestinal mucosa of cats with and without GI signs are broadly similar.[22] Thus the term *lymphoplasmacytic enteritis* is often more helpful at distinguishing this group of patients from those with neutrophilic, granulomatous, or eosinophilic infiltrates, than identifying cats with increased numbers of lymphocytes and plasma cells. The emergence of low-grade, T-cell, alimentary lymphoma in cats means that careful consideration has to be given to the number and regional distribution of mucosa-associated lymphocytes (e.g., clusters of intraepithelial lymphocytes) to enable distinction between lymphoplasmacytic enteritis and lymphoma.[23,24] It is common practice for pathologists to emphasize mucosal cellularity as the dominant histopathologic feature, with scant information provided about mucosal architecture. However, the finding that abnormalities in mucosal architecture, such as villous atrophy and fusion, correlate with proinflammatory cytokine upregulation and the severity of clinical signs in cats with IBD[13] indicates the importance of reporting architecture and cellularity.

There is a paucity of studies that have evaluated the microbiota in cats with GI disease. Studies to date have included client-owned cats with lymphocytic plasmacytic enteritis, less commonly neutrophilic or granulomatous ileitis or colitis; foster kittens with ill thrift; and colony-housed cats with signs of GI disease.

Intestinal Bacteria in the Duodenum of Cats with Lymphocytic Plasmacytic Enteritis

The only culture-independent study of the duodenal mucosal bacteria in cats to date employed FISH analysis to evaluate the numbers and types of bacteria associated with the duodenal mucosa and their relationship to clinical signs, histopathology, and mucosal cytokines.[13] Cats with signs of GI disease had more mucosal Enterobacteriaceae than healthy cats (Figure 10-1). The total number of mucosal bacteria was strongly associated with changes in mucosal architecture and the density of cellular infiltrates, particularly macrophages and T cells. A subset of bacteria comprising Enterobacteriaceae, *Escherichia coli*, and *Clostridium* spp. correlated with abnormalities in mucosal architecture (principally atrophy and fusion), proinflammatory cytokine upregulation (interleukin [IL]-1, -8, and -12), and the number of clinical signs exhibited by the affected cats (Figure 10-2). This study shows that changes in the number and type of mucosa-associated bacteria are related to the presence and severity of IBD in cats and raises the possibility that abnormal mucosal flora are involved in the etiopathogenesis of feline IBD. These changes in the microbiota closely parallel the microbial shifts, termed *dysbiosis,* observed in people, dogs, and murine models of IBD.[10-12,25] From a comparative standpoint, the histopathologic findings, microbial shifts, and cytokine profiles in cats with moderate to severe lymphoplasmacytic IBD resemble those associated with active celiac disease in people.[26-28] The potential for IBD-associated dysbiosis to effect change outside of the gut is increasingly recognized. Studies in cats with inflammatory liver disease[29] and severe pancreatitis (K.W. Simpson and D.C. Twedt, unpublished observations) have revealed the presence of intrahepatic and intrapancreatic bacteria, notably *E.coli* and *Enterococcus* spp., and raise the possibility that the inflamed, dysbiotic gut may be the source for these bacteria (Figure 10-3).[21,29]

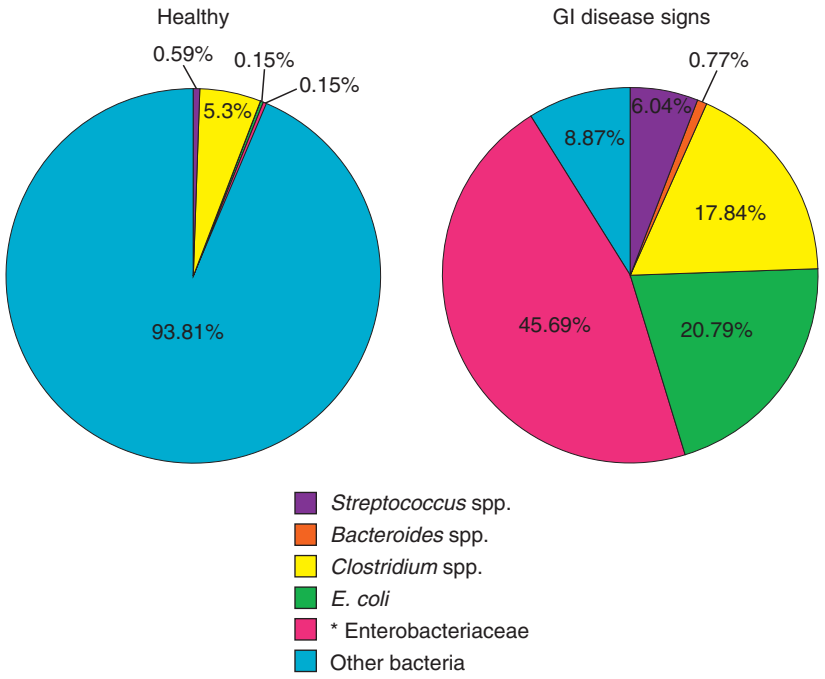

Figure 10-1: Proportion of mucosal *Streptococcus* spp., *Bacteroides* spp., *Clostridium* spp., *E. coli*, and Enterobacteriaceae in healthy cats and cats with signs of gastrointestinal (GI) disease. Numbers indicate the proportion (%) of bacteria recognized by the EUB-338 probe. *Enterobacteriaceae recognized by 1531 minus *E. coli.* (From Janeczko S, Atwater D, Bogel E, et al: The relationship of mucosal bacteria to duodenal histopathology, cytokine mRNA, and clinical disease activity in cats with inflammatory bowel disease. *Vet Microbiol* 128:178-193, 2008.)

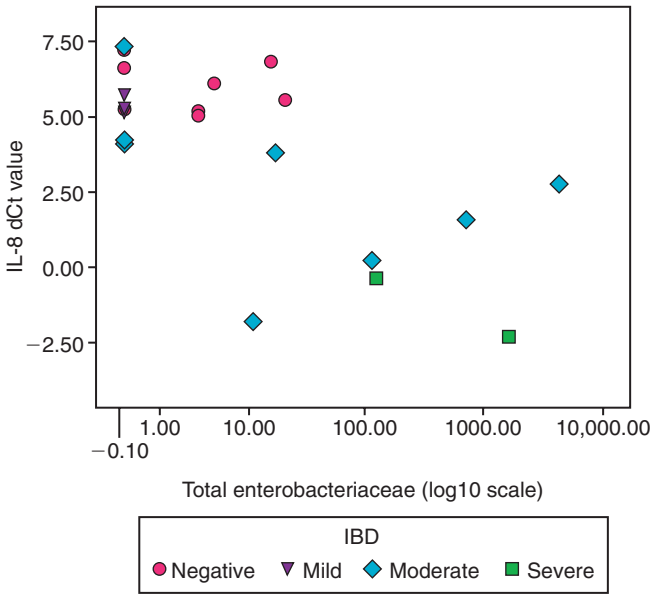

Figure 10-2: Interrelationship of interleukin *(IL)-8* upregulation (lower values for delta cycle threshold (dCT) on the Y axis indicate more upregulation), mucosal Enterobacteriaceae, and histopathologic severity of inflammatory bowel disease *(IBD).* (From Janeczko S, Atwater D, Bogel E, et al: The relationship of mucosal bacteria to duodenal histopathology, cytokine mRNA, and clinical disease activity in cats with inflammatory bowel disease. *Vet Microbiol* 128:178-193, 2008.)

Bacterial Involvement in Neutrophilic and Granulomatous Ileitis or Colitis

There is growing evidence to implicate bacteria and other infectious agents in the development of granulomatous and neutrophilic intestinal inflammation across species. Nonbacterial infections associated with this type of intestinal inflammation in cats include feline infectious peritonitis, fungi, and *Tritrichomonas fetus*.[30,31] Bacterial pathogens such as *Salmonella, Campylobacter,* and *Yersinia* have been associated with neutrophilic enteritis, whereas mycobacteria have been associated with granulomatous inflammation and regional lymphadenopathy.[32] The discovery of invasive *E. coli* in dogs with granulomatous colitis has changed our perception of this disease as an idiopathic immune-mediated condition to a bacterially driven disease in a susceptible host.[10] To date, there is only a single report of granulomatous/histiocytic ulcerative colitis in cats with invasive bacteria that responded to antibiotics.[33] Ileocolitis associated with spiral bacteria identified as an *Anaerobiospirillum* spp. has been described in six cats.[34] Four cats had clinical signs related to the GI tract, whereas two did not. The most significant and consistent histologic change was present in the colon and consisted of marked multifocal to diffuse dilations of the crypt lumina that were filled with large numbers of spiral bacteria, often accompanied by necrotic epithelial cells and degenerate leukocytes or associated with crypt abscesses consisting of

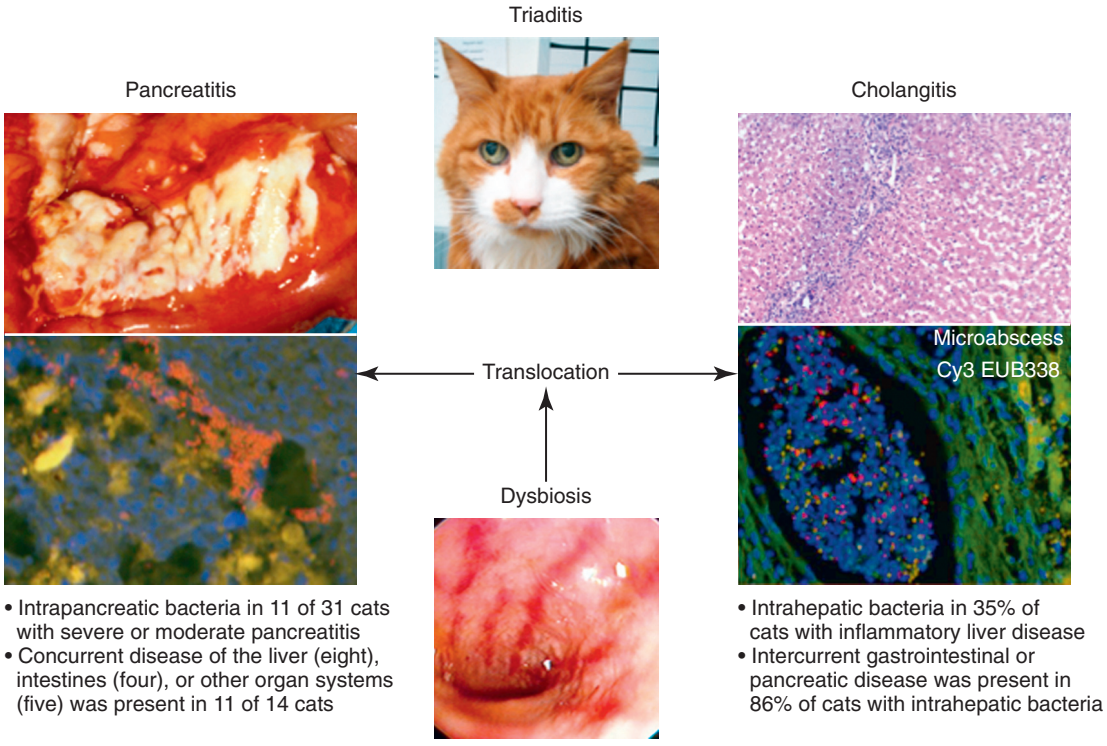

Figure 10-3: Could intestinal dysbiosis promote bacterial translocation to the liver and pancreas? "Intestinal inflammation", most frequently "lymphocytic plasmacytic" or small cell lymphoma, could promote dysbiosis and the translocation of enteric bacteria to the pancreas and liver across the inflamed intestines or pancreatico-biliary papilla. This scenario could lead to the development of triaditis.[21]

necrosis of the crypt epithelium and accumulation of polymorphonuclear neutrophils. Changes in the surface epithelium ranged from focal sloughing to multifocal erosions, particularly over submucosal lymphoid aggregates, to diffuse epithelial necrosis and mucosal ulceration. Inflammatory cells were usually relatively sparse and consisted mainly of lymphocytes, with fewer histiocytes and polymorphonuclear neutrophils. The presence of an *Anaerobiospirillum* spp. was demonstrated by genus-specific 16S PCR and electron microscopy. Nucleotide sequencing of three affected cats showed a close relationship to *Anaerobiospirillum succiniciproducens*. The colons of three clinically healthy cats without lesions and one cat with mild colitis not associated with spiral bacteria were negative for *Anaerobiospirillum* spp. in the same assay. Another study has also correlated the presence of bacteria with spiral morphology with enterocolitis in cats, but the identity of these bacteria was not determined.[35]

It is becoming apparent that routine histopathology is an insensitive means of detecting infectious agents in tissue samples with evidence of neutrophilic and granulomatous inflammation. Although histochemical stains for fungi, argyrophilic and acid-fast bacteria, coronavirus, and culture may improve detection (Figure 10-4A), it is these cases that are likely to benefit most from additional culture-independent evaluation using PCR, 16S sequencing, or FISH analysis.[10,31,34] Eubacterial FISH analysis of formalin-fixed tissues can be performed on the same tissue block used for histopathology. It seems a reasonable first step for screening for the presence of bacteria and their regional distribution within the gut (i.e., luminal, adherent, or invasive).[13] For example, FISH analysis enabled the identification of invasive spiral bacteria in the ileum, colon, and regional lymph nodes of a cat with pyogranulomatous ileocolitis associated with recurrent episodes of fever and leukocytosis whose inflammatory process was considered sterile on the basis of histopathology (see Figure 10-4C). FISH analysis of colonic mucosa from a cat with dilated crypts and neutrophilic infiltrates enabled the detection of bacteria in and around degenerating glands (see Figure 10-4D) that responded to tylosin but not metronidazole or enrofloxacin.

Ileal Biofilm-Forming Bacteria in Foster Kittens

Approximately 15% of foster kittens die before 8 weeks of age, with most of these kittens demonstrating clinical signs or postmortem evidence of enteritis.[36,37] Lesions observed via light microscopy in the GI tract of kittens were largely nonspecific as to etiology and characterized in many cases as

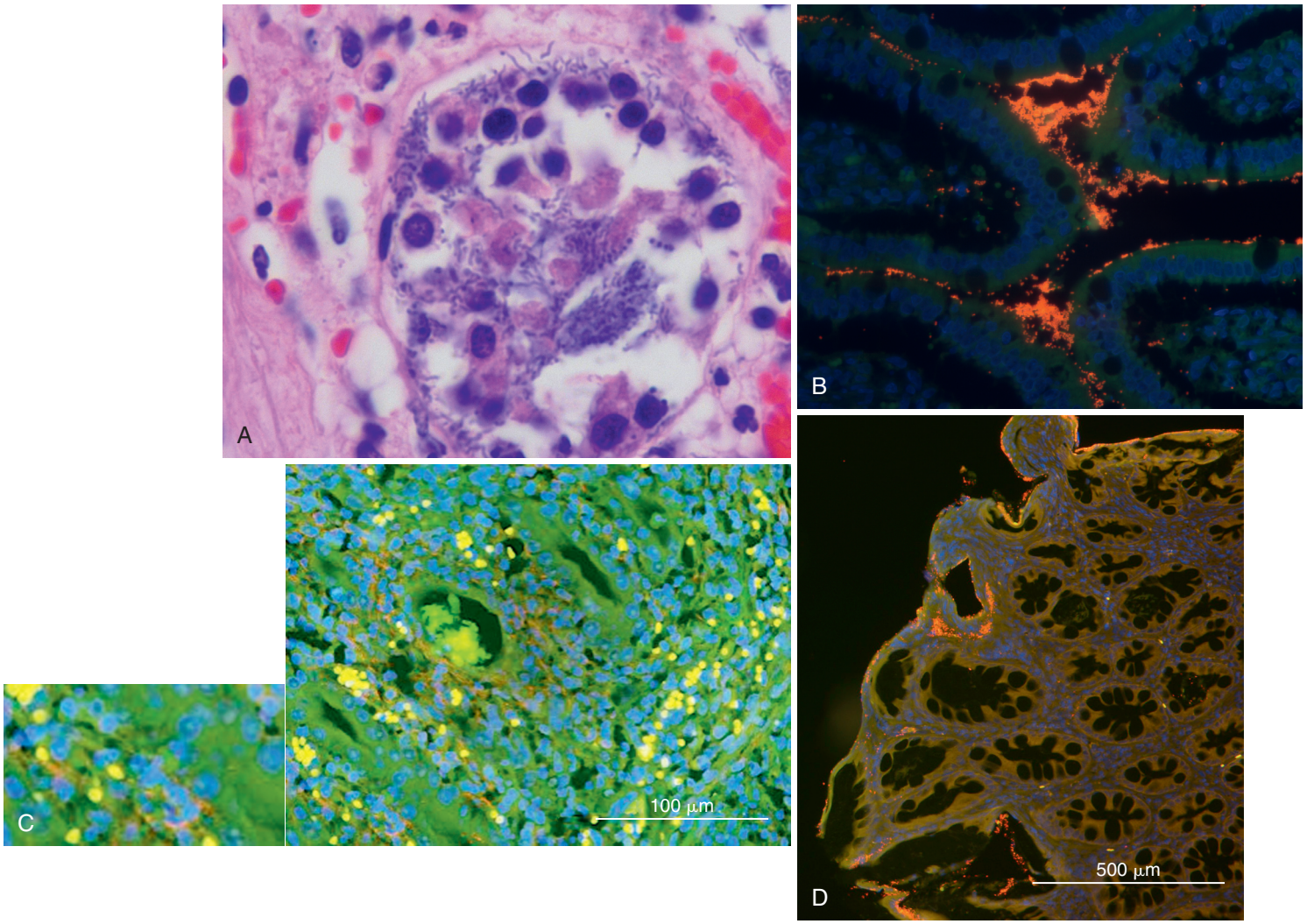

Figure 10-4: Spatial Distribution of Enteric Bacteria. A, Spiral bacteria inside the lumen of a dilated crypt (H&E, magnification, ×120). Histopathology revealed a severe, diffuse lymphoplasmactic, necrotizing enteritis with crypt necrosis and intraluminal spiral bacteria confirmed to be Anaerobiospirillum spp. **B,** Enteroadherent enterococci in the ileum of healthy kittens. Moderate **(C)** to severe **(D)** diffuse bacterial adherence of healthy small intestinal mucosa visualized with fluorescence *in situ* hybridization (FISH) using an oligonucleotide probe specific for eubacteria (Eub-338-FAM) or *Enterococcus* spp. (Enc-221-Cy3). Specimens were nuclear counterstained with 4′,6-diamidino-2-phenylindole (DAPI). **C,** Mucosally invasive spiral bacteria in a cat with pyogranulomatous ileitis visualized with FISH using an oligonucleotide probe specific for eubacteria (bacteria are *red:* Eub-338-Cy3). Specimens were nuclear counterstained with DAPI. Clinical signs resolved in response to doxycycline and enrofloxacin. **D,** Bacteria in and around degenerating crypts in cat with neutrophilic colitis visualized with FISH using an oligonucleotide probe specific for eubacteria (bacteria are *red:* Eub-338-Cy3). Specimens were nuclear counterstained with DAPI. Clinical signs resolved in response to tylosin. (**A,** Image is courtesy of Dr. Stan Marks. **B,** Copyright 2015 Jody Gookin; used with permission.)

consisting of mild inflammatory infiltrates and crypt abscesses. Ghosh and colleagues[37] used culture and FISH analysis to characterize the ileum mucosa-associated enterococcal community of 50 apparently healthy and 50 terminally ill foster kittens. In healthy kittens, *Enterococcus hirae* was the most common species of ileum mucosa-associated enterococci and was often observed to adhere extensively to the small intestinal epithelium (see Figure 10-4B). *E. faecalis*, with numerous virulence traits and multiple antimicrobial resistances, was more commonly isolated from the ileum mucosa of kittens with terminal illness. In addition, attachment of *E. coli* to the intestinal epithelium was significantly associated with terminal illness and was not observed in any kitten with adherent *E. hirae*.

Fecal Bacteria in Colony Cats with Signs of Gastrointestinal Disease

FISH analysis was performed to investigate the microbiota in a colony of cats that developed severe GI disease while undergoing an experimental trial.[38] This colony of cats, which underwent initial clinical investigations at Cornell University, had an ill-defined virus-like enteropathy that was associated with multisystemic and sometimes fatal disease that led to closure of the colony and its relocation to a research facility. These cats are described by Inness and colleagues[38] as having IBD, but they clearly had an unexplained severe enteropathy that is far removed from what is considered IBD in client-owned cats. FISH analysis showed total bacteria, *Bifidobacterium* spp., and *Bacteroides* spp. counts were higher in healthy cats when compared with affected cats, whereas *Desulfovibrio* spp. (producers of toxic sulphides) numbers were found to be significantly higher in affected cats.

The fecal microbiota of 15 colony cats with chronic diarrhea was evaluated using massive parallel 16S sequencing before and after dietary modification to determine the impact of diet change and related improvement in diarrhea.[39] Alterations in the intestinal microbiota were associated with improvement in diarrhea, but their relationship to disease was unclear.

IMPLICATIONS FOR THERAPY

From the limited clinical studies performed to date, it is clear that changes in the microbiota (i.e., dysbiosis) can accompany a variety of GI disorders in cats. It remains to be determined if dysbiosis is a cause or a consequence of GI disease in cats. Recent research indicates that acute, nonspecific intestinal inflammation can induce a consistent shift in the microbiome from Firmicutes to Proteobacteria, accompanied by a reduction in microbial diversity and proliferation of *E. coli* that recapitulates the dysbiosis of IBD.[25] There appears to be an interdependence of inflammation and dysbiosis, with inflammation promoting dysbiosis, and dysbiosis promoting inflammation. Genetic susceptibility may impact the threshold for dysbiosis in response to an external trigger and may also influence the ability of an individual to resolve the self-perpetuating cycle of dysbiosis and inflammation generated by an acute insult.[25]

From a therapeutic standpoint, it is tempting to equate dysbiosis with a need for antimicrobial therapy. However, this may not be required in patients that lack evidence of invasive bacteria (e.g., neutrophilic or granulomatous inflammation, intramucosal bacteria) or enteric translocation (e.g., fever, neutrophilia, regional lymphadenopathy). Because intestinal dysbiosis is an endpoint of many adverse stimuli, simply removing the initiating inflammatory stimulus, without recourse to antimicrobial intervention, may effect clinical resolution. From a mechanistic standpoint, bacteria and diet are frequently separated, but it is important to consider that they are not mutually exclusive, and the ability of diet to alter microbial populations in healthy cats is well established.[40-43] Changes in the microbiome are present in diet-responsive enteropathies such as celiac disease,[26,28] and responses to diet, without recourse to immunomodulatory drugs, have also been observed in people with Crohn's disease. Clinical signs in dogs with lymphoplasmacytic enteritis and concurrent dysbiosis can also resolve in response to a controlled diet without recourse to antimicrobial therapy.[16] With this in mind, it is noteworthy that 49% of 55 cats with chronic GI disease, which would typically be defined as low-grade lymphoplasmacytic enteritis, responded to an antigen-restricted diet without recourse to antimicrobial or immunosuppressive therapy.[17] Similar responses have also been reported in cats fed hydrolyzed diets.[44] Thus it seems prudent that cats with signs of chronic GI disease (that have undergone a thorough workup to exclude infectious or parasitic agents, non-GI disorders, exocrine pancreatic insufficiency, and intestinal structural abnormalities requiring surgery) with biopsy findings that are considered normal, minimal change, or "lymphoplasmacytic enteritis" are treated in a sequential, stepwise manner with progression to more aggressive therapeutics dictated by a lack of response.

For example, dietary modification (e.g., antigen-restricted or hydrolyzed diet) and cobalamin supplementation[45] for 2 weeks could be followed by the addition of an antimicrobial (e.g., tylosin) for 2 weeks, then by the addition of an immunosuppressive agent (e.g., prednisolone). This stepwise approach, which has been very effective in dogs with chronic enteropathies,[16] will hopefully identify subsets of cats that are diet-responsive, antibiotic-responsive, prednisolone-responsive, or unresponsive, and provide a phenotype that is important in ongoing efforts to inform understanding of the pathogenesis, diagnosis, and treatment of IBD. In cats with more severe lymphoplasmacytic enteritis, simultaneous therapy with diet, vitamins, antibiotics, and immunosuppressive agents may be prescribed, with treatments sequentially withdrawn if remission is achieved. In cats with neutrophilic or granulomatous inflammation, infectious agents must be strongly suspected. An aggressive search for organisms in the intestinal mucosa or regional lymph nodes (culture, histopathology and special stains, PCR, and FISH analysis) and potential systemic dissemination is of paramount importance to enable specific therapy. For proven and suspected bacterial infections, antimicrobials are the mainstay of therapy, and choice of treatment should take into consideration the spatial distribution of bacteria (i.e., antimicrobial sensitivities determined *in vitro* have to be reconciled with the ability of an antimicrobial to penetrate tissues and cells harboring the bacteria). Immunosuppression of these patients should be a last resort. Despite great expectations that probiotic bacteria given alone or in combination with prebiotics will alleviate or prevent the dysbiosis associated with IBD or chronic enteropathies, studies that show a positive effect are lacking. Some formulations have been evaluated in healthy cays and cats with chronic diarrhea,[46,47] but clinical trials in cats with IBD have not been reported to date.

SUMMARY

Recent advances in microbiology provide new insights into the composition and spatial distribution of intestinal bacteria, fungi, viruses, and protozoa in health and disease. A picture is emerging that correlates changes in the relative proportions of resident enteric bacterial populations—"dysbiosis"— with clinical signs and mucosal inflammation. The role of dysbiosis in the disease process remains to be elucidated. Is it a cause or a consequence, or a cause and a consequence, of intestinal disease? From a clinical perspective, it seems wise to adopt a stepwise approach to treating cats with lymphocytic plasmacytic enteritis with diet, then antimicrobials, and then immunosuppression as the default approach in most cases. In cats with evidence of neutrophilic or granulomatous intestinal inflammation, the onus is very much on the clinician to search aggressively for infectious agents before defaulting to immunosuppression of an idiopathic disease. Much remains to be learned about the complex interplay among host genetics, the intestinal microenvironment (principally bacteria and dietary constituents), the immune system, and environmental triggers of intestinal inflammation that lead to the development of IBD in cats.

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