



Randomized, controlled trial evaluating the effect of multi-strain probiotic on the mucosal microbiota in  
canine idiopathic inflammatory bowel disease

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## **Abstract**

**Background:** The intestinal microbiota is increasingly linked to the pathogenesis of idiopathic inflammatory bowel disease (IBD) in dogs. While studies have reported alterations in fecal (luminal) microbial populations, only limited information is available about the mucosal microbiota of IBD dogs at diagnosis and in response to medical therapy.

**Aim:** To characterize the mucosal microbiota and determine the clinical, microbiological, and mucosal homeostatic effects of probiotic treatment in dogs with IBD.

**Methods:** Thirty four IBD dogs randomized to receive standard therapy (ST = diet + prednisone) with or without probiotic. Tissue sections from endoscopic biopsies were evaluated by fluorescence in situ hybridization (FISH) on a quantifiable basis. Disease activity and changes in mucosal microbiota and tight junction protein (TJP) expression were assessed before and after 8 weeks of IBD therapy.

**Results:** ST and ST/probiotic therapy modulate the number of mucosal bacteria of IBD dogs in a similar fashion. Both treatments increased the numbers of total bacteria and individual species residing within adherent mucus, with ST therapy increasing *Bifidobacterium* spp. and ST/probiotic therapy increasing *Lactobacillus* spp. ( $P < 0.05$  for both), respectively. Both treatments were associated with rapid clinical remission but not improvement in histopathologic inflammation. Probiotic therapy was associated with up-regulated ( $P < 0.05$ ) expression of TJPs E-cadherin, occludin, and zonulin versus ST.

**Conclusions:** Probiotic effect on mucosal bacteria is similar to that of IBD dogs receiving ST. IBD dogs fed probiotic had increased TJP expression which suggests that the probiotic may have beneficial effects on mucosal homeostasis.

Canine idiopathic inflammatory bowel disease (IBD) is a chronic, immunologically-mediated enteropathy that is poorly understood.<sup>1, 2</sup> Data from humans and animal models of intestinal inflammation indicate that deleterious host-microbiome interactions can incite and perpetuate mucosal inflammation. For example, genetic defects in innate immune sensing and enteric bacterial killing confer increased susceptibility to IBD in German shepherd dogs (GSD) associated with a distinct intestinal dysbiosis.<sup>3</sup> Moreover, bacteria that adhere and invade the intestinal mucosa, including strains of *Escherichia coli* (*E. coli*), may be particularly important for IBD pathogenesis in both humans<sup>4</sup> and companion animals<sup>5</sup>.

Many studies have reported microbial imbalances in canine IBD, characterized by a decrease in diversity, a decreased abundance of some commensal members (such as Clostridium clusters XIVa and IV), and an increase in detrimental bacteria (such as and *E. coli* and the Enterobacteriaceae).<sup>6-8</sup> While these earlier studies have primarily reported changes in fecal microbial populations, only limited information is available about the mucosal microbiota present in dogs with IBD.

Gut microbiota modulation to correct microbial perturbations might be obtained by several therapeutic approaches, including diet, antibiotics, and probiotic supplementation. Probiotics contain live, beneficial bacteria in sufficient amounts to reach the intestine in an active state and exert positive health effects.<sup>9</sup> There is only one investigation reporting on the efficacy of multi-strain probiotic therapy for canine IBD.<sup>10</sup> This earlier study included a small number of patients and reported changes in the fecal, but not the mucosal, microbiota following probiotic administration.

The aim of the present study was compare the effect of standard IBD therapy (ie, elimination diet and oral prednisone) versus standard therapy plus multi-strain probiotic on the composition and spatial distribution of the mucosal microbiota in dogs with idiopathic IBD.

## **Materials and Methods**

### **Ethics statement**

Approval to conduct the study was obtained from the Iowa State University (ISU) Institutional Animal Care and Use Committee (IACUC log # 9-14-7859-K). All clients provided informed written consent permitting enrollment of their animal into the IBD clinical trial.

### **Animals and inclusion/exclusion criteria**

Idiopathic IBD was diagnosed in 34 dogs based on the following criteria: (1) history of persistent or intermittent gastrointestinal (GI) signs of at least 3 weeks duration, (2) exclusion of identifiable underlying disorders, (3) failed response to empiric (parasiticides, diet, antibiotics) therapies, and (4) histopathologic evidence of GI mucosal inflammation.<sup>11</sup> The minimum diagnostic evaluation performed in all dogs included a complete blood count (CBC), serum biochemistry panel, urinalysis, GI panel to Texas A&M University (ie, trypsin-like immunoreactivity [cTLI], canine pancreatic lipase immunoreactivity [cPLI], and serum cobalamin/folate concentrations), direct (wet mount)/indirect (zinc sulfate flotation) examination of feces for nematode and protozoan parasites, resting cortisol/ACTH stimulation test, abdominal radiographs, and histopathologic review of endoscopically-derived biopsy specimens. Abdominal ultrasonography was performed in most dogs (n = 25) at the clinician's discretion.

Prior to trial enrollment, IBD suspect dogs had been treated with an elimination (ie, intact protein or protein hydrolysate) diet fed exclusively for at least 3 weeks followed by treatment with metronidazole (10 mg/kg PO q12h for 3 weeks) and had failed to respond to either therapy. All medications were withdrawn at least 2 weeks before tissue collection. Clinical disease severity was assessed using the canine IBD activity index (CIBDAI) at all visits.<sup>12</sup>

Exclusion criteria included dogs with other causes for GI signs besides idiopathic IBD, and treatment with antimicrobials, anti-inflammatory drugs or both within 14 days of presentation.

### **Study design**

There were three study centers for case recruitment: Iowa State University CVM, Colorado State University CVM, and Veterinary Specialty Hospital – San Diego. Following diagnosis of IBD, dogs were randomized by means of a computer-generated schedule to receive standard IBD therapy (defined as an elimination diet fed

exclusively for the duration of trial and prednisone PO at a dosage of 0.5-1 mg/kg q12h x 3 weeks then 0.5 mg/kg q12h x 3 weeks then maintained or tapered over the 8 week duration of the study = ST), or standard therapy with a multi-strain probiotic (e.g., Visbiome™ which contains the same strains, in the same concentration and proportions, and is therapeutically equivalent to the VSL#3® brand probiotic blend as produced before January 31, 2016 = ST/probiotic). The product contains the following strains: *Lactobacillus plantarum* DSM 24730, *Streptococcus thermophilus* DSM 24731, *Bifidobacterium breve* DSM 24732, *Lactobacillus paracasei* DSM 24733, *Lactobacillus delbrueckii* subsp. *bulgaricus* DSM 24734, *Lactobacillus acidophilus* DSM 24735, *Bifidobacterium longum* DSM 24736, and *Bifidobacterium infantis* DSM 24737 in the specific combination which is currently sold under the brand Vivomixx® in Continental Europe and Visbiome® in the USA and Canada. The percentage in weight of the various ingredients was: *S. thermophilus* 40.55%, Bifidobacteria 12.5%, Lactobacilli 13%, and other excipients 39.05%. Dosing of the probiotic ( $112\text{-}225 \times 10^9$  CFU/10kg; gift of Professor Claudio De Simone) was based on enrollment body weight and was administered using previously published guidelines for treatment of canine IBD.<sup>10</sup> In brief, dogs weighing 10--20 kg body weight and randomized to receive probiotic were administered capsules containing 450 billion probiotic bacteria daily. Dogs randomized to receive ST were administered a placebo consisting of an identical capsule that contained maltodextrin. The probiotic and placebo capsules were mailed from the GI Laboratory at Texas A&M University directly to the client. Both the veterinary gastroenterologist and client were blinded as to whether the dog received probiotic or placebo as a component of their IBD therapy.

Dogs were evaluated at study centers on three separate visits: visit 1 (recruitment), visit 2 (~ 3 weeks after starting trial medications), and visit 3 (~ 8 weeks after starting trial medications; Fig. 1). The diagnostic procedures performed on dogs at each visit are listed in Table 1. The final on-site examination (visit 3 at 8 weeks) was performed while dogs were still receiving trial medications.

### **Intestinal biopsy and histopathologic examination**

Most dogs had diagnostic GI endoscopy performed for the collection of duodenal, ileal and/or colonic biopsy specimens. In some dogs with large bowel GI signs alone, only colonic mucosal biopsies were obtained for analysis. Dogs were prepared for colonoscopy (visit 1) by withholding food overnight and administering an oral colonic electrolyte lavage solution, twice, at a dosage of 20 ml/kg. One or two tepid water enemas (20 ml/kg) were performed in the morning prior to endoscopic examination. Repeat colonoscopy (visit 3) was performed without colonic cleansing by digital evacuation of feces with collection of distal large bowel biopsies from relatively fecal-free areas of colonic mucosa. Prior to endoscopy, the endoscope and biopsy forceps were thoroughly cleaned and sterilized using an activated aldehyde solution and gas sterilization, respectively. Multiple (10-15 tissues from the duodenum; 9--12 tissues from the colon; 3--6 tissues from the ileum) endoscopic biopsies were obtained and fixed in 10% neutral buffered formalin and then paraffin embedded for use in histopathology, using H&E and immunohistochemical (IHC) stains, and for FISH analysis.

Histopathologic examination of endoscopic paraffin-embedded tissue sections was performed by a single pathologist (MA) blinded as to each dog's history and clinical course. Tissues were graded for severity of intestinal mucosal inflammation using simplified WSAVA histopathologic criteria.<sup>13</sup>

### **Fecal microbiota composition**

To evaluate specific bacterial groups of interest to intestinal health (e.g., *Lactobacillus*, *Bifidobacterium*, and *Streptococcus* genera present in the probiotic cocktail) and bacterial species with potential pathogenic roles (e.g., *Escherichia coli*, and *Clostridium perfringens*) quantitative polymerase chain reaction techniques (qPCR) were used as described previously.<sup>14</sup> One hundred mg of feces were aliquoted into a sterile 1.7 ml tube (Microtube, Sarstedt AG & Co, Nümbrecht, Germany) containing 150 µl of 0.1 mm zirconia-silica beads and 100 µl of 0.5 mm zirconia-silica beads (BioSpec Products Inc., Barlesville, OK, USA). Samples were then homogenized (FastPrep-24, MP Biomedicals, USA) for a duration of 1 minute at a speed of 4 m/s. DNA was then extracted with the ZR fecal DNA Mini Prep kit following the manufacturer's instructions (Zymo Research, Irvine CA, USA). Briefly, qPCR reactions were performed using two reaction chemistries. For a

subset of assays SYBR-green based reaction mixtures were used, with a total reaction volume of 10  $\mu$ l. The final mix contained 5  $\mu$ l SsoFast™ EvaGreen® supermix (Bio-Rad Laboratories, CA, USA), 0.4  $\mu$ l each of a forward and reverse primer (final concentration: 400 nM), 2.6  $\mu$ l of high quality PCR water, and 2  $\mu$ l of normalized DNA (final concentration: 5 ng/ $\mu$ l). Conditions for PCR were as follows: initial denaturation at 98°C for 2 min, then 40 cycles with denaturation at 98°C for 3 sec and annealing (see Table 3 for specific annealing temperatures) for 3 sec. Post-amplification, a melt curve analysis was performed using these conditions: 95°C for 1 min, 55°C for 1 min, and increasing incremental steps of 0.5°C for 80 cycles for 5 sec each. All samples were run in duplicate fashion. TaqMan® based reaction mixtures were used in a total reaction volume of 10  $\mu$ l. The final mix contained 5  $\mu$ l TaqMan® Fast Universal PCR master mix (Life Technologies, NY, USA), 0.4  $\mu$ l of a forward and reverse primer (final concentration: 400 nM), 2  $\mu$ l of high quality PCR water, and 2  $\mu$ l of normalized DNA (final concentration: 5 ng/ $\mu$ l). Conditions for PCR were as follows: initial denaturation at 95°C for 20 sec then 40 cycles with denaturation at 95°C and annealing (see Table 3 for specific annealing temperatures) for 3 sec. Post-amplification, a melt curve analysis was performed using these conditions: 95°C for 1 min, 55°C for 1 min, and increasing incremental steps of 0.5°C for 80 cycles for 5 sec each. A complete list of primers and probes used in this study have previously been published.<sup>10</sup> All samples were run in duplicate fashion. The qPCR data was expressed as log amount of DNA (fg) for each individual bacterial group per 10 ng of isolated total DNA.

### **Fluorescence in situ hybridization (FISH)**

The formalin-fixed embedded histopathological tissue sections were mounted on glass slides and evaluated by fluorescence in situ hybridization (FISH) as previously described.<sup>15-17</sup> In brief, paraffin-embedded tissue specimens were deparaffinized using an automated system 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 next rapidly transported in deionized water to the DNA testing laboratory where they were air dried prior to hybridization. FISH probes 5'-labeled with either Cy-3 or FITC (Life Sciences) were reconstituted with DNase-free water and diluted to a working concentration of 5 ng/ $\mu$ L (Table 2).

For total bacterial counts EUB338-FITC was used. For other analyses, specific probes targeting Bifidobacteria, Streptococci, Fecalibacteria, Lactobacilli, and *Enterobacteriaceae* were labeled with Cy-3 and were applied simultaneously with the universal bacterial probe Eub338-FITC. This probe array was selected to identify specific bacterial groups and individual bacterial species previously shown to be relevant in the pathogenesis of IBD in humans and animals.<sup>18-22</sup> Tissue sections were bathed in 30  $\mu$ L of DNA-probe mix in a hybridization chamber maintained at 54° C overnight (12 h). Washing was performed using a wash buffer (hybridization buffer without SDS), the slides were rinsed with sterile water, then allowed to air-dry, and mounted with SlowFade Gold mounting media (Life Technologies, Carlsbad, CA) and 25  $\times$  25-1 cover glass (Fisher Scientific, Pittsburgh, PA).

Probe specificity was confirmed in pilot studies by combining the irrelevant probe non-Eub338-FITC with Eub338-Cy-3, and through hybridization experiments with pure bacterial isolates to screen for non-selective hybridization.

#### **In situ quantification of mucosal bacteria**

The bacteria were visualized by FISH and 4,6-diamidino-2-phenylindole (DAPI) staining using a 60x Plan Apo oil objective in conjunction with an optional 1.5x multiplier lens on an Eclipse TE2000-E fluorescence microscope (Nikon Instruments Inc., Melville NY) and photographed with a CoolSnap EZ camera (Photometrics, Tuscon, AZ) controlled by MetaMorph software (Nashville, TN). Quantification was only performed when the hybridization signals were strong and could clearly distinguish intact bacteria morphologically by either 2-color (universal and bacterial specific FISH probe) or 3-color (FISH probes and DAPI stain) identification. A minimum of 4 different endoscopic biopsy specimens/organs were evaluated for their mucosal bacterial content. Bacterial quantification was performed in 10 representative fields at a final observed magnification of 600x or 900x. Each counting field generally included bacteria found within mucosal and adjacent supra-mucosal regions. The ten fields included bacteria located within 4 well-defined mucosal compartments: (1) bacteria contained within the mucosa, (2) bacteria attached to the surface epithelium, (3) bacteria localized within adherent mucus, and (4) bacteria found within free mucus

(Supplemental Fig.1).<sup>23</sup> The number of bacteria per compartment summed across the 10 counting fields for each probe in each dog was used in statistical analysis.

### **Immunohistochemistry for tight junction protein expression**

Paraffin tissue sections were rehydrated and neutralized for endogenous peroxidases using 3% hydrogen peroxide for 5 minutes then rinsed for 5 minutes in distilled water. For antigen retrieval, slides were incubated in an antigen retrieval solution of 0.01 M Tris-EDTA buffer (pH 9.0) for claudin 2, occludin and E-cadherin in a steamer (Black & Decker, Towson, MD, USA) for 20 minutes. For zonulin stain, slides were immersed in a staining dish containing Sodium Citrate Buffer (10mM Sodium Citrate, 0.05% Tween 20, pH 6.0) which was heated to 95--100°C in a water bath and with the lid placed loosely on the staining dish for an optimal incubation of 35 minutes. Following incubation, the slides were cooled for 20 minutes then washed in PBS-Tween 20 for 2 × 2 minutes. For all tissue sections, non-specific binding was blocked by incubation with a protein-blocking agent (Protein-blocking agent, Dako, Carpinteria, CA, USA) for 10 minutes before application of the primary antibodies. Slides were incubated overnight in a moist-chamber (4°C) with the following primary antibodies: Polyclonal rabbit anti-claudin-2 (Polyclonal rabbit anti-claudin-2 (PAD: MH44), Invitrogen Ltd., Paisley, UK) and anti-occludin (anti-occludin PAD: Z-T22, Invitrogen Ltd., Paisley, UK) antibodies and monoclonal mouse anti-E-cadherin IgG2 $\alpha$  (Monoclonal mouse anti-E-cadherin IgG2 $\alpha$  (clone: 36), BD Biosciences, Oxford, UK) as described previously.<sup>10</sup> For zonulin stain, the primary antibody was a rabbit derived polyclonal antibody (anti-Zonulin pAb, LS-C132998, LSBio Inc., USA, diluted 1:300). The immunohistochemistry stain LS-C132998 pAb was validated previously using a panel of 21 formalin-fixed, paraffin-embedded (FFPE) human and canine tissues after heat-induced antigen retrieval in pH 6.0 citrate buffer. Following incubation with the primary antibodies, slides were incubated with biotinylated secondary antibodies. These antibodies included 1) goat anti-rabbit biotinylated immunoglobulin (E0432, Dako, Glostrup, Denmark) used at a dilution of 1:250 and incubated for 1 hour to bind polyclonal rabbit-derived anti-zonulin, claudin-2 and occludin antibodies; and 2) goat polyclonal anti-mouse biotin-coupled secondary antibody (E 0443, Dako, Glostrup, Denmark) used at dilution of 1:200 and incubated for 1 hour to

bind monoclonal murine-derived anti-E-cadherin antibody. The incubation with secondary antibodies was followed by an avidine-biotin complex (ABC *elite*, Vector, Burlingame, UK) incubation of 45 minutes and a chromogen (DAB and Vector VIP, Vector) incubation generally of 10 minutes, but under direct microscope-control, evaluating the degree of intensity of the stain.

To assess expression of claudin-2, occludin, E-cadherin, and zonulin proteins in endoscopic biopsies obtained before and after either treatment, stained tissue sections were evaluated at  $\times 200$  and  $\times 630$  (oil immersion) magnification to identify areas of consistent staining and acceptable orientation. Immunostaining was evaluated along the length of multiple enteric/colonic crypts and in areas of intact luminal epithelium. Stain intensity was subjectively graded as absent (-), weak (+), moderate (++) , or strong (+++), and the localization and distribution of chromogen were noted. For quantification of the number of TJP expressing cells, the intestinal epithelium was divided into luminal, proximal, and distal gland/crypt regions, and the intercellular junction was divided into apical and basolateral compartments. All IHC positive cells were quantified using a light microscope (Carl Zeiss), a  $\times 40$  objective, a  $\times 10$  eyepiece, and a square eyepiece graticule ( $10 \times 10$  squares, with a total area of  $62,500 \mu\text{m}^2$ ). Ten appropriate sites were chosen for each compartment and arithmetic means were calculated for each intestinal region. Results were expressed as the number of IHC positive cells per  $62,500 \mu\text{m}^2$ , regardless of staining intensity. The IHC stained slides were evaluated in a blinded manner by a single pathologist (GR) to confirm staining specificity and to perform quantification of the number of TJP expressing cells.

### **Outcome measures**

The primary endpoints were changes in mucosal bacteria, induction of clinical remission, and immunohistochemical evaluation of tight junction protein expression in intestinal biopsies. Clinical remission was defined as a decrease in the CIBDAI of 50% or more from baseline (week 0) to visit 2, and a decrease in the CIBDAI of 75% or more from baseline to visit 3.<sup>11</sup>

Secondary measures included total histopathologic score pre- versus post-therapy, fecal microbiota composition, the need for pharmacologic treatments outside the study regimen, and any adverse drug or probiotic reactions.

### **Statistical analysis**

Tabular data were organized by mucosal compartment and treatment group of dogs. Mean, median, minimum, and maximum values were calculated from the bacterial counts. Median values were compared among groups using the Wilcoxon rank sum tests. Similar summary statistics were performed on cells expressing individual TJPs with median values compared between the duodenum and colon before and after treatment using Wilcoxon rank sum tests. Associations between bacterial numbers and histopathologic score were assessed using linear mixed models with dogs as a random effect. Histopathologic scores were compared between dog groups over time using a one way ANOVA and student's T test. Association among other variables were assessed using Spearman's rank correlation and tested for significance.

For statistical comparisons of temporal qPCR data, differences between time points were evaluated within either the ST or ST/probiotic group. Log DNA was tested for normality using the Shapiro-Wilk test and the normal assumption was rejected for most data sets. Therefore, a Friedman's test was used to evaluate repeated measures within either of the IBD treatment groups.

A Dunn's post-test was then used where Friedman's test was found to be significant. For the qPCR data analyses, 9 dogs were evaluated in the ST group while 10 dogs were evaluated in the ST/probiotic group. The level of significance for all comparisons was set at  $P < 0.05$ .

Sample size for enrollment was calculated following consultation with a statistician ( $n = 32$  total dogs over 36 months which included a 20% "washout") and was based on initial power calculations using variability in CIBDAI scores between IBD treatments.<sup>11</sup> Randomization of 16 dogs per group would give a power of 80% to detect this difference at the 0.05 significance level. Additional dogs were enrolled to allow for a noncompliance rate up to 20%.

### **Results**

### **Trial enrollment and completion**

Thirty four dogs were enrolled in the trial with 17 dogs assigned to the ST group and 17 dogs assigned to the ST/probiotic group (Fig.2). No dog was withdrawn prior to treatment assignment. Five dogs in the ST group did not complete the trial. One dog was withdrawn because of severe refractory GI disease associated with protein-losing enteropathy (PLE) and was euthanized. A second dog never returned after enrollment and was lost to follow-up. Three other dogs were excluded following diagnosis of alimentary neoplasia (two dogs with small intestinal lymphoma [LSA] and one dog with colonic adenocarcinoma) on repeat endoscopy at visit 3. In the ST/probiotic group, 3 dogs failed to complete the trial due to possible adverse medication(s) reaction (n = 1), severe refractory GI disease requiring antibiotics (n = 1), and small intestinal LSA diagnosed on visit 3 repeat endoscopy with histopathology.

Ultimately, 12 dogs in the ST group and 14 dogs in the ST/probiotic group completed the 8 week clinical trial.

There was no significant difference in total histopathologic scores between treatment groups at visit 1. Both dog groups combined (e.g., 69% of total dogs) were diagnosed with moderate-to-severe intestinal inflammation characterized by a predominant lymphocytic-plasmacytic infiltrate which was variably increased within the intestinal lamina propria. Post-treatment histology scores (visit 3) failed to differ significantly between treatment groups nor to improve significantly with either therapy as compared to visit 1. There were 2 dogs that required drug therapy outside the study regimen. One dog (ST/probiotic cohort) with PLE and steroid refractory disease was placed on cyclosporine (5mg/kg PO q24h) from visit 2 to visit 3; a second dog (ST cohort) developed a bacterial urinary tract infection (UTI) at visit 2 and was placed on systemic antibiotics. Following urine culture confirmation of infection, treatment with placebo was stopped, the dog was administered a penicillin plus clavulanic acid antibiotic for 14 days, and repeat urinalysis/urine culture was performed to confirm eradication of the bacterial UTI. Following a 2 week washout period where the dog was maintained only on the elimination diet and prednisone (0.25 mg/kg PO q 12h), the dog was placed back on placebo for the remaining 5 weeks of the trial.

No major adverse effects were reported. Two dogs in the ST/probiotic arm had soft stools during the first week of therapy; one other dog in the same group had increased vomiting during week 2 of therapy and withdrew from the study. In each instance, it could not be determined whether GI signs were attributable to the medical intervention or underlying IBD activity (e.g., disease flare).

### **Baseline clinical data**

The majority (24/26, 92%) of dogs completing the trial had clinical signs of enteritis or enterocolitis; 2 dogs were enrolled with signs of large bowel diarrhea alone. Dogs in both groups comprised a diverse mix of predominantly pure breed dogs with German shepherd dogs, Labrador retrievers, Beagles, Welsh Pembroke Corgis, and English bulldogs comprising 2 enrollees each. The treatment groups did not differ in any patient demographic at visit 1 (Table 3). Individual patient metadata may be found as supplemental figure 2. Eight dogs had serum cobalamin concentrations below reference range at enrollment and were treated for cobalamin deficiency using a standard treatment regimen.<sup>24</sup> Ultrasonographic abnormalities were observed in 8/25 dogs and included mild abdominal effusion (n = 4), loss of normal intestinal wall layering (n = 3), increased intestinal wall thickness (n = 8), and/or enlarged and hypoechoic mesenteric lymph nodes (n = 6), with some dogs having multiple sonographic abnormalities.

### **Primary end points**

Significant differences in the number of colonic total bacteria (e.g., bacteria summed across all 4 mucosal compartments) and mucosal bacteria (e.g., bacteria summed across 3 mucosal compartments including bacteria found within the mucosa, attached to the surface epithelium, and/or localized within adherent mucus) as determined by FISH were observed post-treatment (see supplemental Figs 3A and 3B.). There were no differences between treatment groups in the number of total bacteria hybridizing against 5 of the 6 probes at visit 1. Only the total number of Eub338-positive bacteria were increased in dogs randomized to receive ST/probiotic ( $P < 0.02$ ) at enrollment. Similarly, the total number of most bacteria increased significantly ( $P < 0.03$  to  $P < 0.001$ , depending on the bacterial group evaluated) regardless of the IBD treatment

administered (visit 1 → visit 3; Fig.3). Dogs treated with ST/probiotic showed increased total numbers of *Lactobacillus* spp. versus dogs treated with ST alone ( $P < 0.001$ ).

Changes in the number of mucosal bacteria mirrored those observed for total bacteria except that Eub338-positive mucosal bacteria failed to increase ( $P = 0.13$ ) in dogs treated with ST/probiotic. When evaluating changes in the spatial distribution of mucosal bacteria following IBD therapy, significant increases in bacteria hybridizing to probes against *Eubacteria*, *Bifidobacterium* spp., *Fecalibacterium* spp., and Enterobacteriaceae were observed within adherent mucus of dogs treated with ST; while, these same bacterial groups and *Lactobacillus* spp. were increased within the adherent mucus of IBD dogs receiving ST/probiotic therapy ( $P < 0.001$  for each; Fig. 4). A comparison of treatment effects involving adherent mucus bacteria showed that ST therapy preferentially increased mucosal *Bifidobacteria* spp. ( $P < 0.05$ ) while ST/probiotic therapy preferentially increased mucosal *Lactobacillus* spp. ( $P < 0.001$ ).

Clinical disease severity (CIBDAI) at enrollment (visit 1) was scored as moderate-to-severe in both treatment groups. Similarly, both treatments were associated with significantly reduced CIBDAI scores at visits 2 and 3 as compared to visit 1 ( $P < 0.001$ ; Fig. 5). At visit 2, clinical remission was observed in 12/14 (86%) and 10/12 (83%) of dogs receiving ST/probiotic and ST therapy, respectively. Only 1 dog in each treatment group failed to achieve full clinical remission at visit 3 (e.g., 8 weeks post-treatment).

Since probiotics may effect intestinal barrier integrity, we examined tight junction protein expression using IHC in intestinal tissues of IBD dogs treated with ST and ST/probiotics. Probiotic therapy was associated with up-regulated ( $P < 0.05$ ) expression of TJPs E-cadherin, occludin, and zonulin when compared to dogs receiving ST (Table 4; Fig. 6).

### **Correlation between total histopathologic score and numbers of mucosal bacterial**

Total histopathologic scores did not differ pre- versus post-treatment in dogs treated with either ST ( $P = 0.18$ ) or ST/probiotic ( $P = 0.08$ ). There was no association observed between bacterial numbers and total histopathologic score for either treatment group (data not shown).

## Quantitative PCR analysis performed on feces

Quantitative PCR was used to target select bacterial groups known to often be altered during health and disease states. Additionally, qPCR was used to target specific genera found in the probiotic cocktail. In the ST group, *Turicibacter*, was found to be significantly different ( $p = 0.0476$ ) when comparing the baseline, 3 week, and 8 week time points. However, post-testing revealed no significant differences between time points. In the ST/probiotic group, *Bifidobacterium* was found to be significantly increased at 3 weeks post-treatment compared to baseline (log DNA mean [standard deviation]: 6.8 [0.7] and 4.9 [1.6], respectively). Statistical analysis also revealed a difference in the abundance of *Lactobacillus* in the ST/probiotic group, however, this did not reach statistical significance ( $P = 0.0665$ ). Supplemental figures 4 and 5 include graphical representation for all bacterial groups analyzed by qPCR.

## Discussion

There is growing medical evidence in humans that the consumption of probiotics promotes GI health, representing a promising new therapy for canine gastroenteritis including idiopathic IBD.<sup>25-27</sup> Current treatments for canine IBD include the administration of nonspecific anti-inflammatory drugs (e.g., corticosteroids and others) which may confer serious side effects and do not address the underlying suspected basis for disease, namely, altered microbial composition. Use of probiotics (viable, non-pathogenic bacteria that exert health benefits beyond basic nutrition) offers an attractive, physiologic, and non-toxic alternative to potentially shift the balance from harmful to protective bacterial species and treat IBD.

The probiotic product (Visbiome™, ExiGi Pharma, which contains the De Simone Formulation probiotic) used in the present study contained  $\sim 1.8 \times 10^{12}$  live bacteria, including *L. plantarum* DSM24730, *L. paracasei* DSM24733, *L. delbrueckii* subsp. *bulgaricus* DSM24734, *L. acidophilus* DSM 24735, *S. thermophilus* DSM24731, *B. breve* DSM24732, *B. longum* DSM24736, and *B. infantis* DSM24737. This same probiotic formulation has been effective in the prevention and treatment of colitis in different rodent models of intestinal inflammation<sup>28-30</sup>, and is useful in the management of a subset of human IBD patients

diagnosed with ulcerative colitis<sup>31, 32</sup> and pouchitis<sup>33, 34</sup>. The exact mechanism underlying the therapeutic effect of the probiotic is currently unknown. It has been hypothesized that the beneficial activities might include altering the composition of the microbiota by competition for adhesion sites and nutrients or by secretion of bacteriocins and lactic acids, enhancement of intestinal barrier function (via increased production of mucus and anti-microbial peptides by epithelia), and modulation of immune cell responses.<sup>35, 36</sup>

There are only few reports on the use of probiotic bacteria in dogs with gastroenteritis. Earlier *in vitro* studies have confirmed the ability of a lyophilized probiotic cocktail (e.g., three different *Lactobacillus* spp. strains) to modulate the expression of regulatory versus pro-inflammatory cytokines in dogs with chronic enteropathies.<sup>37</sup> However, a clinical trial using this same probiotic cocktail fed to dogs with food-responsive diarrhea failed to induce consistent patterns of beneficial cytokine expression in spite of clinical improvement.<sup>38</sup> Still others have reported variable clinical efficacy of *E. faecium* strain SF68 in eradicating giardial cyst shedding, fecal giardial antigen and immune responsiveness in dogs<sup>39</sup>, while its use was associated with shortened time to improved stool consistency in dogs having acute gastroenteritis<sup>40</sup>. Treatment of CE dogs with *E. faecium* plus a prebiotic (FOS) alongside dietary intervention had no effect on inflammasome gene expression in a separate trial.<sup>41</sup>

More recently, a multi-strain probiotic was shown to enhance clinical remission and reduce histopathologic inflammation in IBD dogs when administered continuously for 8 weeks.<sup>10</sup> While this earlier study and the present report were both associated with robust clinical remission, we were unable to confirm significant histopathologic improvement associated with probiotic use in the IBD dogs our study. This difference might be explained by variation in clinical disease duration and severity and/or differences in the magnitude of histopathologic inflammation observed at diagnosis in IBD dogs of the present study. Moreover, in contrast to this earlier report, we did not evaluate the expression of mucosal TGF- $\beta$ + or the number of CD3+ T cells and FoxP3+ cells in response to either IBD therapy. Our qPCR results showed that the abundance of *Turicibacter* spp. (ST group) and *Bifidobacterium* spp. (ST/probiotic group) in feces were increased in IBD dogs post-treatment while the earlier trial showed that only the abundance of *Fecalibacterium* spp. was increased in

IBD dogs receiving combination probiotic. No significant changes were observed for any other bacterial groups in response to treatment.

In this study, we show now that ST (e.g., elimination diet and oral prednisone dosed at anti-inflammatory levels) and ST + probiotic modulate the number of mucosal bacteria of dogs with IBD in a similar fashion following 8 weeks of continuous administration. Moreover, both IBD therapies increased the numbers of bacterial species residing within the adherent mucus compartment. Comparison of the effects between IBD treatments showed that ST therapy significantly increased *Bifidobacterium* spp. and ST/probiotic therapy significantly increased *Lactobacillus* spp. in the adherent mucus compartment of colonic tissues versus the other treatment, respectively. Probiotic therapy was associated with up-regulated expression patterns of TJPs in duodenal and colonic biopsies suggesting potential effects of combination probiotic on intestinal barrier function.<sup>42</sup>

It is now well established that canine chronic enteropathies, including IBD, are associated with broad shifts in the intestinal microbiota which includes reduced microbial diversity (e.g., reduction in Firmicutes, class Clostridia including Clostridium clusters IX and XIV, genus Bacteroides, and Fusobacteria with increases in Proteobacteria and *Enterobacteriaceae*).<sup>43</sup> These data are derived from separate investigations where microbial composition was investigated by culture-independent methods such as 454 pyrosequencing<sup>6</sup>, gene clone libraries<sup>8, 44, 45</sup>, and quantitative PCR techniques.<sup>10, 46</sup> Direct assessment of mucosal bacteria using FISH has provided compelling new evidence on the association between altered composition and spatial organization of intestinal microbes and disease pathogenesis in dogs with granulomatous colitis<sup>47</sup> and other forms of IBD and colorectal cancer<sup>23</sup>. Furthermore, eradication of invasive *E. coli* with antibiotic therapy was associated with histopathologic improvement and resolution of GI signs, suggesting that interventions targeting harmful mucosal bacterial species are important treatment considerations.<sup>48</sup>

This is the first study utilizing FISH methods to localize, quantify, and directly compare different medical treatments on mucosal bacterial populations in dogs with IBD. Our experimental design allowed assessment of steroid-induced changes in mucosal microbiota which had not been previously reported. The most

important finding of this study obtained using FISH was the absence of a significant difference in the numbers of colonic microbiota following treatment of IBD dogs with ST versus ST/probiotic. Our results are difficult to compare to previous studies since only one other trial has investigated probiotic therapy in IBD dogs. Here, Rossi et al showed that increased fecal concentrations of *Fecalibacterium* spp. and *Turicibacter* spp. were present in IBD dogs fed a multi-strain probiotic continuously for 8 weeks.<sup>10</sup> Similar to Rossi, we observed increased luminal concentrations of select beneficial bacterial species, including *Bifidobacterium* spp., in the IBD dogs treated with ST/probiotic. Our results are also consistent with those obtained using terminal restriction fragment length polymorphism (T-RFLP) methods to investigate the impact of a probiotic mixture on the mucosal-adherent colonic microbiota of TNBS-induced colitis in rats.<sup>29</sup> In this trial, probiotic consumption did not significantly affect species richness or biodiversity of the mucosal microbiota but was associated with decreased severity of colitis. In contrast, a separate study showed that multi-strain probiotic therapy could alter luminal and mucosal microbial composition but did not protect against colitis-associated inflammation and tumorigenesis in susceptible mice.<sup>49</sup>

We hypothesized that probiotic effects in IBD dogs would preserve epithelial barrier function through increased number of cells expressing TJPs as previously shown in other IBD animal models.<sup>10, 50, 51</sup> As compared to ST, IBD dogs fed probiotic had increased number of cells expressing E-cadherin, occludin, and zonulin TJPs which suggests that the probiotic may have beneficial effects on mucosal homeostasis. In support of this notion, we investigated whether increased number of cells expressing TJPs would be associated with histopathologic improvement in dogs treated with probiotic, similar to an earlier report.<sup>10</sup> Results indicated that while histopathologic scores improved following probiotic therapy in IBD dogs, the cumulative mean score approached ( $P = 0.086$ ) but did not reach statistical significance similar to dogs treated with ST. One difference between our study and the early report was that we evaluated temporal changes (visit 1 → visit 3) in the number of TJP expressing cells while Rossi reported treatment-dependent differences in the number of TJP expressing cells only post-treatment. We now extend these initial observations to show that the number of cells expressing TJP molecule zonulin is increased in the small and

large intestines of IBD dogs treated with combination probiotic. Our studies have important translational implications since the spontaneous canine model shares remarkable homology to human IBD including known genetic basis, pivotal role for gut bacteria in disease pathogenesis, similar clinical presentation and disease activity indices, method of definitive diagnosis (biopsy), and positive response to immunosuppressive therapy.<sup>52</sup>

There were no major adverse effects reported with either IBD therapy. Clients whose dogs were randomized to receive ST/probiotic reported that the product was palatable and well tolerated by their pet. As expected, some large dogs (>35 kg body weight) receiving glucocorticoids exhibited transient panting, polydipsia, and/or polyuria which resolved as prednisone dosages were reduced, accordingly.

There are some potential limitations to this study. It is possible that mucosal bacteria other than those microbes targeted by our 6 probe array were significantly altered as a consequence of ST or ST/probiotic therapy. All dogs in the trial received routine colonic cleansing prior to collection of ileal and colonic mucosal biopsies. In these instances, the administration of oral colonic electrolyte lavage solutions and enemas might have disrupted mucus compartments and reduced bacterial populations available for mucosal counting.<sup>53</sup> However, we have previously investigated bacterial populations by FISH in pilot studies using untreated colonic specimens and found that mucus compartments do not differ appreciably between purged versus non-purged dogs.<sup>23</sup> Another potential factor impacting quantification of mucosal bacteria might be mechanical artifacts associated with tissue processing (microtome cutting) and/or non-intended wash of biopsy specimens by formalin solutions during transport to the pathology laboratory.<sup>54</sup> Our previous experiences have allowed us to readily identify these tissue artifacts and to avoid these areas, if present, when performing mucosal bacterial counts. Finally, gut microbial populations may vary by age, gender, breed, and dietary consumption. Our own studies, evaluating the potential impact of age, body weight, and/or diet have not identified any significant associations of microbial abundances with these variables in dogs to date.<sup>6, 46, 55</sup>

Finally, our trial lacked sufficient power to definitively define the effect of treatment between dog groups. Sample size was derived following consultation with a statistician (n = 32 total dogs over 36 months which

included a 20% “washout”) and was based on initial power calculations using variability in CIBDAI scores between IBD treatments.<sup>11</sup> The number of dogs (n = 34) initially enrolled in the trial met this minimum requirement. While the dog population in total completing the trial failed to reach statistical power for definitive conclusion, it is probably likely that our results regarding the effect of treatment on mucosal bacterial populations are correct.

In conclusion, this is the first study utilizing FISH methods to localize, quantify, and directly compare different medical treatments on mucosal bacterial populations in canine IBD. There were no differences in the numbers of mucosal bacteria following treatment of IBD dogs with ST versus probiotic. Changes in mucosal bacteria in probiotic treated IBD dogs were accompanied by increased number of cells expressing select tight junction proteins in intestinal tissues. Microbiota from mucosal samples more clearly represent the underlying microbial dysbiosis, at diagnosis and in response to treatment, as compared to fecal samples.

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Table 1. Diagnostic procedures performed during trial visits. CIBDAI = canine IBD activity index; CBC/BC/UA = complete blood count, biochemistry profile, urinalysis; TLI/cPLI = trypsin like immunoreactivity and canine pancreatic lipase immunoreactivity.

Visit	History/Physical examination	CIBDAI	CBC/BC/UA	TLI/cPLI	Folate/cobalamin	Cortisol or ACTH stimulation	Fecal examination	Endoscopy with biopsy
V1	X	X	X	X	X	X	X	X
V2	X	X			As needed			
V3	X	X			As needed			X

**Table 2.** Probes used for fluorescence in situ hybridization

Probe	Sequence (5' → 3')	Target	Reference
Eub338	GCT GCC TCC CGT AGG AGT	Bacteria	Amann (1990)
Bif164	CAT CCG GCA TTA CCA CCC	<i>Bifidobacterium</i> spp.	Harmsen (2000)
Ebac1790	CGT GTT TGC ACA GTG CTG	Enterobacteriaceae	Poulsen (1994)
Fecali698	GTG CCC AGT AGG CCG CCT TC	<i>Fecalibacterium</i> spp.	Garcia-Mazcorro (2012)
Lab158	GGT ATT AGC ATC TGT TTC CA	<i>Lactobacillus</i> spp.	Harmsen (2000)
Strc493	GTT AGC CGT CCC TTT CTG G	<i>Streptococcus</i> spp.	Franks (1998)

Table 3. Baseline parameters of IBD dogs completing the treatment trial. CIBDAI = canine IBD activity index; ST = standard therapy. <sup>a</sup> Mean disease activity at diagnosis, range 0--18. <sup>b</sup> Mucosal lesions of increased granularity, friability, and/or erosions visualized. <sup>c</sup> Histopathologic severity of mucosal inflammation.

Parameter	ST Group (n = 12)	ST/probiotic Group (n = 14)
Mean age (yr.)	6.2	4.6
Range	(1.5-10)	(1-8)
Mean weight (kg.)	26.7	18.8
Range	(6.3-47.3)	(5-34.1)
Male sex, n (%)	7 (58)	8 (57)
Disease duration (mo.)	10.5	7.3
CIBDAI score <sup>a</sup>	8.1	6.7
Endoscopic lesions <sup>b</sup>	100%	100%
Histopathologic grade <sup>c</sup>		
Mild IBD	37%	40%
Moderate-severe IBD	63%	60%

Table 4. Quantification of the number of epithelial cells expressing select TJPs in the intestinal mucosa of IBD dogs treated with ST or ST/probiotic. Intestinal biopsies were evaluated before and after 8 weeks of IBD treatment. Data expressed as the median number of IHC positive cells/62,500  $\mu\text{m}^2$  of mucosa. Median values compared between the duodenum and colon before and after treatment using Wilcoxon rank sum tests.

	Claudin-2	E-cadherin	Occludin	Zonulin
<b>Duodenum</b>				
Pre-ST/probiotic	1011	827	133	452
Post-ST/probiotic	741 <sup>*</sup>	997	947 <sup>*</sup>	1125 <sup>*</sup>
Pre-ST	782	1017	1098	371
Post-ST	751	1287	1413 <sup>*</sup>	117 <sup>*</sup>
<b>Colon</b>				
Pre-ST/probiotic	1212	575	131	61
Post-ST/probiotic	82	902 <sup>*</sup>	859 <sup>*</sup>	326 <sup>*</sup>
Pre-ST	248	NP	NP	NP
Post-ST	192	NP	NP	NP

<sup>\*</sup>Indicates significant difference ( $P < 0.05$ ). NP = not performed

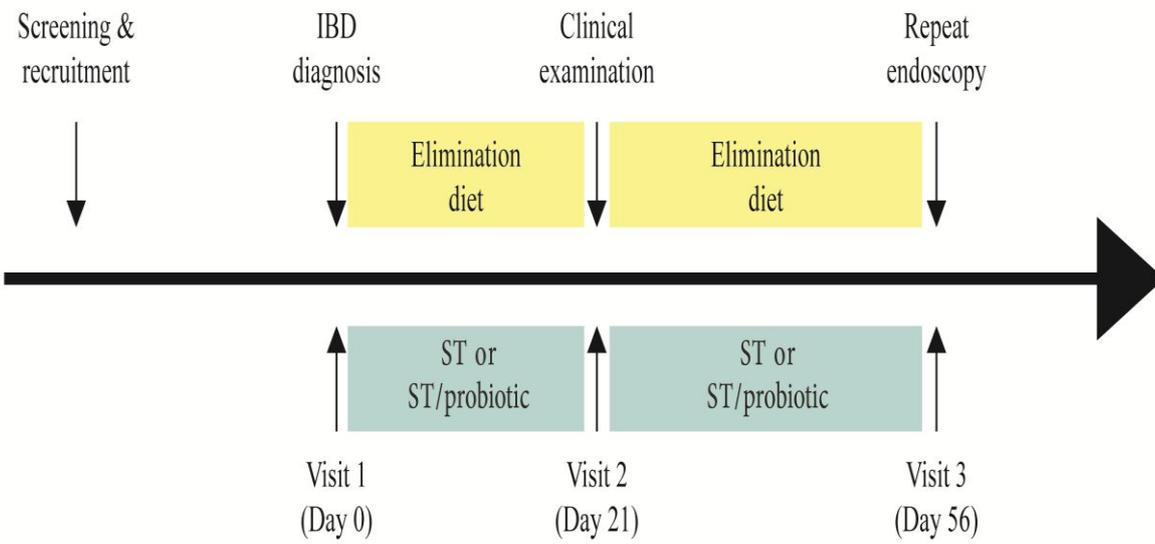


Figure 1. Trial design and flow of IBD dogs. ST = standard therapy.

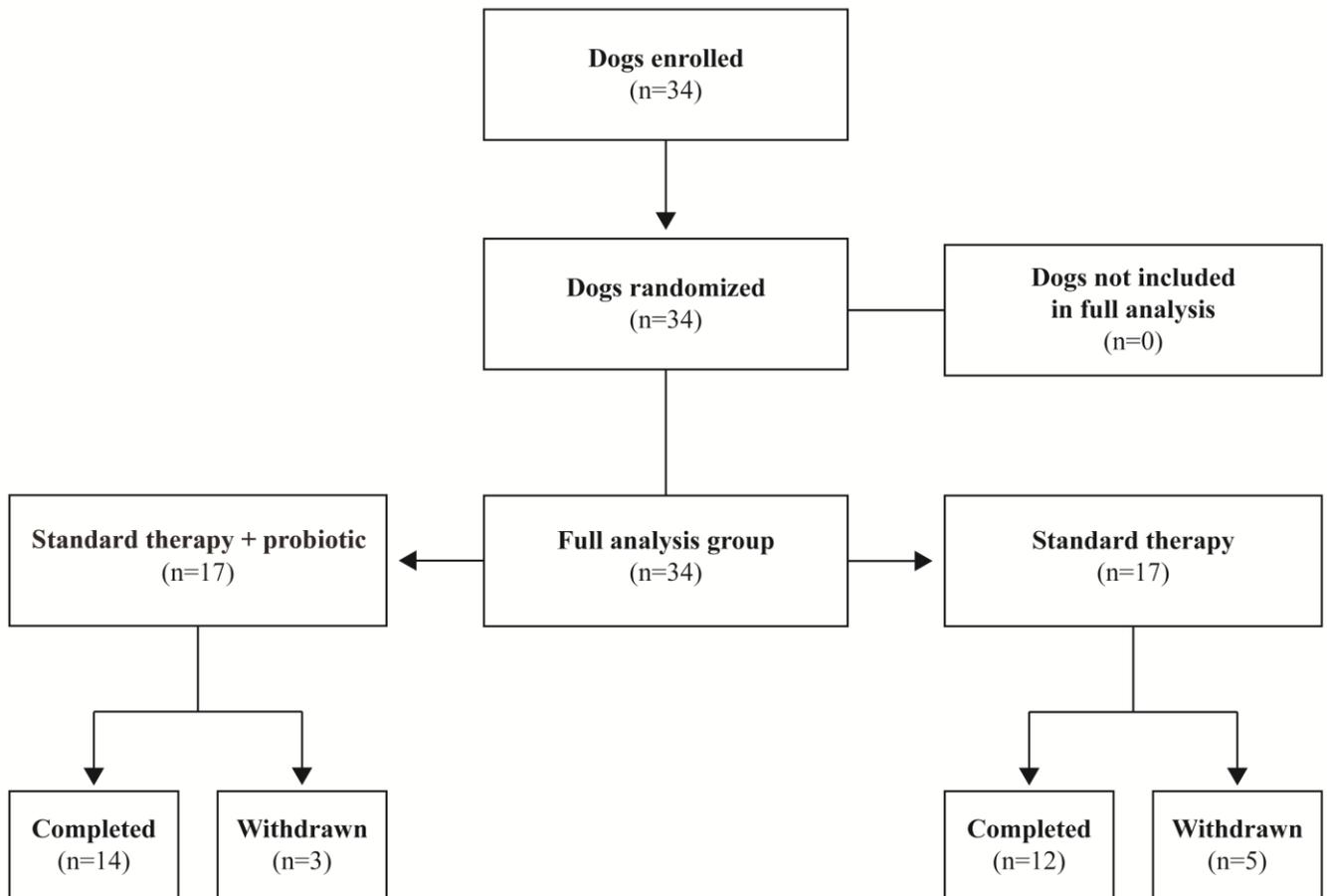


Figure 2. Dog enrollment and disposition.

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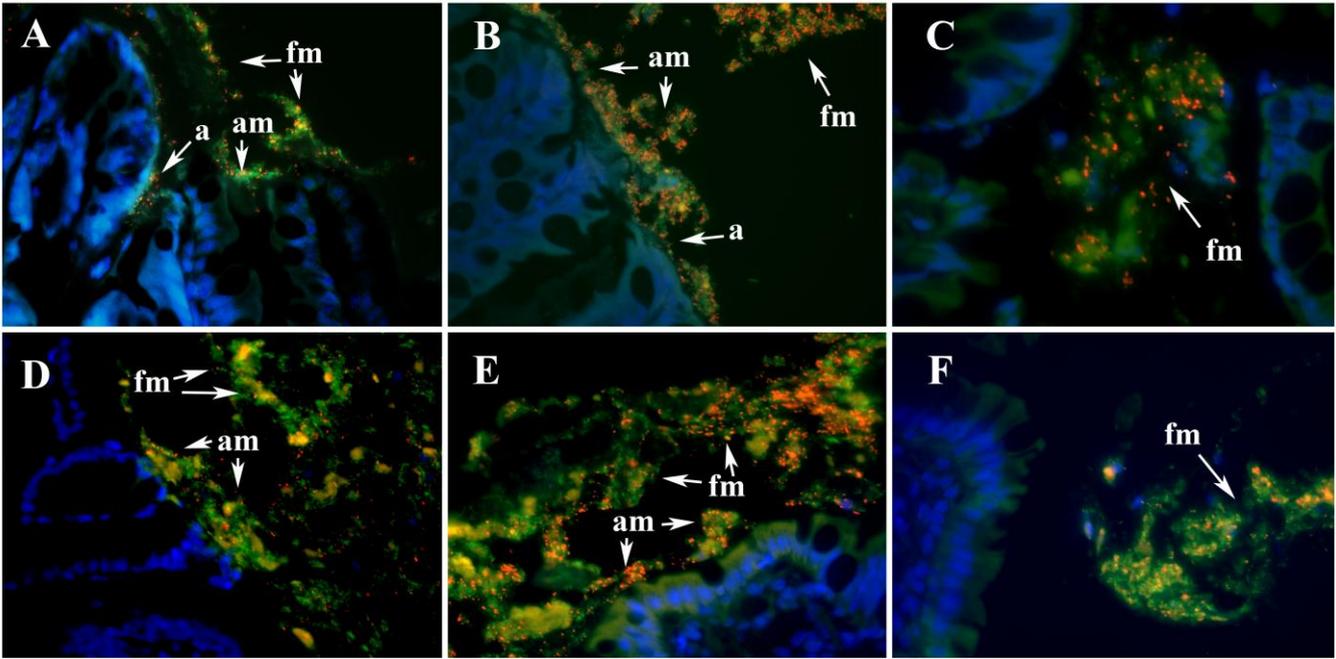
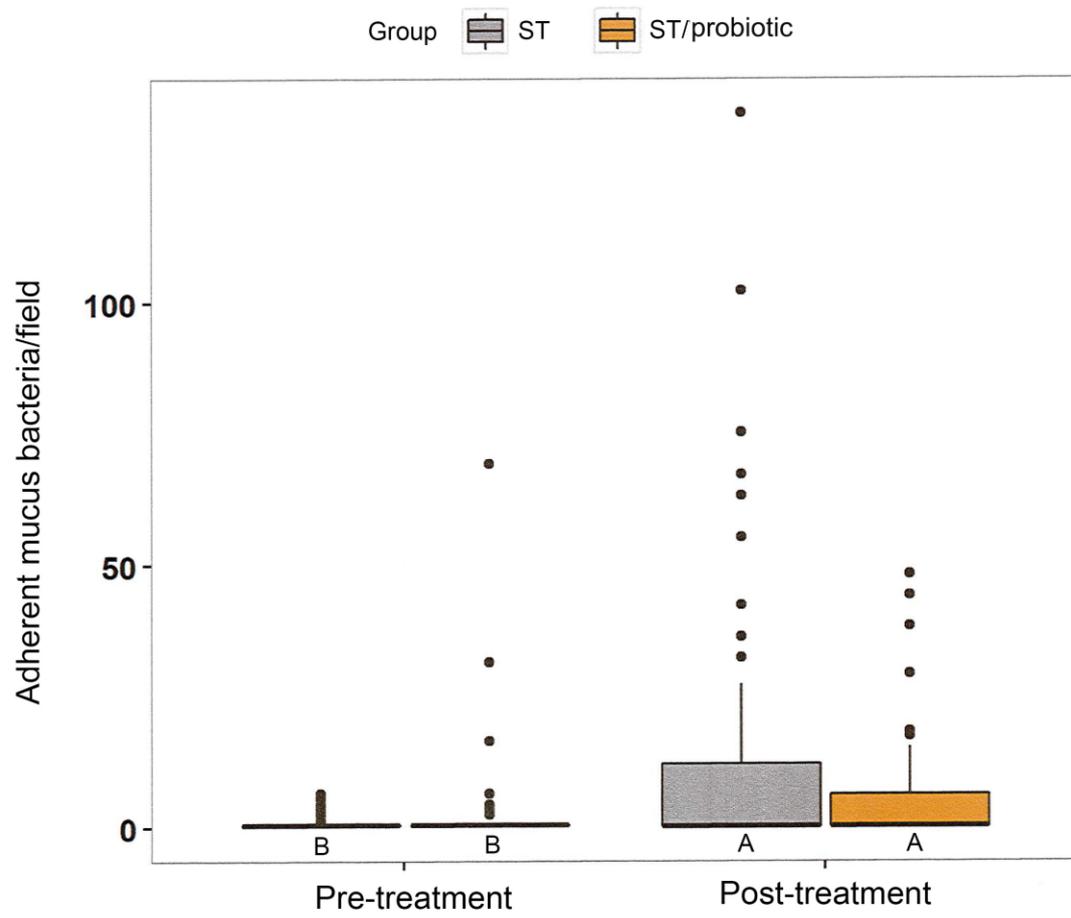


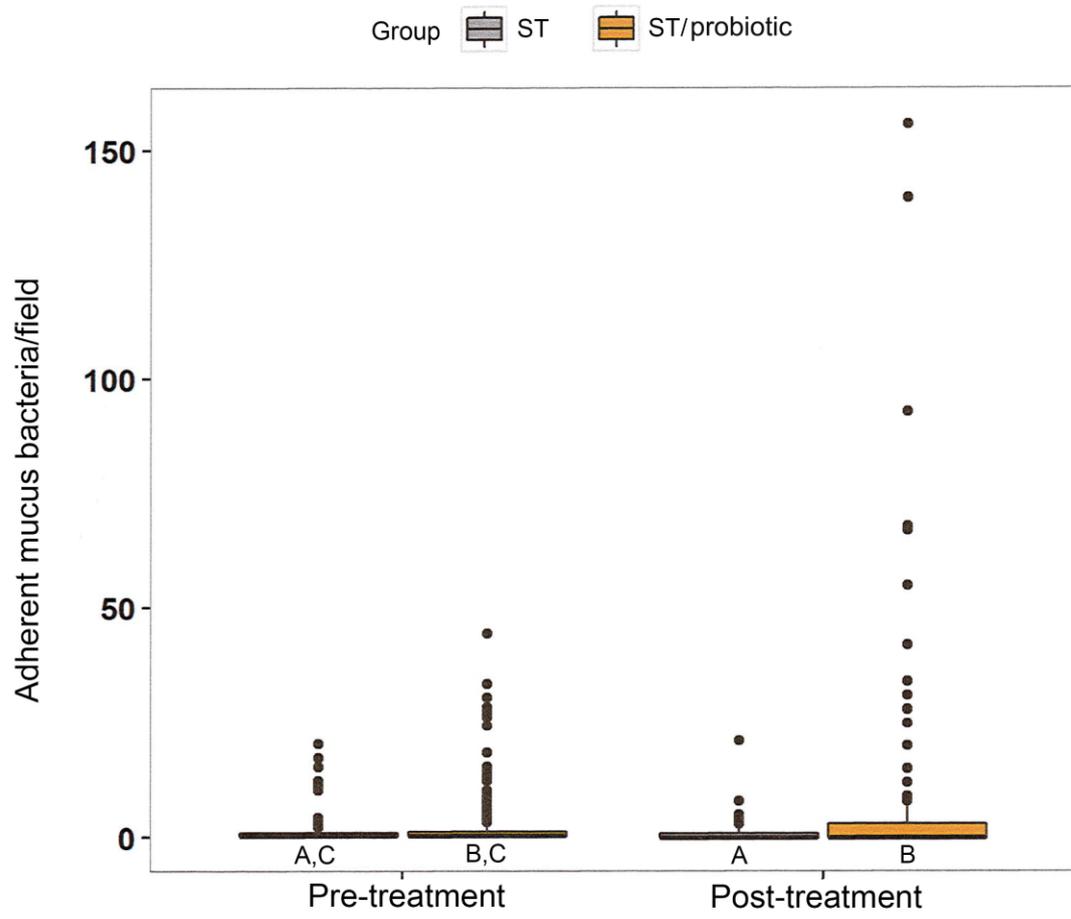
Figure 3. FISH of canine IBD endoscopic biopsies. Triple color FISH identifies colonic microbiota within mucosal compartments of endoscopic biopsies. Panels A-C = ST/probiotic group and panels D-F = ST group. Panel A = tissue hybridized with probe Cy3-Strc493- Panel B = tissue hybridized with probe Cy3-Ebac; Panel C = tissue hybridized with probe Cy3-Fecal698; Panel D = tissue hybridized with probe Cy3-Ebac; Panel E = tissue hybridized with probe Cy3-Bif164; Panel F = issue hybridized with probe Cy3-Strc493. All other bacteria that hybridize exclusively with the universal probe (Eub338-FITC) appear green. DAPI-stained colonic mucosa with goblet cells appears blue. All images at 600x magnification. a = attaching bacteria; am = adherent mucus compartment; fm = free mucus compartment.

### Bif164



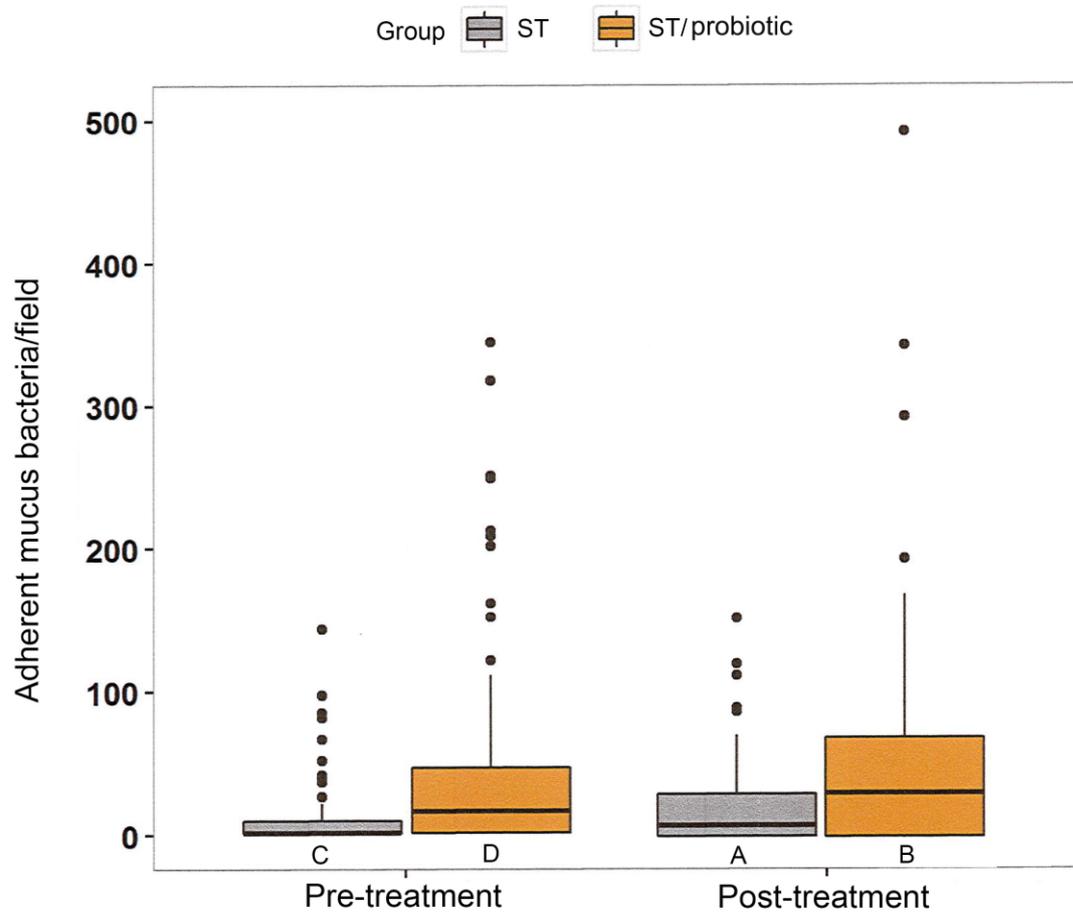
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### Ebac1790



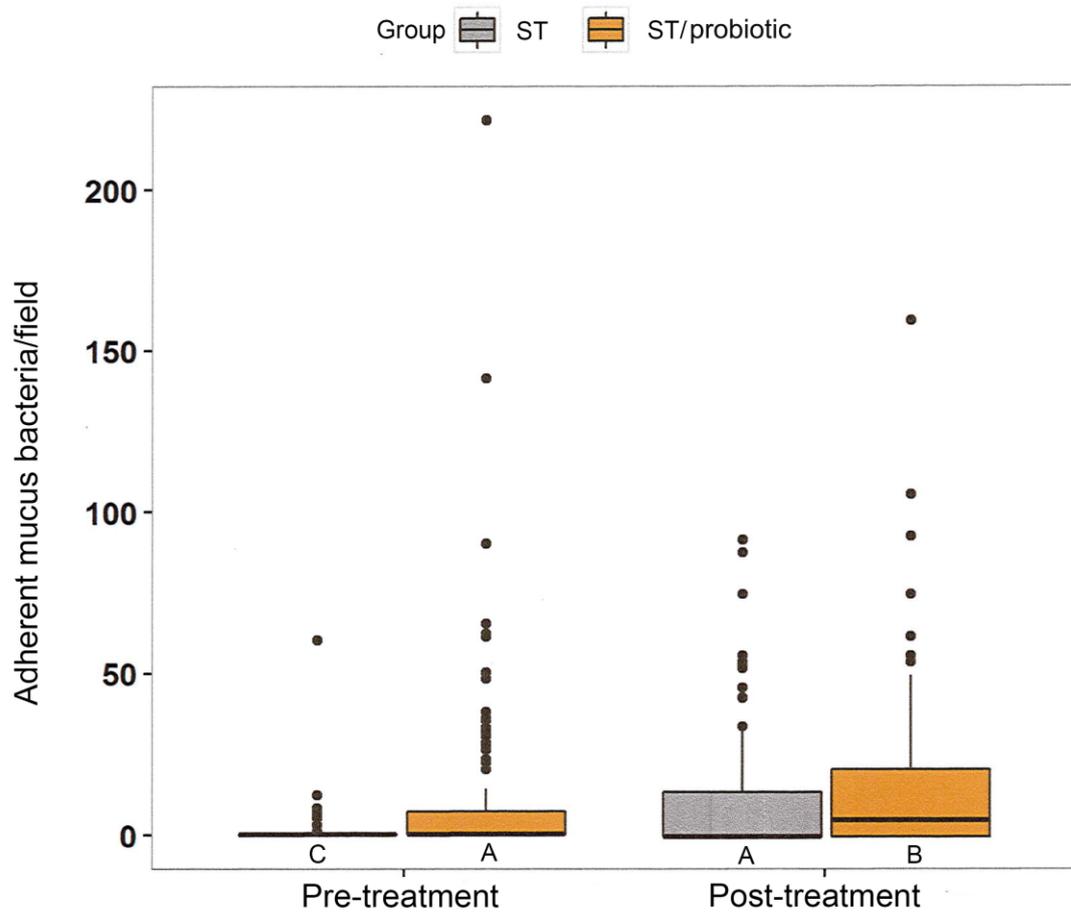
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### Eub338



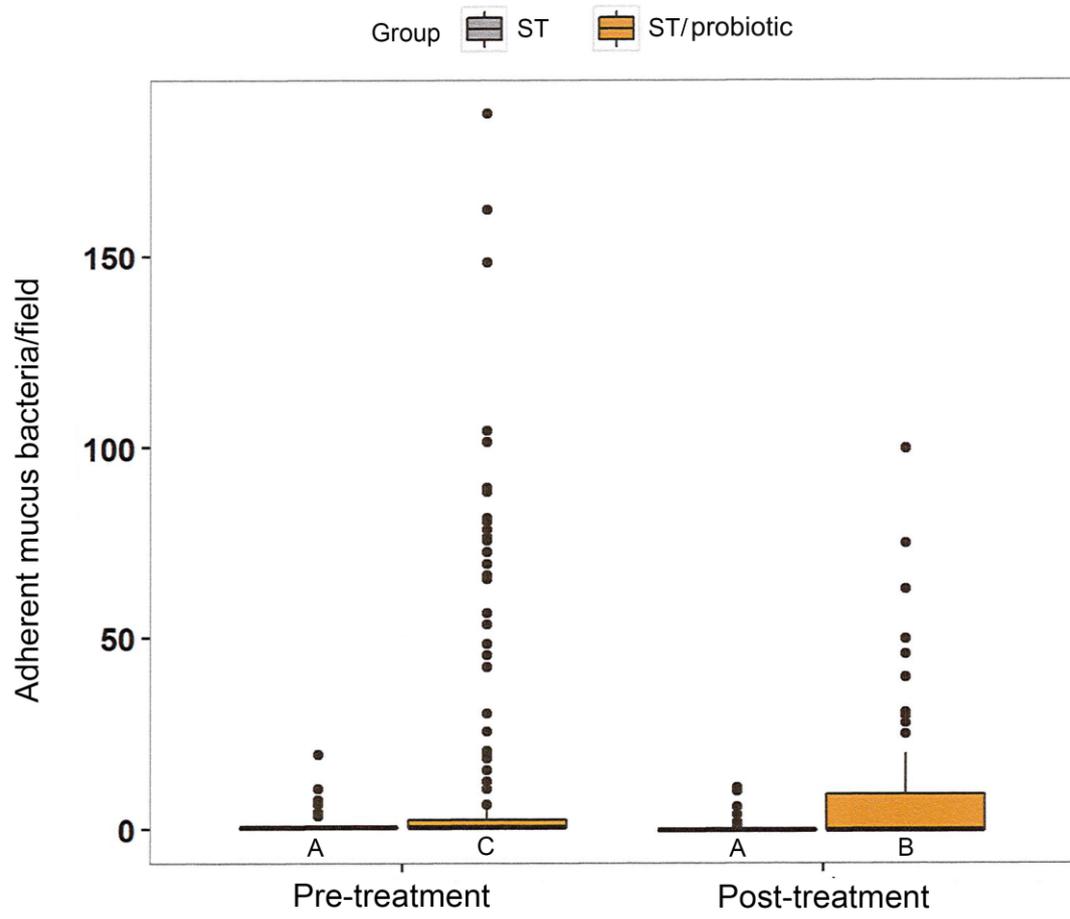
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### Fecali698



Accepted

### Lab158



Accepted

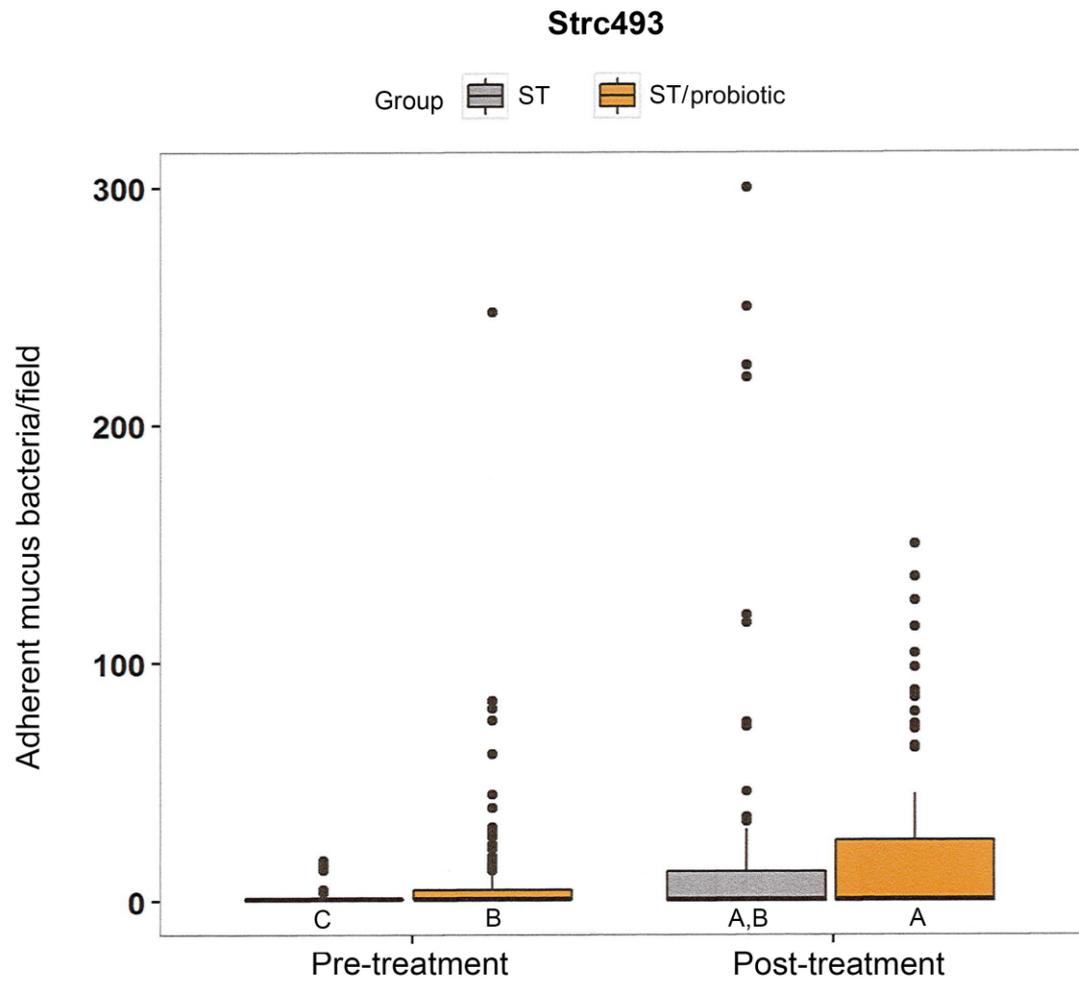


Figure 4. Box plots showing the effect of treatment on the number of colonic microbiota within adherent mucus of IBD dogs treated with ST or ST/probiotic. Figures A- F show the number of mucosal bacteria which hybridize against each probe: Bif164 → *Bifidobacterium* spp., Ebac1790 → Enterobacteriaceae, Eub338 → all bacteria, Fecali698 → *Fecalibacterium* spp., Lab158 → *Lactobacillus* spp., and Strc493 → *Streptococcus* spp. Differences ( $P < 0.05$ ) in the numbers of bacteria between treatment groups are indicated by the letters A, B, C, and D. Groups with the same letter are not statistically different.

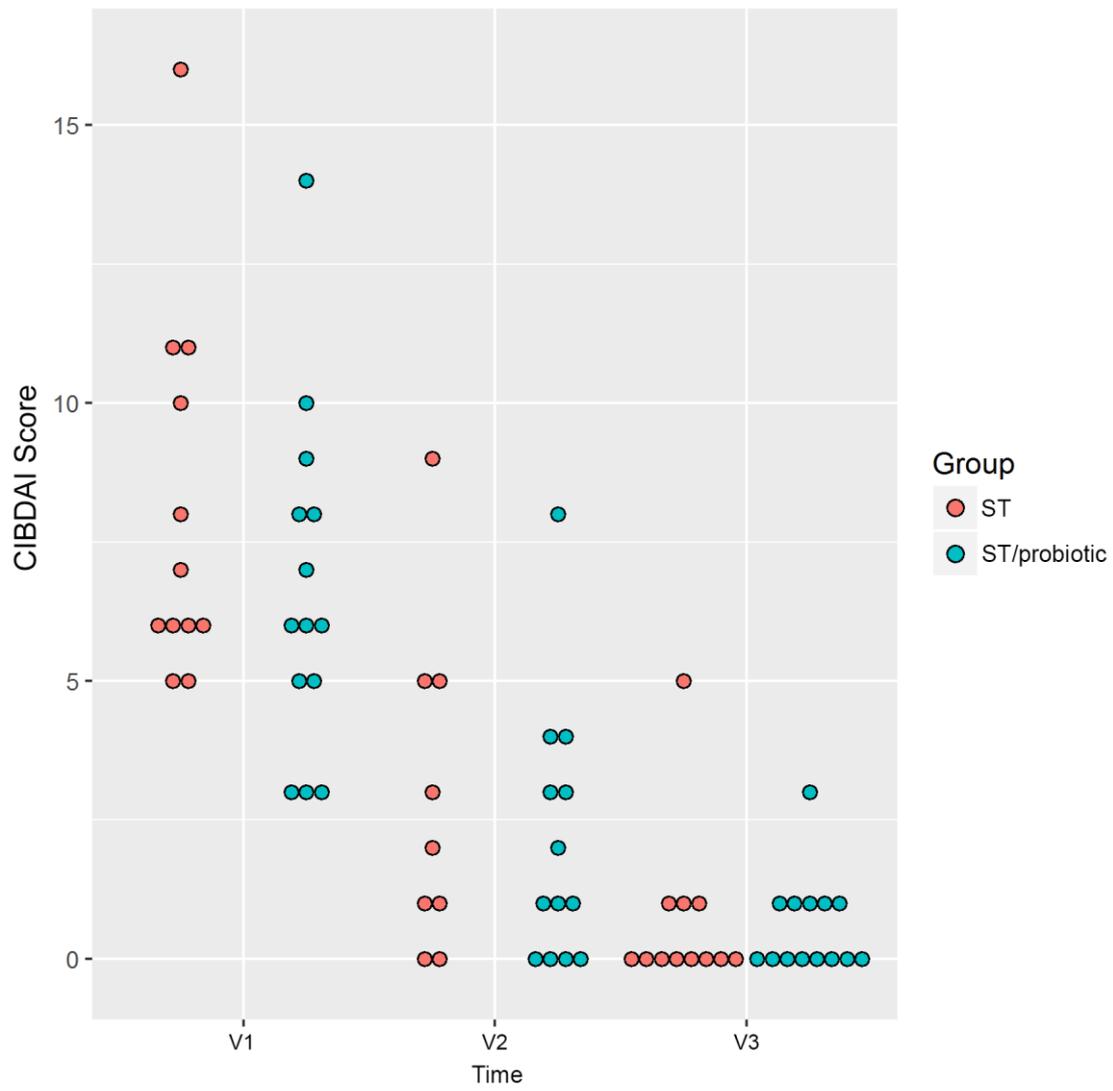


Figure 5. Temporal evaluation of clinical (CIBDAI) scores by IBD treatment group. CIBDAI = canine IBD activity index; ST = standard therapy; V = visit.

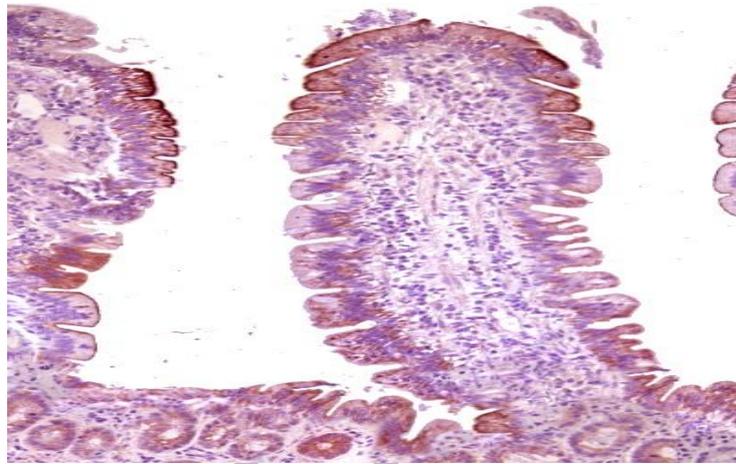
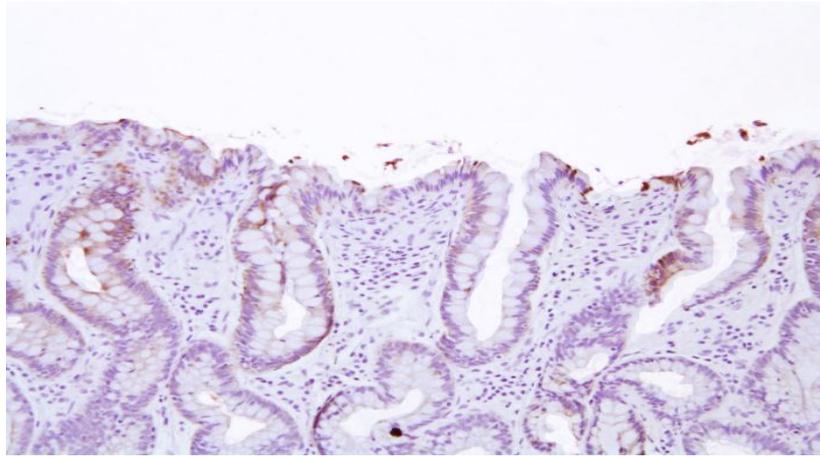


Figure 6. Tight junction protein (TJP) expression in intestinal epithelia of canine IBD endoscopic biopsies. (A) Panel shows immunohistochemical (IHC) staining for TJPs in duodenal biopsies of ST/probiotic treated dogs as compared to H&E (control) tissue. (B) Panel shows IHC staining for TJP in colonic biopsies of dogs treated with ST. Protein expression was defined by the number of epithelial cells within the mucosa expressing a select TJP. See [Table 4](#) for additional information.