

The relationship of mucosal bacteria to duodenal histopathology, cytokine mRNA, and clinical disease activity in cats with inflammatory bowel disease

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Abstract

Feline inflammatory bowel disease (IBD) is the term applied to a group of poorly understood enteropathies that are considered a consequence of uncontrolled intestinal inflammation in response to a combination of elusive environmental, enteric microbial, and immunoregulatory factors in genetically susceptible cats. The present study sought to examine the relationship of mucosal bacteria to intestinal inflammation and clinical disease activity in cats with inflammatory bowel disease.

Duodenal biopsies were collected from 27 cats: 17 undergoing diagnostic investigation of signs of gastrointestinal disease, and 10 healthy controls. Subjective duodenal histopathology ranged from normal (10), through mild (6), moderate (8), and severe (3) IBD. The number and spatial distribution of mucosal bacteria was determined by fluorescence in situ hybridization with probes to 16S rDNA. Mucosal inflammation was evaluated by objective histopathology and cytokine profiles of duodenal biopsies.

The number of mucosa-associated Enterobacteriaceae was higher in cats with signs of gastrointestinal disease than healthy cats ($P < 0.001$). Total numbers of mucosal bacteria were strongly associated with changes in mucosal architecture ($P < 0.001$) and the density of cellular infiltrates, particularly macrophages ($P < 0.002$) and CD3⁺ lymphocytes ($P < 0.05$). The number of Enterobacteriaceae, *E. coli*, and *Clostridium* spp. correlated with abnormalities in mucosal architecture (principally atrophy and fusion), upregulation of cytokine mRNA (particularly IL-1, -8 and -12), and the number of clinical signs exhibited by the affected cats.

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These data establish that the density and composition of the mucosal flora is related to the presence and severity of intestinal inflammation in cats and suggest that mucosal bacteria are involved in the etiopathogenesis of feline IBD.

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1. Introduction

Feline inflammatory bowel disease (IBD) is the term applied to a group of poorly understood intestinal disorders that are associated with vomiting, diarrhea and weight loss in cats. Diagnosis is usually based upon subjective analysis of intestinal mucosal biopsies and qualified according to the dominant mucosal infiltrate, typically lymphocytes and plasma cells (Jergens, 2002; Jergens et al., 1992; Waly et al., 2004). However, more objective studies have demonstrated increased expression of MHC class II antigen by leukocytes in the lamina propria and enterocytes, and upregulation of pro-inflammatory and immunoregulatory cytokines (Nguyen Van et al., 2006; Waly et al., 2004), rather than an increase in mucosal cellularity. Abnormalities in mucosal architecture, such as crypt distortion, villous blunting and fusion, and fibrosis have also been described, and have been associated with the severity of clinical signs (Baez et al., 1999; Hart et al., 1994), and the subjective histological grade of IBD (Baez et al., 1999; Dennis et al., 1992; Hart et al., 1994; Jergens, 2002). The cause of feline IBD has not been determined, but it is suspected that IBD in cats, like IBD in people, is a consequence of uncontrolled intestinal inflammation in response to a combination of elusive environmental, enteric microbial, and immunoregulatory factors in genetically susceptible individuals (Hanauer, 2006; Sartor, 2006). Genetic susceptibility in people is linked increasingly to defects in innate immunity, exemplified by mutations in the innate immune receptor NOD2/CARD15, that in the presence of the enteric microflora may lead to up-regulated mucosal cytokine production, delayed bacterial clearance and increased bacterial translocation, thereby promoting and perpetuating intestinal inflammation (Hanauer, 2006; Sartor, 2006). This possibility is supported by studies showing the pivotal importance of the enteric microflora in the development of IBD in rodents with engineered susceptibility (Elson et al., 2005; Kim et al., 2005),

and those demonstrating an abnormal mucosa-associated flora, considered to interact most closely with the innate immune system, in people with IBD (Kleessen et al., 2002; Swidsinski et al., 2005). Knowledge of genetic susceptibility and the enteric microflora in cats with IBD is limited, with some studies reporting a predisposition for purebred cats (Dennis et al., 1992), and culture based studies that show fewer luminal microaerophilic bacteria in the duodenal juice of cats with clinical signs of gastrointestinal disease than healthy cats (Johnston et al., 2001).

It is against this background that we sought to examine the relationship of the mucosal flora (determined by fluorescence in situ hybridization with labeled oligonucleotides to bacterial 16S rDNA) to intestinal inflammation (determined by objective histopathology and measurement of cytokine mRNA) and clinical disease activity in cats with and without inflammatory bowel disease. We found that total numbers of mucosal bacteria were strongly associated with changes in mucosal architecture and the density of cellular infiltrates, particularly macrophages. A subset of bacteria comprised of Enterobacteriaceae, *E. coli*, and *Clostridium* spp. correlated with abnormalities in mucosal architecture (principally atrophy and fusion), cytokine upregulation (particularly IL-1, -8 and -12), and the number of clinical signs exhibited by the affected cats. These data establish that the density and composition of the mucosal flora are related to the presence and severity of intestinal inflammation in cats and suggest that mucosal bacteria are involved in the etiopathogenesis of feline IBD.

2. Materials and methods

2.1. Cats

Seventeen cats presented to the Cornell University Hospital for Animals (CUHA) for investigation of

signs of gastrointestinal disease (vomiting 13, weight loss 11, anorexia 7, or diarrhea 6) were enrolled in this study. All cats had completed a thorough diagnostic evaluation consisting of physical examination, clinicopathological testing (complete blood count, biochemistry profile and serum T₄) and abdominal ultrasonography to exclude non-gastrointestinal causes for their clinical signs. Mean age (\pm S.D.) was 10 ± 4.3 years (range 5 months to 17 years) and both sexes were equally represented (9 castrated males, 8 spayed females). Nine cats were domestic shorthairs, while the remaining eight cats were various purebreds (3 Siamese, 1 Oriental Shorthair, 1 Bengal, 1 Ocicat, 1 Sphinx and 1 Maine Coon). As Oriental cats represented only 2.98% of the 4332 cats examined at CUHA during the same period our findings are consistent with previous reports of breed predisposition to IBD (Dennis et al., 1992).

Of the 17 cats with signs of gastrointestinal disease 9 had not received antibiotics within 2 weeks of endoscopy, and 7 had received antibiotics within 2 weeks of referral (1 ampicillin, 3 amoxicillin, 1 cephalexin, 3 metronidazole) without resolution of clinical signs. Three cats had received prednisolone prior to referral without resolution of clinical signs.

Abnormalities detected on physical examination included thickened gut loops (5/17), mesenteric lymphadenomegaly (4/17) and thin body condition (4/17). Clinicopathological abnormalities included panhypoproteinemia (5/17), neutrophilia (5/17), lymphopenia (5/17) elevated AST (5/17), hypoalbuminemia (4/17), hypokalemia (4/17), hyponatremia (4/17), hypochloremia (4/17), hypoglobulinemia (3/17), hyperglycemia (3/17), increased ALP and ALT (2/17), hemoconcentration (2/17), mild anemia (1/17), and hyperglobulinemia (1/17). Ultrasonographic examination revealed an abnormal intestine in 6 cats and mesenteric lymphadenomegaly in 3. Upper gastrointestinal endoscopy showed an abnormal duodenum in 6 of 13 cats examined. All of these clinical findings are consistent with a diagnosis of IBD in cats (Baez et al., 1999; Dennis et al., 1992).

A control group of 10 clinically normal Domestic Shorthair cats from a cat colony was included in the study (7 females and 3 males, ranging in age from 0.5 to 10 years, mean \pm S.D. = 2.8 ± 3.9 years). These cats were from eight different litters born over a 9.5 years timespan. These cats were free from FeLV, FIV

and FIP. Previous studies have shown that the duodenal bacterial flora is similar in colony cats and pet cats (Johnston et al., 2001). These cats were housed in a barrier facility with a controlled environment (lighting, 12 h on, 12 h off; temperature 20–21 °C; humidity 35–45%) at Cornell University, were fed a standard commercial diet (Teklad lab cat diet) ad libitum and had constant access to water throughout the study. None of the control cats had received antibiotics. Cornell University operates under an approved Animal Welfare Assurance (A3347-01, A-3125-01) and is fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care. The Institutional Animal Care and Use committee at Cornell University approved this project.

2.2. Intestinal biopsy

Biopsy samples were collected during upper GI endoscopy in 23 cats (13 with signs of gastrointestinal disease and 10 controls). All cats were fasted overnight. The endoscope was thoroughly cleaned and sterilized using an activated aldehyde solution (Metrex, Parker CO). Biopsy forceps were sterilized in a similar fashion and the biopsy cups were immersed in sodium hypochlorite (.53%) for 10 min to destroy residual DNA. Surgical biopsies were obtained during exploratory laparotomy in four cats by use of a sterile 4 mm dermatological punch biopsy. Four to seven endoscopic biopsies, or one surgical biopsy, from the duodenum were fixed in 4% neutral buffered formalin and paraffin-embedded for histopathology and FISH analysis. Two endoscopic biopsy samples, or one surgical biopsy, were placed in RNA-later on ice or snap frozen in liquid nitrogen prior to storage at -70 °C pending cytokine analysis.

2.3. Fluorescence in situ hybridization (FISH)

Formalin-fixed paraffin embedded histological sections (4 μ) were mounted on Probe-On Plus slides (Fisher Scientific, Pittsburgh, Pa.) and evaluated by fluorescence in situ hybridization (FISH) as previously described (Priestnall et al., 2004). Briefly, paraffin embedded biopsy specimens were deparaffinized by passage through xylene (3 \times 10 min), 100% alcohol (2 \times 5 min), 95% ethanol (5 min) and finally 70%

ethanol (5 min). The slides were air-dried. FISH probes 5'-labeled with either Cy3 or 6-FAM (Integrated DNA Technologies, Coralville, IA) were reconstituted with sterile water and diluted to a working concentration of 5 ng/ μ L with a hybridization buffer appropriate to the probe. For total bacterial counts EUB338 Cy-3 was combined with the irrelevant probe non-EUB-338-FAM (ACTCC-TACGGGAGGCAGC) to control for non-specific hybridization. For subsequent analyses a specific bacterial probe labeled with Cy-3 and the universal bacterial probe EUB338 labeled with 6-FAM were applied simultaneously. Specific probes, directed against *Clostridium*, *Bacteroides/Prevotella*, Enterobacteriaceae, *E. coli* and *Streptococcus* (Table 1), were selected on the basis of their dominance in previous studies of cultivable bacteria present in duodenal juice and duodenal mucosa (Johnston et al., 1993; Papsasoulotis et al., 1998; Uchida and Terada, 1971). A combination of two probes against *Helicobacter* spp. (Table 1) was used to determine if these difficult to culture bacteria were present in the feline duodenum. Sections were allowed to hybridize with 30 μ L of DNA probe mix in hybridization chamber overnight (12–14 h). Washing was performed with the appropriate wash buffer (hybridization buffer without SDS), and the samples were then rinsed in sterile water, allowed to air-dry, and mounted with ProLong[®] Antifade Gold (Molecular Probes Inc., Eugene, OR).

Probe specificity was controlled by evaluating a battery of slides prepared from cultured *E. coli* DH5 α , *Shigella sonnei* (ATCC 25931), *Salmonella typhimurium* (ATCC14028), and clinical isolates of *Yersinia enterocolitica*, *Proteus vulgaris*, *Klebsiella pneumoniae*, *Bacteroides fragilis*, *Pseudomonas aeruginosa*, *Enterococcus fecium*, *Streptococcus equi*, *Streptococcus bovis*, *Clostridium perfringens*, *Clostridium difficile*, *Lactobacillus plantarum*, *Listeria monocytogenes* and *Helicobacter pylori*. Sections of gastric mucosa from cats or dogs with *Helicobacter* infections were used as additional controls for *Helicobacter* FISH (Priestnall et al., 2004). Probe specificity was additionally evaluated by including positive and negative control slides in each assay.

Sections were examined on an Olympus BX51 (Olympus America, Melville, NY) epifluorescence microscope and images captured with an Olympus DP-7camera.

The number of bacteria (total and different bacterial species) and their spatial distribution (free mucus, adherent mucus, glandular mucus, glandular epithelium, superficial epithelium, within mucosa) was determined in ten 60 \times fields of each section and results expressed as bacteria/mm².

2.4. Assessment of Intestinal Inflammation

2.4.1. Histopathology and Immunohistochemistry

Formalin-fixed, paraffin-embedded biopsy samples were sectioned at 4 μ m and stained with H&E (cellular infiltrates and architecture), Masson's trichrome (fibrosis), and Alcian blue pH 0.4 (mast cells). Semi-quantitative evaluation of mucosal cellular infiltrates (neutrophils, plasma cells, lymphocytes, eosinophils, macrophages, intraepithelial lymphocytes, mast cells, and large granular lymphocytes) architecture (epithelial erosion, goblet cell hyperplasia, villous fusion, villous atrophy, cryptal hyperplasia, lymphangectasia, and fibrosis) and overall IBD grade was performed by a pathologist (SPM), blinded to the origin of the sections, who assigned a grade (normal [0], mild [1], moderate [2], and severe [3]).

Immunohistological staining for B (BLA36 and CD79 α) and T (CD3⁺) lymphocytes was performed with streptavidin–biotin–horseradish peroxidase and a catalyzed signal amplification system according to the manufacturer's instructions (Dako, Carpinteria, Ca). The chromogen was 3,3'-diaminobenzidine (Sigma, St. Louis, Mo), and all slides were lightly counterstained with Gills' hematoxylin. The specificity, antigen retrieval method, and source of primary antibodies have been reported previously (Straubinger et al., 2003). Positive tissue controls consisted of normal feline spleen and lymph node. Negative technique controls were performed by substituting the primary antibody with phosphate-buffered saline, normal rabbit serum, or an isotype-matched irrelevant mouse monoclonal antibody. The total number of T and B cells present in ten 60 \times fields was determined for each section.

2.4.2. Mucosal cytokine mRNA

RNA was extracted from biopsy samples with a kit according to the manufacturer's instructions (Rneasy: QIAamp DNA Mini Kit). After elution of RNA from the spin columns with 40 μ L of diethylpyrocarbonate-

Table 1
Probes and hybridization conditions for fluorescence in situ hybridization

Probe	Sequence (5' → 3')	Target	Hybridization conditions	Washing conditions	Reference
EUB 1531	GCT GCC TCC CGT AGG AGT CAC CGT AGT GCC TCG TCA	Eubacteria 23S RNA of <i>E. coli</i> , <i>Shigella</i> , <i>Salmonella</i> , <i>Klebsiella</i>	As used for specific probe 100 mM Tris, 0.9 M NaCl, 0.01% SDS, 35% formamide, pH 7.5, 46 °C overnight	As used for specific probe 100 mM Tris, 0.9 M NaCl, pH 7.5, 48 °C for 20 min	Amann et al. (1990) Poulsen et al. (1994)
<i>E. coli</i>	GCA AAG GTA TTA ACT TTA CTC CC	<i>E. coli</i> and <i>Shigella</i>	20 mM Tris, 0.9 M NaCl, 0.1% SDS, pH 7.2, 50 °C overnight	20 mM Tris, 0.9 M NaCl, pH 7.2, 54 °C for 20 min	McGregor et al. (1996)
Clit135	GTT ATC CGT GTG TAC AGG G	<i>Clostridium lituseburense</i> group	20 mM Tris, 0.9 M NaCl, 0.1% SDS, 20% formamide, 10% dextran sulfate, pH 7.2, 50 °C overnight	20 mM Tris, 0.9 M NaCl, pH 7.2, 55 °C for 20 min	Franks et al. (1998)
Chis150	TTA TGC GGT ATT AAT CTY CCT TT	<i>Clostridium histolyticum</i>	20 mM Tris, 0.9 M NaCl, 0.1% SDS, 20% formamide, 10% dextran sulfate, pH 7.2, 50 °C overnight	20 mM Tris, 0.9 M NaCl, pH 7.2, 55 °C for 20 min	Franks et al. (1998)
BAC303	CCA ATG TGG GGG ACC TT	<i>Bacteroides</i>	20 mM Tris, 0.9 M NaCl, 0.01% SDS, pH 7.4, 46 °C overnight	20 mM Tris, 0.9 M NaCl, pH 7.4, 48 °C for 20 min	Manz et al. (1996)
Strep	CAC TCT CCC CTT CTG CAC	<i>Streptococcus</i> and <i>Lactobacillus</i> spp.	20 mM Tris, 0.9 M NaCl, 0.1% SDS, pH 7.2, 50 °C overnight	20 mM Tris, 0.9 M NaCl, pH 7.2, 50 °C for 10 min	Kempf et al. (2000)
Hel 274	GGC CGG ATA CCC GTC ATW GCC T	<i>Helicobacter</i> spp.	20 mM Tris, 0.9 M NaCl, 0.1% SDS, 40% formamide, pH 7.2, 46 °C overnight	20 mM Tris, 0.9 M NaCl, pH 7.2, 48 °C for 20 min	Chan et al. (2005)
Hel 717	AGG TCG CCT TCG CAA TGA GTA	<i>Helicobacter</i> spp.	20 mM Tris, 0.9 M NaCl, 0.1% SDS, 40% formamide, pH 7.2, 46 °C overnight	20 mM Tris, 0.9 M NaCl, pH 7.2, 48 °C for 20 min	Chan et al. (2005)

treated water, each sample was treated with 1 U of DNAase I, following the manufacturer's protocol. Immediately after this treatment, samples were stored at -80°C .

Reverse transcription reactions were carried out in a personal thermocycler. Five microliters of total RNA (approximately 0.5 μg total RNA) in 55- μL reaction volumes consisting of 1 \times PCR Buffer II, 5.5 mM of MgCl_2 , 500 μM each of deoxynucleoside triphosphates (dNTPs), 0.4 U/ μL of RNasin ribonuclease inhibitor, 5.0 U/ μL of Moloney murine leukemia virus reverse transcriptase (RT), and 2.5 μM random hexamers were transcribed into cDNA at 25°C for 10 min, followed by 48°C for 30 min, and at 95°C for 5 min.

PCR primers and probes against feline IFN- γ , IL-1 β , IL-6, IL-8, IL-10, IL-12 and GAPDH were used to amplify and quantify their respective cDNA, as described previously (Straubinger et al., 2003). Amplification was performed in MicroAmp 96-well plates using the ABI Prism 7700 Sequence Detection System standard amplification protocol recommended by the manufacturer (2 min at 50°C ; 10 min at 95°C ; 40 cycles with 15 s at 95°C , and 1 min at 6°C) and signals were recorded with a charge-couple device camera, controlled by the Sequence Detection Software, Version 1.6. Recorded cycle threshold (CT) values for feline cytokines were normalized with corresponding CT values for GAPDH, a housekeeping gene. ΔCT values correspond to the difference in cytokine mRNA expression relative to a housekeeper gene (GAPDH) and served to normalize the individual sample.

2.5. Statistical analysis

Bacterial numbers in healthy cats and cats with clinical signs of gastrointestinal disease were compared by use of the Mann–Whitney test. Differences in spatial distribution and the relative number of different bacterial species were examined with the Friedman test. The total number of clinical signs (vomiting, diarrhea, anorexia, weight loss, with each allocated a value of 1 = present, 0 = absent) at presentation was used as an indicator of clinical disease activity (CDA). The relationship of CDA to bacterial numbers was determined by Spearman's rho correlation.

Evaluation of the interrelationship of the mucosa-associated flora, duodenal histopathology and cyto-

kine mRNA was preceded by principle components analysis (PCA) to combine multiple correlated variables into a lesser number of still-meaningful component factors. Reducing data in this way reduces the likelihood of type II error when performing multiple computations, and can also provide information on data structure. Cytokine expression data (IFN- γ , IL-1 β , IL-6, IL-8, IL-10, IL-12) was reduced by conventional PCA with varimax factor rotation. Because architecture data (epithelial erosion, goblet cell hyperplasia, villous fusion, villous atrophy, cryptal hyperplasia, lymphangiectasia, and fibrosis) was ordinal, a categorical principle components analysis more appropriate for nonparametric data was used for data reduction (CATPCA). In each case, the Kaiser criterion (excluding factors with Eigen values <1.000) was used to determine the number of components to extract. The cell count data and serological data were not evaluated by PCA and CATPCA due to the restricted distribution of the data and lack of strong correlations among the measured variables.

Spearman's rho correlations were used to evaluate relationships among architecture component scores, cytokine component scores, total bacterial densities, cell counts, IBD, and CDA. To examine which variables could be used in combination to predict clinical features, pair-wise combinations of the variables that strongly correlated with CDA were used as predictor variables in ordinal regressions against CDA. In the case of cytokines and architecture, the constituent variables that contributed most powerfully to the component scores were used instead of the component scores themselves. Statistical analyses were performed using SPSS software (SPSS, Chicago).

3. Results

3.1. Mucosa-associated bacteria

The mucosa-associated bacterial flora (EUB-338) in the duodenum of healthy cats was most abundant in free and adherent mucus (Figs. 1A, 2). Sub-populations of bacteria hybridized with probes directed against *Clostridium* spp., *Bacteroides* spp., *Streptococcus* spp., Enterobacteriaceae and *E. coli* but not

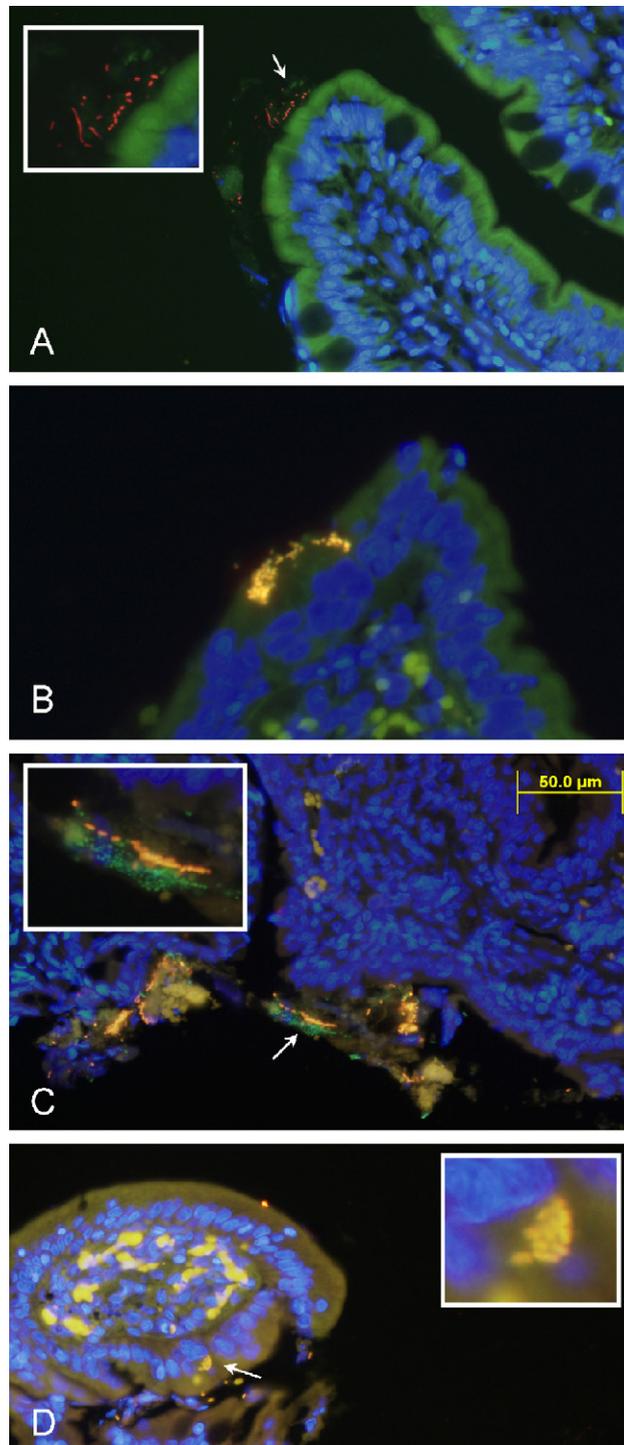


Fig. 1. Analysis of the duodenal mucosa-associated flora in situ. In situ hybridization (FISH) with oligonucleotide probes against bacteria in general (EUB 338), a subset of *Enterobacteriaceae* (1531, 23S rRNA), *E. coli/Shigella* (16S rRNA) and *Clostridium* spp. (Chis150/Clit135 16S

Table 2
Number of mucosal bacteria determined by FISH

		Bacteria/mm ²					
		EUB-338	Enterobacteriaceae	<i>E. coli</i>	<i>Bacteroides</i>	<i>Clostridium</i>	<i>Streptococcus</i>
Healthy (<i>N</i> = 10)	Median	48	0	0	0	0	0
	Range	0–399	0–3	0–2	0–0	0–46	0–6
GI disease (<i>N</i> = 17)	Median	127	17* [#]	0	0	0	0
	Range	7–7244	0–4219	0–997	0–39	0–1206	0–540

* $P < 0.001$ vs. healthy.

$P < 0.002$ vs. other bacteria.

Helicobacter spp. (Table 2). There was no significant difference in the number and spatial distribution of these bacterial species within the mucosa of healthy cats ($P > 0.05$). The total numbers of bacteria hybridizing to probes against *Clostridium* spp., *Bacteroides* spp., *Streptococcus* spp., Enterobacteriaceae represented only 6% of bacteria hybridizing to EUB-338 (Fig. 3).

The total number of EUB-338 positive bacteria in cats with clinical signs of gastrointestinal disease was not significantly different from healthy cats (Fig. 1, Table 2). However, cats with signs of gastrointestinal disease had many more Enterobacteriaceae ($P < 0.001$), localized predominantly within adherent mucus, than healthy cats (Table 2, Figs. 1B, 2). Enterobacteriaceae were also more numerous ($P < 0.002$) than *Bacteroides*, *Clostridium* and *Streptococcus* spp. within biopsies of cats with signs of GI disease (Table 2, Fig. 3). The spatial distribution of bacteria in the mucosa of cats with signs of gastrointestinal disease was significantly different ($P < 0.05$) from healthy cats, with higher numbers of Enterobacteriaceae, *Clostridium* spp. and *Streptococcus* spp. detected within the free mucus compared to other regions. Invasive *Clostridium* spp. accounted for 8% of total *Clostridium* spp. in sections from cats with

signs of gastrointestinal disease (Fig. 1D). In contrast to healthy cats, the proportion of the flora hybridizing to EUB338 recognized by probes to *Clostridium* spp., *Bacteroides* spp., *Streptococcus* spp., Enterobacteriaceae was 91% (Fig. 3), with *E. coli* making up about 30% of the Enterobacteriaceae (Fig. 3). *Helicobacter* spp. were not detected in samples from cats with signs of gastrointestinal disease. There was no significant effect (i.e. $P > 0.05$) of age or prior antibiotic treatment on the number of EUB-338 positive mucosal bacteria.

3.2. The relationship of mucosal bacteria to duodenal histopathology

Principle components analysis was used to derive two factors that described 79% of the variability in mucosal architecture. These factors consisted of epithelial erosion, villous fusion, villous atrophy, cryptal hyperplasia (Arc-Fac-1), and fibrosis (ArcFac 2) (Fig. 4).

Total bacterial load (EUB338) correlated with ArcFac1 ($P < 0.001$, rho 0.765), the density of the cellular response ($P < 0.016$, rho 0.495: predominantly T lymphocytes, $P < 0.05$, rho 0.448) and macrophages ($P < 0.002$, rho 0.607).

rRNA) was used to examine the number and spatial distribution of intact bacteria within the duodenal mucosa of healthy cats and cats with signs of gastrointestinal disease. (A) Fluorescence in situ hybridization with probe Cy3-EUB338 (red) shows that bacteria in normal duodenal mucosa are localized predominantly in the superficial mucus covering a villus. DAPI stained DNA (blue). Bacteria are 2–3 μ long, original magnification 600 \times . (B) Fluorescence in situ hybridization with Cy3-Enterobacteriaceae-1531 (red) and FAM-EUB338 (green) shows a group of Enterobacteriaceae (orange/yellow) on the duodenal mucosal surface of a cat with inflammatory bowel disease. DAPI stained DNA (blue). Bacteria are 2–3 μ long, original magnification 600 \times . (C) Fluorescence in situ hybridization with Cy3-*E.coli/Shigella* (red) and FAM-EUB338 (green) shows a mixed population of *E. coli* (orange/yellow) and other bacteria (green) on the mucosal surface of the eroded and inflamed duodenal mucosa. DAPI stained DNA (blue). Bacteria are 2–3 μ long, original magnification 600 \times . (D) Fluorescence in situ hybridization with Cy3-Chis150/Clit135 (red) and FAM-EUB338 (green) shows a cluster of *Clostridium* spp. (orange/yellow) within the superficial mucosa of a cat with inflammatory bowel disease. DAPI stained DNA (blue). Bacteria are 2–3 μ long, original magnification 600 \times (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.).

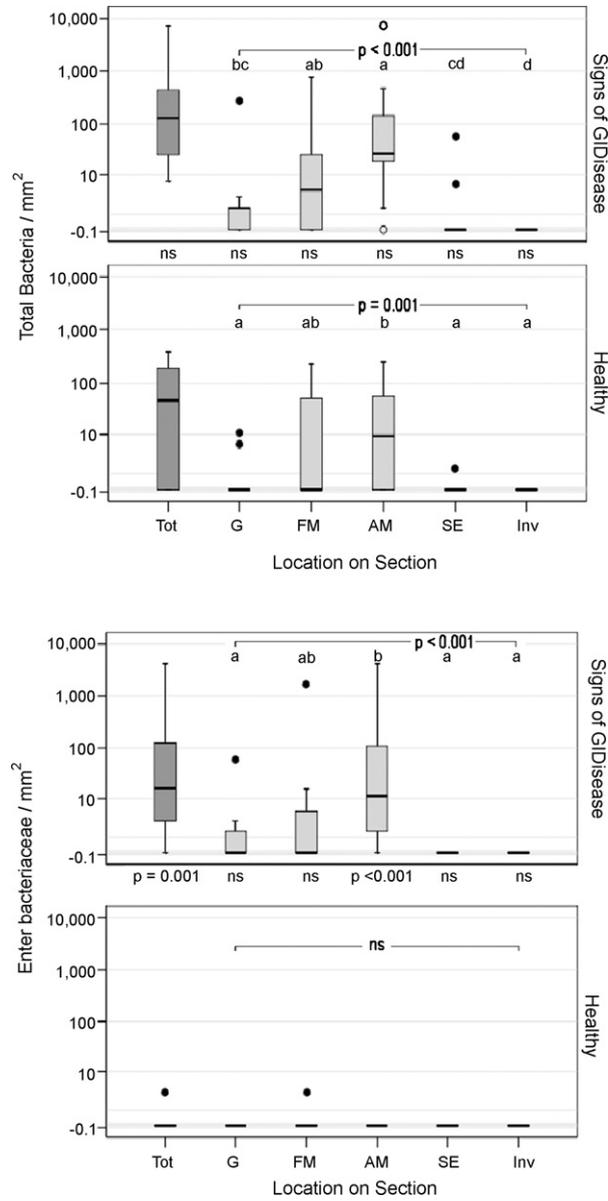


Fig. 2. Spatial distribution of bacteria in the duodenal mucosal bacteria of healthy cats and cats with signs of gastrointestinal disease. Tot = total bacteria, G = glands, FM = free mucus, AM = adherent mucus, SE = adherent to the superficial epithelium, Inv = intramucosal. Differences in the numbers of bacteria in different regions of the mucosa are indicated by the letters a, b, c and d. Groups with the same letter are not statistically different. Comparisons between cats with signs of GI disease and healthy cats are indicated by the *P* values between box plots. NS = no significant difference.

Total counts for Enterobacteriaceae correlated with ArcFac1 ($P < 0.05$, rho 0.417). *E. coli* and *Streptococcus* in free mucus, and invasive *Clostridium* spp. were positively correlated with changes in ArcFac 1 (*E. coli* $P < 0.01$, rho 0.544; *Streptococcus* $P < 0.05$,

rho 0.467; *Clostridium* $P < 0.01$, rho 0.524). The number of *E. coli* and *Clostridium* spp. were negatively correlated with changes in ArcFac2 (*E. coli* $P < 0.05$, rho 0.490; *Clostridium* $P < 0.05$, rho 0.499).

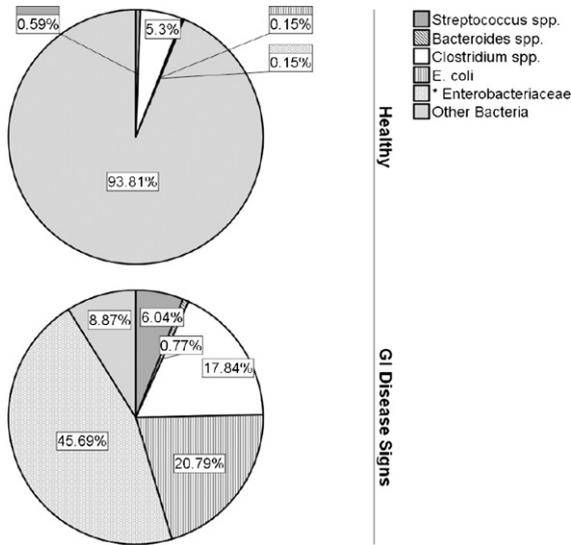


Fig. 3. Proportion of mucosal *Streptococcus* spp., *Bacteroides* spp., *Clostridium* spp., *E. coli* and Enterobacteriaceae in healthy cats and cats with signs of GI disease. Numbers indicate the proportion (%) of bacteria recognized by EUB 338. * = Enterobacteriaceae recognized by 1531 minus *E. coli*.

Subjective evaluation of duodenal histopathology for the presence and grade of IBD, independent of presenting complaint or clinical signs, categorized 10 samples as normal, and 6 with mild, 8 with moderate and 3 with severe IBD. The subjective IBD grade correlated with total bacterial load (EUB338, $P < 0.0001$, rho 0.643) and total counts for Enterobacteriaceae ($P < 0.05$, rho 0.450) (predominantly in adherent mucus).

3.3. The relationship of mucosal bacteria to duodenal cytokine mRNA

Principle components analysis was used to derive three factors that accounted for 87% of the variability in cytokine levels (IFN- γ , IL-1 β , IL-6, IL-8, IL-10, IL-12: Fig. 5). All cytokines grouped together (cyt fac1), upregulation of IL-1 β , IL-8 and IL-12 relative to IL-10 and IFN- γ (cyt fac 2), and upregulation of IL-6 (cyt fac3).

There was a correlation between CytFac2 and total numbers of *Clostridia* ($P < 0.001$, rho 0.624), Enterobacteriaceae ($P < 0.05$, rho 0.405) and *E. coli* ($P < 0.01$, rho 0.522). However, total numbers of Enterobacteriaceae and those in adherent mucus were

inversely correlated with CytFac 3 ($P < 0.02$, rho 0.491).

3.4. Relationships between mucosal histopathology and cytokine mRNA

CytFac1, which grouped all cytokines together, correlated with the numbers of mucosal neutrophils ($P < 0.021$, rho 0.450) and macrophages ($P < 0.030$, rho 0.426), the severity of architectural change (ArchFac1 $P < 0.01$, rho 0.514), and the subjective IBD score ($P < 0.01$, rho 0.530). That is, increasing cytokine levels were correlated with more severe architectural changes, and increased numbers of neutrophils and macrophages, and more severe grades of IBD. CytFac1 was negatively correlated with ArchFac2 ($P < 0.05$, rho 0.393); thus, cytokine levels decreased as the amount of fibrosis in a biopsy sample increased. CytFac3 (IL-6) was strongly correlated with mast cells – as the number of mast cells in a biopsy increased, the amount of IL-6 tended to decrease. Individual cytokine and architectural parameters were also evaluated singly against a small number of variables to better investigate the relationships present. The number of macrophages correlated with levels of IL-1 ($P < 0.05$, rho 0.484), IL-8 ($P < 0.05$, rho 0.441) and IL-12 ($P < 0.01$, rho 0.540). The subjective grade of IBD was strongly associated with IL-8 upregulation ($P < 0.001$, rho 0.665).

Mucosal architecture (ArchFac1) correlated with T cells ($P < 0.02$, rho 0.518), the density of the cellular response ($P < 0.001$, rho 0.672), and macrophages ($P < 0.001$, rho 0.635).

3.5. The relationship of mucosal bacteria and inflammation to clinical disease activity

The interrelationship of clinical disease activity, mucosal IL-8 levels and mucosa-associated bacteria are illustrated in Fig. 6. Total counts for Enterobacteriaceae (adherent mucus $P < 0.001$, rho 0.832), *E. coli* (free mucus $P < 0.011$, rho 0.554) and *Clostridium* spp. (free mucus and invasive, $P < 0.011$, rho 0.554) correlated with clinical disease activity (Fig. 6A and B).

Changes in mucosal architecture (ArchFac1 $P < 0.001$, rho 0.639), particularly atrophy ($P < 0.004$,

Architecture Categorical PCA		
Model Summary		
Factor	1	2
Eigenv.	2.787	1.182
Variance	55.74%	23.65%
Cum. Var.	55.74%	79.39%
Epcellch	0.737	-0.424
Atrophy	0.908	-0.062
Fusion	0.935	-0.059
Hyperpla	0.725	0.410
Fibrosis	0.139	0.910
Variable Principal Normalization.		

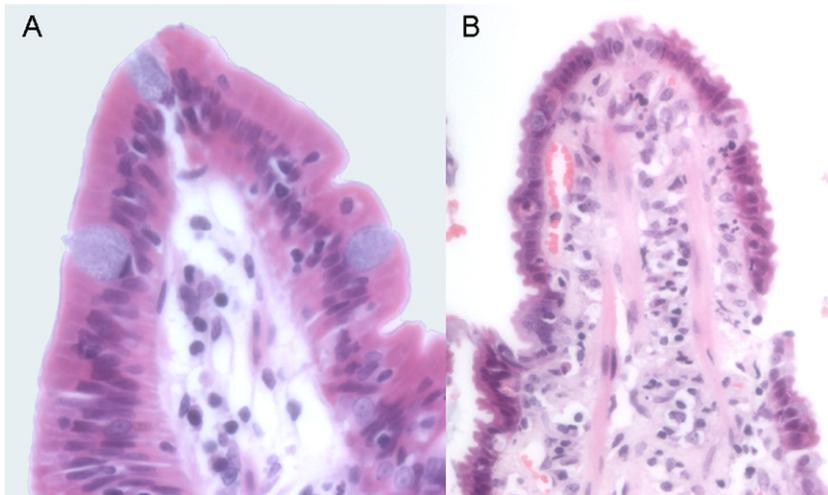
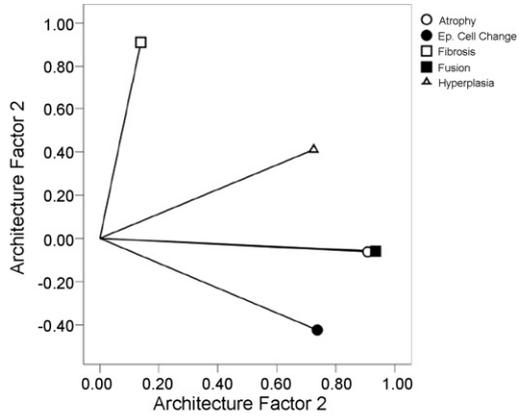


Fig. 4. Analysis of mucosal architecture. The table and graph show the results of principal components analysis of histopathological abnormalities in the duodenal mucosa. Two factors consisting of epithelial erosion, villus fusion, villous atrophy, cryptal hyperplasia (ArcFac-1), and fibrosis (ArcFac 2) described 79% of the variability in mucosal architecture. (A) A normal villus tip with an appropriate shape and intact epithelium (H&E, $\times 600$). (B) An atrophied, rounded villus with epithelial erosion (H&E, $\times 600$).

Cytokine PCA			
Model Summary			
Factor	1	2	3
Eigenv.	3.585	0.874	0.731
Variance	59.75%	14.57%	12.19%
Cum. Var.	59.75%	74.32%	86.51%
IL1	0.860	-0.422	-0.031
IL8	0.837	-0.094	0.229
IFN	0.736	0.391	-0.372
IL6	0.594	0.465	0.629
IL10	0.750	0.338	-0.379
IL12	0.830	-0.452	0.022
Unrotated factor solution			

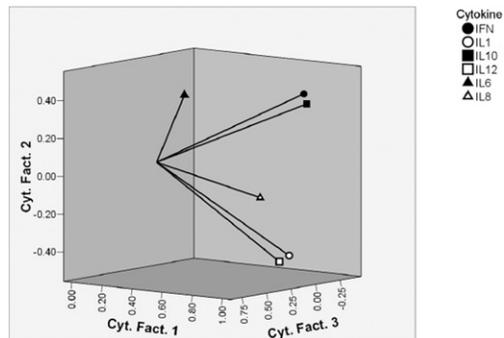


Fig. 5. Analysis of mucosal cytokines. Principle components analysis was used to derive three factors that accounted for 87% of the variability in cytokine levels (IFN- γ , IL-1 β , IL-6, IL-8, IL-10, IL-12: Fig. 5). All cytokines grouped together (cyt fac1), upregulation of IL-1 β , IL-8 and IL-12 relative to IL-10 and IFN- γ (cyt fac 2), and upregulation of IL-6 (cyt fac3).

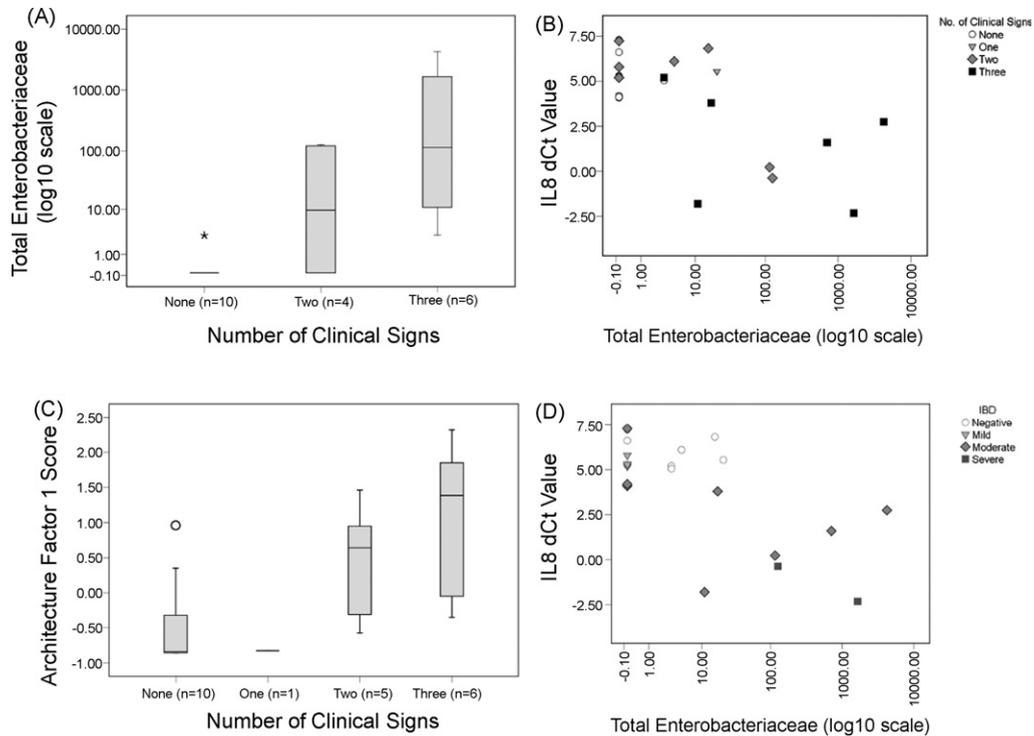


Fig. 6. The relationship of mucosal bacteria and inflammation to clinical disease activity. (A) Total counts for Enterobacteriaceae correlated with the number of clinical signs ($P < 0.001$, ρ 0.832). (B) Inter-relationship of IL-8 upregulation (lower Δ CT values indicate more upregulation), clinical signs and mucosal Enterobacteriaceae. (C) Changes in mucosal architecture (ArchFac1 $P < 0.001$, ρ 0.639), particularly atrophy ($P < 0.004$, ρ 0.589) and fusion ($P < 0.001$, ρ 0.675), and upregulation of IL-8 ($P < 0.002$, ρ 0.624) were correlated with clinical disease activity. (D) Inter-relationship of IL-8 upregulation (lower Δ CT values indicate more upregulation), mucosal Enterobacteriaceae and histopathological severity of IBD.

ρ 0.589) and fusion ($P < 0.001$, ρ 0.675), and upregulation of IL-8 ($P < 0.002$, ρ 0.624) were correlated with clinical disease activity (Fig. 6C and D).

4. Discussion

The enteric flora is implicated increasingly as a pivotal factor in the development of intestinal inflammation in people and experimental animals (Elson et al., 2005; Sartor, 2006). While the specific bacterial characteristics that drive the inflammatory response remain elusive, the discovery that defects in innate immunity are related to intestinal inflammation suggests that bacteria inhabiting the interface between the luminal environment and the intestinal mucosa may play an important role in the inflammatory

process (Hanauer, 2006; Sartor, 2006). In the present study we used fluorescence in situ hybridization to examine the relationship of the mucosal flora to intestinal inflammation and clinical disease activity in cats. We found that cats with signs of gastrointestinal disease had many more mucosal Enterobacteriaceae than healthy cats. 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 comprised of Enterobacteriaceae, *E. coli*, and *Clostridium* spp. correlated with abnormalities in mucosal architecture (principally atrophy and fusion), proinflammatory cytokine upregulation (IL-1, -8 and -12), and the number of clinical signs exhibited by the affected cats. These data establish that changes in the number and type of mucosa-associated bacteria are related to the presence and severity of IBD in cats

and raise the possibility that an abnormal mucosal flora is involved in the etiopathogenesis of feline IBD.

Previous investigations of the bacteria flora in the small intestine of cats have focused on bacteria living within luminal contents (Johnston et al., 1993; Papasouliotis et al., 1998; Sparkes et al., 1998). The present study reveals that the duodenal mucosa of healthy cats and cats with signs of gastrointestinal disease is colonized by bacteria that are predominantly localized to the superficial and adherent mucus. The composition of the mucosal flora differed markedly in healthy versus sick cats, with probes to Enterobacteriaceae, *E. coli*, *Streptococcus*, *Clostridiales* and *Bacteriodes* accounting for only 6% of mucosal bacteria in healthy cats, compared to 91% of the mucosal flora in cats with signs of gastrointestinal disease. Enterobacteriaceae were the dominant mucosal bacteria in cats with signs of gastrointestinal disease, representing more than 66% of mucosal bacteria. This selective enrichment in Enterobacteriaceae relative to a depletion in normal flora parallels reports of the fecal and intestinal mucosal flora of people with IBD (Gophna et al., 2006; Martinez-Medina et al., 2006), and has been termed “dysbiosis” (Seksik et al., 2005; Tamboli et al., 2004). While the dominant bacterial species in the mucosa of healthy cats remains to be determined, it is noteworthy that *Clostridiaceae* such as *Fecalibacteria* are decreased in people with IBD (Gophna et al., 2006; Martinez-Medina et al., 2006). This has important implications as these bacteria are the major source of butyrate which is considered important for optimal mucosal health (Bartholome et al., 2004; Pryde et al., 2002).

To further define the Enterobacteriaceae colonizing feline duodenal mucosa we used a probe that is specific for *E. coli* and *Shigella*. This probe hybridized with 20% of the mucosal bacteria in cats with signs of GI disease compared with less than 1% in healthy cats. Interestingly, *E. coli* are increasingly linked with IBD in people (Barnich and Darfeuille-Michaud, 2007), and have recently been identified in Boxer dogs with granulomatous colitis (Simpson et al., 2006). The *E. coli* strains associated with IBD in people and Boxer dogs with colitis share the ability to replicate in cultured epithelial cells and macrophages and belong to a novel pathogroup of adherent and invasive *E. coli* (AIEC) (Barnich and Darfeuille-Michaud, 2007). *E. coli* in cats with IBD were most abundant in the

adherent mucus overlying the superficial epithelium, a localization that could enable participation in the mucosal inflammatory response, and further study is required to determine their potential virulence.

An important finding of the present study was the detection of a shift in the spatial distribution of *Clostridium* spp. in cats with signs of gastrointestinal disease. In healthy cats *Clostridium* spp. were restricted to free and adherent mucus, whereas affected cats had approximately 10% of these bacteria on or in the surface epithelium and deeper tissues. This may represent opportunistic invasion by *Clostridium* spp. that are the dominant anaerobe in feline duodenal juice (Johnston et al., 1993; Papasouliotis et al., 1998), or perhaps the presence of a pathogenic strain of *Clostridium* spp. related to IBD.

To circumvent the subjectivity of conventional histopathology mucosal inflammation was assessed by measurement of mucosal cytokine mRNA and objective histopathology. The use of principal components analysis enabled us to gain insights into data structure and to reduce the numbers of variables prior to statistical analysis, which has been a limiting factor in previous studies of dogs with intestinal disease (Peters et al., 2005). Interestingly, we found that the total number of mucosal bacteria was strongly associated with changes in mucosal architecture, particularly villus atrophy and fusion, and the density of cellular infiltrates, especially T cells and macrophages. A subset of bacteria composed of Enterobacteriaceae spp., *E. coli*, and *Clostridium* spp. was also associated with significant changes in mucosal architecture, upregulation of IL-1, IL-8 and IL-12 relative to IFN- γ , IL10 and IL-6, and the severity of clinical disease based on the number of clinical signs. The possibility that mucosal bacteria are eliciting the mucosal inflammatory response is supported by the ability of enteric bacteria including, *E. coli* (Arikawa et al., 2005), *Salmonella* (Cho and Chae, 2003) and *Clostridium* spp. (Linevsky et al., 1997), to induce the secretion of proinflammatory cytokines. While the mechanisms of cytokine induction remain to be fully elucidated, it is known that mucosal invasion is not essential to induce the production of pro-inflammatory cytokines (Gewirtz et al., 1999), and that bacterial motility, adherence and invasion, possibly acting through Toll-like receptors and the translocation of NF- κ B are involved (Akhtar et al., 2003; Arikawa

et al., 2005; Cario, 2005). It is also becoming increasingly apparent that cross-talk between superficial epithelial cells and mononuclear cells in the lamina propria is important in modulating inflammatory responses to enteric bacteria, by down-regulating pro-inflammatory gene expression by intestinal epithelial cells in response to non-pathogenic enteric flora (Haller et al., 2004).

The design of the present study enabled us to examine the relationship between mucosal histopathology and cytokine mRNA. Our findings that cytokine upregulation in the feline duodenum and clinical disease activity correlate with the severity of architectural changes (atrophy, fusion, cryptal hyperplasia, and epithelial cell changes) and provide objective support for the use of histological grading schemes that relate changes in morphology to the severity of mucosal inflammation (Baez et al., 1999; Dennis et al., 1992; Hart et al., 1994; Jergens, 2002). Cytokine upregulation was also related to increases in the density of the cellular reaction, and the number of neutrophils and macrophages present in a specimen, and correlated with the histological severity of subjective IBD grade assigned by a pathologist, which takes into account a combination of architectural changes and cellularity. Our observation that cytokine levels were inversely correlated with fibrosis suggests downregulation as a result of chronicity, or loss and/or replacement of cytokine-producing cells with fibroblasts. The concurrent upregulation of the immunomodulatory cytokine IL-10 and proinflammatory cytokine IFN- γ observed in the present study parallels the mixed pattern of cytokine upregulation reported in cats with IBD in the UK (Nguyen Van et al., 2006). If cytokines are considered individually, it is notable that IL-8 had the strongest association with IBD score and clinical disease activity, suggesting that mucosal or fecal IL-8 may be a potential surrogate marker for disease activity in cats, as it is in people with intestinal inflammation (Banks et al., 2003; McCormack et al., 2001).

In conclusion, the present study establishes that the composition and number of mucosa-associated bacteria correlate with the presence and severity of inflammatory bowel disease in cats. It raises the possibility that an abnormal mucosal flora is involved in the pathogenesis of feline IBD and suggests that therapeutic intervention directed at the

mucosal flora may abrogate the mucosal inflammatory response.

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References

- Akhtar, M., Watson, J.L., Nazli, A., McKay, D.M., 2003. Bacterial DNA evokes epithelial IL-8 production by a MAPK-dependent NF-kappaB-independent pathway. *FASEB J.* 17, 1319–1321.
- Amann, R.L., Binder, B.J., Olson, R.J., Chisholm, S.W., Devereux, R., Stahl, D.A., 1990. Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. *Appl. Environ. Microbiol.* 56, 1919–1925.
- Arikawa, K., Meraz, I.M., Nishikawa, Y., Ogasawara, J., Hase, A., 2005. Interleukin-8 secretion by epithelial cells infected with diffusely adherent *Escherichia coli* possessing Afa adhesin-coding genes. *Microbiol. Immunol.* 49, 493–503.
- Baez, J.L., Hendrick, M.J., Walker, L.M., Washabau, R.J., 1999. Radiographic, ultrasonographic, and endoscopic findings in cats with inflammatory bowel disease of the stomach and small intestine: 33 cases (1990–1997). *J. Am. Vet. Med. Assoc.* 215, 349–354.
- Banks, C., Bateman, A., Payne, R., Johnson, P., Sheron, N., 2003. Chemokine expression in IBD Mucosal chemokine expression is unselectively increased in both ulcerative colitis and Crohn's disease. *J. Pathol.* 199, 28–35.
- Barnich, N., Darfeuille-Michaud, A., 2007. Adherent-invasive *Escherichia coli* and Crohn's disease. *Curr. Opin. Gastroenterol.* 23, 16–20.
- Bartholome, A.L., Albin, D.M., Baker, D.H., Holst, J.J., Tappenden, K.A., 2004. Supplementation of total parenteral nutrition with butyrate acutely increases structural aspects of intestinal adaptation after an 80% jejunioileal resection in neonatal piglets. *JPEN J. Parenter. Enteral. Nutr.* 28, 210–222 discussion 222–213.
- Cario, E., 2005. Bacterial interactions with cells of the intestinal mucosa: Toll-like receptors and NOD2. *Gut* 54, 1182–1193.
- Chan, V., Crocetti, G., Grehan, M., Zhang, L., Danon, S., Lee, A., Mitchell, H., 2005. Visualization of *Helicobacter* species within the murine cecal mucosa using specific fluorescence in situ hybridization. *Helicobacter* 10, 114–124.
- Cho, W.S., Chae, C., 2003. Expression of inflammatory cytokines (TNF-alpha, IL-1, IL-6 and IL-8) in colon of pigs naturally

- infected with *Salmonella typhimurium* and *S. choleraesuis*. *J. Vet. Med. A Physiol. Pathol. Clin. Med.* 50, 484–487.
- Dennis, J.S., Kruger, J.M., Mullaney, T.P., 1992. Lymphocytic/plasmacytic gastroenteritis in cats: 14 cases (1985–1990). *J. Am. Vet. Med. Assoc.* 200, 1712–1718.
- Elson, C.O., Cong, Y., McCracken, V.J., Dimmitt, R.A., Lorenz, R.G., Weaver, C.T., 2005. Experimental models of inflammatory bowel disease reveal innate, adaptive, and regulatory mechanisms of host dialogue with the microbiota. *Immunol. Rev.* 206, 260–276.
- Franks, A.H., Harmsen, H.J., Raangs, G.C., Jansen, G.J., Schut, F., Welling, G.W., 1998. Variations of bacterial populations in human feces measured by fluorescent in situ hybridization with group-specific 16S rRNA-targeted oligonucleotide probes. *Appl. Environ. Microbiol.* 64, 3336–3345.
- Gewirtz, A.T., Siber, A.M., Madara, J.L., McCormick, B.A., 1999. Orchestration of neutrophil movement by intestinal epithelial cells in response to *Salmonella typhimurium* can be uncoupled from bacterial internalization. *Infect. Immun.* 67, 608–617.
- Gophna, U., Sommerfeld, K., Gophna, S., Doolittle, W.F., Veldhuyzen van Zanten, S.J., 2006. Differences between tissue-associated intestinal microfloras of patients with Crohn's disease and ulcerative colitis. *J. Clin. Microbiol.* 44, 4136–4141.
- Haller, D., Holt, L., Parlesak, A., Zanga, J., Bauerlein, A., Sartor, R.B., Jobin, C., 2004. Differential effect of immune cells on non-pathogenic Gram-negative bacteria-induced nuclear factor-kappaB activation and pro-inflammatory gene expression in intestinal epithelial cells. *Immunology* 112, 310–320.
- Hanauer, S.B., 2006. Inflammatory bowel disease: epidemiology, pathogenesis, and therapeutic opportunities. *Inflamm. Bowel Dis.* 12 (Suppl. 1), S3–S9.
- Hart, J.R.S.E., Patnaik, A.K., Garvey, M.S., 1994. Lymphocytic-plasmacytic enterocolitis in cats: 60 cases (1988–1990). *JAAHA* 30, 505–514.
- Jergens, A.E., 2002. Feline inflammatory bowel disease—current perspectives on etiopathogenesis and therapy. *J. Feline Med. Surg.* 4, 175–178.
- Jergens, A.E., Moore, F.M., Haynes, J.S., Miles, K.G., 1992. Idiopathic inflammatory bowel disease in dogs and cats: 84 cases (1987–1990). *J. Am. Vet. Med. Assoc.* 201, 1603–1608.
- Johnston, K., Lamport, A., Batt, R.M., 1993. An unexpected bacterial flora in the proximal small intestine of normal cats. *Vet. Rec.* 132, 362–363.
- Johnston, K.L., Swift, N.C., Forster-van Hijfte, M., Rutgers, H.C., Lamport, A., Ballevre, O., Batt, R.M., 2001. Comparison of the bacterial flora of the duodenum in healthy cats and cats with signs of gastrointestinal tract disease. *J. Am. Vet. Med. Assoc.* 218, 48–51.
- Kempf, V.A., Trebesius, K., Autenrieth, I.B., 2000. Fluorescent In situ hybridization allows rapid identification of microorganisms in blood cultures. *J. Clin. Microbiol.* 38, 830–838.
- Kim, S.C., Tonkonogy, S.L., Albright, C.A., Tsang, J., Balish, E.J., Braun, J., Huycke, M.M., Sartor, R.B., 2005. Variable phenotypes of enterocolitis in interleukin 10-deficient mice mono-associated with two different commensal bacteria. *Gastroenterology* 128, 891–906.
- Kleessen, B., Kroesen, A.J., Buhr, H.J., Blaut, M., 2002. Mucosal and invading bacteria in patients with inflammatory bowel disease compared with controls. *Scand. J. Gastroenterol.* 37, 1034–1041.
- Linevsky, J.K., Pothoulakis, C., Keates, S., Warny, M., Keates, A.C., Lamont, J.T., Kelly, C.P., 1997. IL-8 release and neutrophil activation by *Clostridium difficile* toxin-exposed human monocytes. *Am. J. Physiol.* 273, G1333–G1340.
- Manz, W., Amann, R., Ludwig, W., Vancanneyt, M., Schleifer, K.H., 1996. Application of a suite of 16S rRNA-specific oligonucleotide probes designed to investigate bacteria of the phylum cytophaga-flavobacter-bacteroides in the natural environment. *Microbiology* 142 (Pt 5), 1097–1106.
- Martinez-Medina, M., Aldeguer, X., Gonzalez-Huix, F., Acero, D., Garcia-Gil, L.J., 2006. Abnormal microbiota composition in the ileocolonic mucosa of Crohn's disease patients as revealed by polymerase chain reaction-denaturing gradient gel electrophoresis. *Inflamm. Bowel Dis.* 12, 1136–1145.
- McCormack, G., Moriarty, D., O'Donoghue, D.P., McCormick, P.A., Sheahan, K., Baird, A.W., 2001. Tissue cytokine and chemokine expression in inflammatory bowel disease. *Inflamm. Res.* 50, 491–495.
- McGregor, D.P., Forster, S., Steven, J., Adair, J., Leary, S.E., Leslie, D.L., Harris, W.J., Titball, R.W., 1996. Simultaneous detection of microorganisms in soil suspension based on PCR amplification of bacterial 16S rRNA fragments. *Biotechniques* 21 (468), 461–470 463–466.
- Nguyen Van, N., Taglinger, K., Helps, C.R., Tasker, S., Gruffydd-Jones, T.J., Day, M.J., 2006. Measurement of cytokine mRNA expression in intestinal biopsies of cats with inflammatory enteropathy using quantitative real-time RT-PCR. *Vet. Immunol. Immunopathol.* 113, 404–414.
- Papasoulotis, K., Sparkes, A.H., Werrett, G., Egan, K., Gruffydd-Jones, E.A., Gruffydd-Jones, T.J., 1998. Assessment of the bacterial flora of the proximal part of the small intestine in healthy cats, and the effect of sample collection method. *Am. J. Vet. Res.* 59, 48–51.
- Peters, I.R., Helps, C.R., Calvert, E.L., Hall, E.J., Day, M.J., 2005. Cytokine mRNA quantification in duodenal mucosa from dogs with chronic enteropathies by real-time reverse transcriptase polymerase chain reaction. *J. Vet. Intern. Med.* 19, 644–653.
- Poulsen, L.K., Lan, F., Kristensen, C.S., Hobolth, P., Molin, S., Krogfelt, K.A., 1994. Spatial distribution of *Escherichia coli* in the mouse large intestine inferred from rRNA in situ hybridization. *Infect. Immun.* 62, 5191–5194.
- Priestnall, S.L., Wiinberg, B., Spohr, A., Neuhaus, B., Kuffer, M., Wiedmann, M., Simpson, K.W., 2004. Evaluation of “*Helicobacter heilmannii*” subtypes in the gastric mucosae of cats and dogs. *J. Clin. Microbiol.* 42, 2144–2151.
- Pryde, S.E., Duncan, S.H., Hold, G.L., Stewart, C.S., Flint, H.J., 2002. The microbiology of butyrate formation in the human colon. *FEMS Microbiol. Lett.* 217, 133–139.
- Sartor, R.B., 2006. Mechanisms of disease: pathogenesis of Crohn's disease and ulcerative colitis. *Nat. Clin. Pract. Gastroenterol. Hepatol.* 3, 390–407.
- Seksik, P., Lepage, P., de la Cochetiere, M.F., Bourreille, A., Sutren, M., Galmiche, J.P., Dore, J., Marteau, P., 2005. Search for localized dysbiosis in Crohn's disease ulcerations by temporal

- temperature gradient gel electrophoresis of 16S rRNA. J. Clin. Microbiol. 43, 4654–4658.
- Simpson, K.W., Dogan, B., Rishniw, M., Goldstein, R.E., Klaessig, S., McDonough, P.L., German, A.J., Yates, R.M., Russell, D.G., Johnson, S.E., Berg, D.E., Harel, J., Bruant, G., McDonough, S.P., Schukken, Y.H., 2006. Adherent and invasive *Escherichia coli* is associated with granulomatous colitis in boxer dogs. Infect. Immun. 74, 4778–4792.
- Sparkes, A.H., Pappasouliotis, K., Sunvold, G., Werrett, G., Clarke, C., Jones, M., Gruffydd-Jones, T.J., Reinhart, G., 1998. Bacterial flora in the duodenum of healthy cats, and effect of dietary supplementation with fructo-oligosaccharides. Am. J. Vet. Res. 59, 431–435.
- Straubinger, R.K., Greiter, A., McDonough, S.P., Gerold, A., Scanziani, E., Soldati, S., Dailidene, D., Dailide, G., Berg, D.E., Simpson, K.W., 2003. Quantitative evaluation of inflammatory and immune responses in the early stages of chronic *Helicobacter pylori* infection. Infect. Immun. 71, 2693–2703.
- Swidsinski, A., Weber, J., Loening-Baucke, V., Hale, L.P., Lochs, H., 2005. Spatial organization and composition of the mucosal flora in patients with inflammatory bowel disease. J. Clin. Microbiol. 43, 3380–3389.
- Tamboli, C.P., Neut, C., Desreumaux, P., Colombel, J.F., 2004. Dysbiosis in inflammatory bowel disease. Gut 53, 1–4.
- Uchida, K., Terada, A., 1971. Intestinal microflora of cats. Nippon Vet. Zootechnical Coll. Bull. 20, 11–16.
- Waly, N.E., Stokes, C.R., Gruffydd-Jones, T.J., Day, M.J., 2004. Immune cell populations in the duodenal mucosa of cats with inflammatory bowel disease. J. Vet. Intern. Med. 18, 816–825.