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Enterocolic increase of cannabinoid receptor type 1 and type 2 and clinical improvement after probiotic administration in dogs with chronic signs of colonic dysmotility without mucosal inflammatory changes

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Abstract

Background: Colonic dysmotility in dogs can cause different GI signs. Sometimes, histology of enterocolic biopsies does not reveal inflammatory infiltrates or mucosal lesions that are typically associated with clinical disease activity. It is speculated that, similarly to humans, colonic dysmotility may be anxiety-based, although recent data demonstrate that irritable bowel syndrome (IBS) could result from acute infectious enteritis. Specific *Lactobacillus* spp. strains administered orally in humans induced the expression of μ-opioid and cannabinoid receptors in mucosal enterocytes, modulating intestinal morphine-like analgesic functions. We investigated the potential association of GI signs caused by colonic dysmotility and mucosal expression of cannabinoid receptors in intestinal epithelial cells and the number of mucosal mast cells.

Methods: Ten to 15 endoscopic biopsies were collected from colonic mucosa of 20 dogs diagnosed with dysmotility disturbances before and after probiotic (Slab51 bacterial blend; Sivoy®) administration (3-month period). Number and distribution of mast cells (MCs), and cannabinoid receptor type 1 (CB1) and type 2 (CB2) were evaluated by immunohistochemistry and PCR. Results were compared to data obtained from five clinically healthy dogs (archive samples).

Key results: Decreased numbers of MCs ($P < .0001$) and increased CB1- and CB2-positive epithelial cells ($P < .0001$) in diseased dogs were positively associated with post-treatment CCECAI scores ($P < .0001$).

Conclusions and inferences: Our results suggest that probiotic administration can reduce signs of colonic dysmotility, possibly due to microbiota modulation and epithelial cell receptor–mediated signaling in intestinal mucosa.

KEYWORDS

diarrhea, endocannabinoid system, enterocolitis, irritable bowel syndrome, probiotics

1 | INTRODUCTION

Abdominal pain, colonic dysmotility, and constipation/diarrhea are frequent symptoms characterizing irritable bowel syndrome (IBS) in man. It is a chronic gastrointestinal disorder reported as the most frequent gastrointestinal (GI) problem referred at primary and secondary care. IBS is a chronic GI disease defined as a functional bowel disorder according to the recent Rome IV criteria.[1] These conditions can be frequently accompanied also by other symptoms like bloating or abdominal distention. Colonic biopsies in affected patients usually reveal only low-grade mucosal/submucosal inflammation[2]; similarly, in dogs, the absence or the presence of minimal/mild mucosal inflammation and other morphologic lesions is usually reported with colonic motility disorders.[3] In addition, anxiety and stress (both mental and physical) have also been implicated in disease pathogenesis in both humans and animals.[4,5]

In dogs, a condition defined as chronic idiopathic large bowel diarrhea (CILBD) bears many similarities to IBS.[6] In dogs, this condition can be diagnosed once other causes for chronic enterocolitis (including inflammatory bowel disease)[7] are eliminated and if histopathology on endoscopic biopsies reveals no inflammation and/or neoplasia.[6] Recently, Cerquetella et al hypothesized that an IBS-like condition occurs in dogs,[8] describing a series of studies in which the pathology is well documented and "atypical behavior" (eg, aggressiveness, fearful, hyperreactivity) or triggering factors (eg, visit at the veterinary clinic, routines modification, the presence of strangers in the house) were present in 30% of affected animals.[9] In the dog, the presence of clinical signs of colitis, characterized by episodes of constipation (typically reported in IBS in man)[1] following often, acute episodes of diarrhea,[10] are described. In these animals, the finding of normal histology in colon biopsies has also been formerly described by Henroteaux, who reported a "spastic colon" in 7 dogs (out of 40 patients),[11] even though it could be not excluded that canine IBS may originate by an infectious disease,[10] similarly to the postinfectious IBS described in man.[12]

Motility and secretion are some of the GI functions that are regulated by the endocannabinoid system both in health and in disease. The activation of cannabinoid receptor type 1 (CB1) and type 2 (CB2) decreases motility, secretions, and hypersensitivity in the gut.[13-16] CB2 receptors are expressed by mast cells (MCs), and these cells function in response to cannabinoid stimulation.[17-19] In vitro, suppression of mast cells (MCs) pro-inflammatory mediator release by endocannabinoids has been described.[19] These cells are also likely involved in autocrine regulatory activity, as suggested by their production of endocannabinoids, like anandamide, palmitoylethanolamide (PEA), and 2-arachidonylglycerol.[19] Studies conducted in animal models of chronic colonic hypersensitivity describe probiotic modulation of the gut microflora which affects the expression of mucosal opioid and cannabinoid receptors and the perception of visceral pain.[20] In a systematic review of the literature, different lactobacilli species, alone or in association with bifidobacteria, are shown to ameliorate the clinical signs of IBS.[21]

While there are no published studies on the use of probiotics for the treatment of canine colonic motility disturbances, especially in CILBD/IBS, our study evaluated whether oral administration of a specific probiotic mixture attenuated GI signs of dysmotility, and whether probiotic administration modulated the expression of cannabinoid receptors and the number of resident MCs in intestinal mucosa.

Key Points

- Few data are available to understand mechanisms underlying canine chronic gastrointestinal dysmotility. We investigated in affected dogs the effect of probiotic administration on GI signs and mucosal expression of cannabinoid receptors and the number of resident mast cells.
- We observed decreased numbers of mucosal mast cells and increased expression of cannabinoid receptor positive cells; these changes were positively associated with improved post-treatment clinical scores.
- Our results suggest that probiotic administration may lead to reduced signs of colonic dysmotility.

TABLE 1 Enrolled dogs

	SG (n = 20)	CG (n = 5)
Breed	Mixed Breed (4), Golden Retriever (3), Bolognese (2), Boxer (2), Beagles (2), German shepherd (2), Rottweiler (1), Greyhound (1), Papillon (1), Jack Russell Terrier (1), Shih Tzu (1)	Mixed Breed (1), Epagneul Breton (1), English Pointer (1), English Setter (1), Weimaraner (1)
Sex	m = 9, mn = 2, f = 2, fs = 7	m = 2, f = 1, fs = 2
Median age (range) in years	6.3 (2-10)	5.5 (1-9)
Body weight (range) in kg	15.7 (3.5-38)	25.4 (8-40)
Median (range) time to remission (days)	23.4 (17-28)	n/a

Abbreviations: f, female; fs, spayed female; m, male; mn, neutered male; n/a, not applicable.

2 | MATERIALS AND METHODS

2.1 | Ethical animal use

Collection and analysis of intestinal biopsies obtained endoscopically from dogs included in the study were performed for clinical purposes, using routine techniques, respecting National Laws on Studies Involving Animals (DL 4 marzo 2014, n. 26). The enrolled dogs and their owners received written information on methods, according to previous studies,[22] and written informed consent was obtained from all owners of dogs enrolled in separate trials. The collection of colonic samples from healthy dogs was performed in five animals immediately after death, spontaneous or humanely induced (during the time of the study or archive samples), due to traumatic causes (car investment in three cases, and hunting accident in two cases).

2.2 | Animals

Two groups of dogs were included in the study; group 1 (study group = SG) comprised a cohort of 20 dogs (11 males and nine females; age range, 2 to 10 years) (Table 1) referred for chronic signs of enterocolitis, and diagnosed with CILBD/IBS[8]; group 2 (control group = CG) (Table 1) served as controls and included five adult healthy dogs (two males and three females; age range, 1 to 9 years).

For dogs with IBS, the inclusion criteria comprised persistent (>3 weeks) GI signs, the absence of response to dietary or symptomatic therapies (eg, propantheline, ranitidine), and the exclusion of other causes potentially able to determine chronic diarrhea. Regarding histopathology, all cases enrolled in the SG showed a substantial absence or a minimal/mild mucosal inflammation and other morphologic lesions (see below) as previously reported.[3,23]

For all IBS dogs, a complete medical history was recorded, and one or more clinical evaluations performed, plus hemato-biochemical analyses, urinalysis, direct (wet mount) and indirect (flotation) examination of feces for endoparasites, diagnostic imaging (including abdominal sonography), and histopathology on GI mucosal biopsies (duodenal and colonic, or only colonic samples).

Dogs included in group 2/CG were all not referring GI signs over several months prior to samplings (after death, the GI tract was removed). Moreover, samples included in the CG were considered normal also thanks to normal results on physical, postmortem, examination, hemato-biochemical analyses (performed as diagnostic routine workup while still alive), urinalysis, fecal examination, and dirofilaria antigen assay.

Mucosal samples of both small and large intestine were collected in all dogs. Histopathology performed on full-thickness intestinal biopsies in CG dogs resulted normal as previously stated.

2.3 | Therapeutic intervention

The trial was a 90-day open-label evaluation of the effects of probiotic (Slab51 bacterial blend; Sivoy®) administration in 20 dogs of the SG, regardless the histological aspect of colonic mucosa, the levels of expression of endocannabinoids receptors, and the presence of MCs. Between 112 and 225 billion (112 to 225 × 10^9), lyophilized bacteria per 10 kg body weight were administered to patients, once a day, for 90 consecutive days, in the drinking water.

2.4 | Clinical disease activity

The severity of clinical presentation at diagnosis was scored using the canine chronic enteropathy clinical activity index (CCECAI).[24] In this index, the final total score results from the summation of individual parameters (each scored 0-3) (ie, attitude/activity, appetite, vomiting, stool consistency, stool frequency, weight loss, albumin levels, ascites and peripheral edema, and pruritus) that comprise it. The disease can be then considered clinically insignificant (with a score from 0 to 3); mild (4 to 5); moderate (6 to 8); severe (9 to 11), or very severe (greater than or equal to 12). The CCECAI was assessed at baseline (T0) and after 90 days (T1) of enrollment, and then results compared to MCs, CB1, and CB2 counts (see below).

2.5 | Tissue samplings

After enrollment (time point T0) and after 90 days (T1), multiple (10-15 specimens) colonic mucosal biopsy specimens were obtained endoscopically from all SG dogs (n = 20). All these dogs had clinical signs of enterocolitis (ie, tenesmus, hematochezia, mucoid feces, and/or frequent defecation). Thirteen cases underwent colonoscopy, and seven cases had upper and lower endoscopic examinations due to concurrent clinical signs. Biopsy specimens were obtained directly from mucosal alterations (ie, areas of edema, reddening, mildly increased granularity, and/or friability) if present, as well as from areas of normal-appearing mucosa. The biopsies were placed in Carnoy solution to well preserve intestinal mucosal and connective MCs[25] and then paraffin-embedded; serial 3-μm-thick sections were prepared. At the same time, tissue samples were also frozen at −80°C for PCR procedures. For CG dogs, full-thickness intestinal and colonic biopsies (two or three, 3-5 cm long intestinal tracts), identically processed, were used.

2.6 | Histopathologic assessment

Hematoxylin and eosin (H&E)-stained tissue sections of Carnoy solution-fixed, paraffin-embedded endoscopic biopsies from the colon of each dog were evaluated for histopathologic lesions by a single pathologist, who was blinded regarding history, clinical signs, or endoscopic observations. A severity score was assigned for each dog, by using a standardized and previously described histological grading system, based on the extent of architectural disruption and mucosal epithelial changes,[26,27] as proposed by the WSAVA for the diagnosis of gastrointestinal inflammation.[28]

Tissues were also evaluated for expression patterns of CB1 and CB2 endocannabinoid receptors, and MCs, in both dog groups, comparing the results of cellular counts before and after the end of the therapy for the SG, and then comparing these values with those obtained from CG.

TABLE 2 Primer sequences

Gene symbol	Accession number	Forward primer 5′ to 3′′	Reverse primer 5′ to 3	Product length (bp)	Tm (°C)
RPL8	XM_532360	CCATGAATCCTGTGGAGC	GTAGAGGGTTTGCCGATG	64	55
GAPDH	NM_001003142	TGTCCCCACCCCCAATGTATC	CTCCGATGCCTGCTTCACTACCTT	100	58
CB1	AY_011618.1	CCTCTGTAGGCAGCCTGTTC	GGCAGCACAGCAATCACAAT	148	60
CB2	NM_001284480	TTGGTCTCCTACTTGCCCCT	GCAATGAACAGGAGCCAACC	110	60

2.7 | Histochemistry (HC) and Immunohistochemistry (IHC) analysis

Since mucosal, but not connective infiltrating MCs are sensitive to routine formalin fixation and cannot be histochemically identified in standard histological sections, all biopsies were fixed in Carnoy solution, a non-aldehyde solution that totally preserves the metachromatic nature of MC granules, after sequential staining with alcian blue and safranin, or toluidine blue, according to standard methods.[29,30] After these two procedures, MCs stain blue and are easily evidenced and counted in histological colonic section from SG and CG dogs. The cellular count was performed as reported below. For IHC evaluations, paraffin sections of dog GI biopsies were rehydrated and neutralized for endogenous peroxidases with 3% hydrogen peroxide for 5 minutes followed by rinsing for 5 minutes in distilled water. Non-specific binding of antibody was prevented by incubation of slides for 10 minutes at room temperature with a protein-blocking agent (Dako) before application of the primary antibody. Slides were washed and incubated with anti-CB1 or anti-CB2 primary antibody over-night (approx 16 hours) at 4°C. Because the canine CB1 protein sequence is homologous to the human CB1 protein sequence, tissue samples were labeled with a commercially available polyclonal rabbit anti-human-CB1antibody (rabbit polyclonal anti-CB1, catalog No. ab23703, Abcam, diluted 1:100 in 0.1 mol/L phosphate-buffered saline solution). Similarly, the CB2 protein sequence is conserved among species of mammals; therefore, tissue samples were labeled with a polyclonal rabbit anti-human-CB2 antibody (rabbit polyclonal anti-CB2, catalog No. ab45942, Abcam, diluted 1:100 in 0.1 mol/L PBS solution).[31] Slides were washed and incubated for 30 minutes at room temperature with a peroxidase polymer–conjugated secondary antibody. Negative control tissue samples were prepared, firstly, by the substitution of the primary antibody with rabbit serum (1:3000; R4505; Sigma-Aldrich), using the same gamma-globulin concentration as in the primary antibody formulation. Negative control tissues were also obtained, by the replacement of the primary antibody with an unrelated peptide and preabsorption of the unrelated peptide for 20 minutes at room temperature with CB1 or CB2 blocking peptide (diluted 1:500 in the PBS solution containing the primary antibody that corresponded to the blocking peptide) (CB11-P human cannabinoid receptor [CB1] control/blocking peptide # 1, and human cannabinoid receptor [CB21 P] control/blocking peptide # 1—human cannabinoid receptor, Alpha Diagnostic International) according to the method reported by Campora et al.[32]

Since in humans and laboratory animals, CB1 and CB2 are strongly expressed in hippocampus and lymph nodes, respectively,[32] samples of canine hippocampus and lymph nodes were used as positive control samples for CB1 (hippocampus) and CB2 (lymph nodes). These tissues were obtained from archived formalin-fixed and paraffin-embedded tissues of healthy dogs retrieved from the University of Camerino Veterinary Pathology Unit archives.

The immunoreaction with streptavidin-immunoperoxidase (streptavidin-immunoperoxidase, Black & Decker) was visualized with 3,3′-diaminobenzidine substrate (Vector). Tissues were counterstained with Mayer's hematoxylin.

All cell types were evaluated using a light microscope (Carl Zeiss), a × 40 objective, a × 10 eyepiece, and a square eyepiece graticule (10 × 10 squares, having a total area of 62 500 μm^2). Ten appropriate fields were chosen for each compartment, and arithmetic means were calculated for each colonic region. Results were expressed as IHC-positive cells per 62 500 μm^2. For all parameters, cells on the margins of the tissue sections were not considered for evaluation to avoid inflation of positive cell numbers.

For the evaluation of different CB1 and CB2 and MC subsets in the same histological sections, consecutive 3-μm-thick bioptic cross sections were cut. Sections were placed consecutively on each of eight separate slides, after which the ninth section was placed on the first slide, next to the first section, continuing for 48 sections. A single slide, upon which were six bioptic cross sections from each dog, was analyzed for any given immunostain. Numbers of CB1+, CB2+, and MCs were quantified by using an image analysis system consisting of a light microscope (Carl Zeiss) attached to a Javelin JE3462 high-resolution camera and a personal computer equipped with a Coreco-Oculus OC-TCX frame grabber and high-resolution monitor. Computerized color image analysis was performed using Image-Pro Plus software (Media Cybernetics). The area of each biopsy in all six cross sections in every dog was recorded, as also was the total number of epithelial positive cells determined by immunostaining as previously described. For each dog, the total immunostained cells were counted per section, and stained cell densities were expressed as the number of immunostained/epithelial cells per square millimeter of analyzed bioptic area.[33]

2.8 | RNA extraction and cDNA synthesis

Total RNA was isolated from intestinal tissues using Mini Kit RNAeasy® (Qiagen) extraction kits, following the manufacturer's

TABLE 3 Summary statistics for evaluated markers

	SG (20 dogs)		CG (5 dogs)		
	T0	T1	C		*P* value
Histology score	1.0 (0-2)	1.0 (0-2)	0.0 (0-1)	—	.8125
CCECAI score	8.0 (4-13)	0.0 (0-3)	—	—	<.0001*
Mast cells[a]	9.6 (±4.63)	6.5 (±1.49)	3.2 (±0.7)	—	.0001*
CB1+ cells[a]	6.4 (±5.17)	66.9 (±123.46)	262.0 (±38.6)	—	<.0001*
CB2+ cells[a]	205.7 (±114.28)	575.7 (±333.15)	1374.6 (±197.6)	—	<.0001*

Note: Numerical data are expressed as median (range) (±SD). Histology score and CCECAI are expressed as median (range) (min-max).
[a]Cells per 62 500 μm^2.
*Significant differences between T0 and T1.

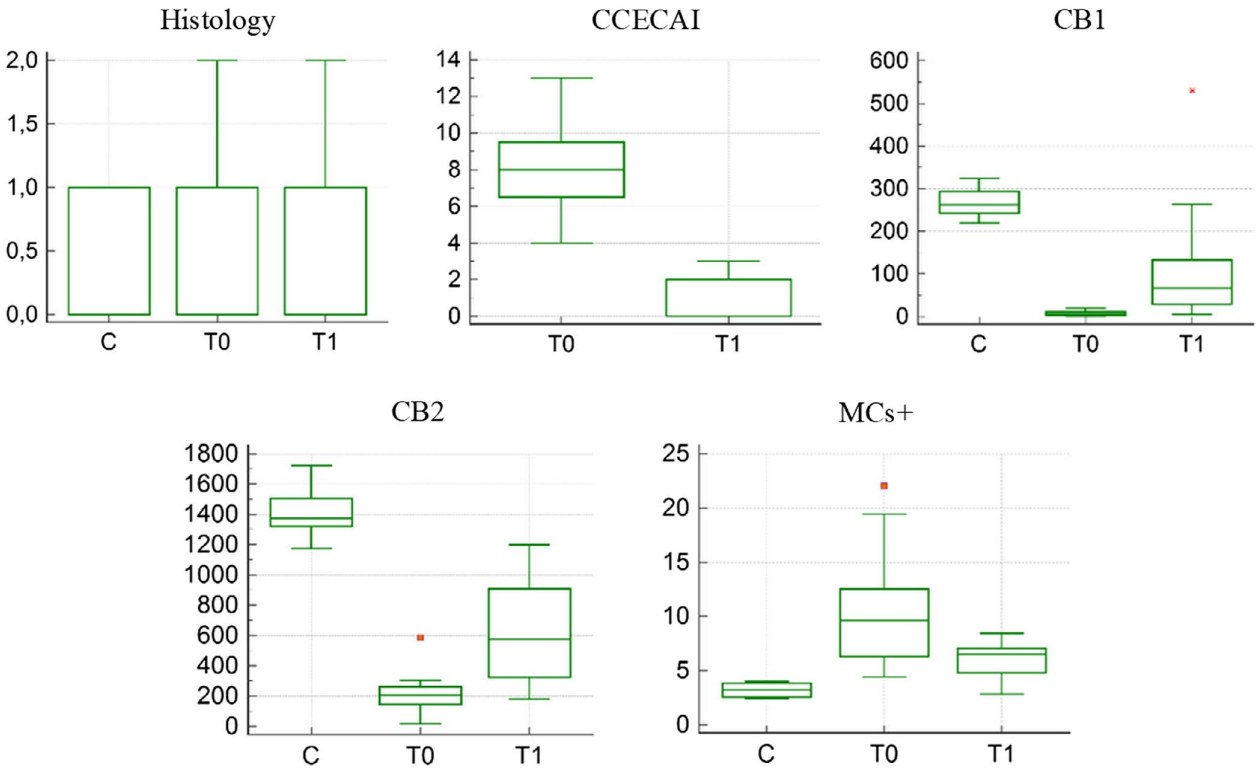

FIGURE 1 Results for histology scores, CCECAI, CB1 + cells, and CB2 + cells, MCs+. Significant differences between baseline (T0) and after 90 days of therapy (T1) were observed for all parameters except for histological score (P = .8125). While CB1 and CB2 increased significantly in treatment group (P < .0001), the number of MCs decreased significantly (P = .0001) in IBS suffering dogs, after probiotic treatment. Data for MCs+, CB1 + cells, and CB2 + cells are expressed as cells per 62,500 μm2

protocol. Final RNA concentrations were determined with a NanoDropTM 1000 Spectrophotometer (Thermo Scientific), and the RNA integrity was verified by ethidium bromide staining of 28 S and 18 S ribosomal RNA bands on 1% agarose gel. RNA was stored at −80°C until use. Total RNA was treated with DNAse (10 IU at 37°C for 10 minutes, MBI Fermentas). A total amount of 1 μg of RNA was used for cDNA synthesis, employing the iScript cDNA Synthesis Kit (Bio-Rad).

2.9 | Real-time quantitative PCR (qPCR)

PCRs were performed with the SYBR green method in a CFX96 Real-Time PCR System (Bio-Rad) following Gioacchini and coworkers.[34] Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and ribosomal protein L8 (RPL8) were used as internal standards in each sample in order to standardize the results by eliminating variation in mRNA and cDNA quantity and quality. No amplification products were

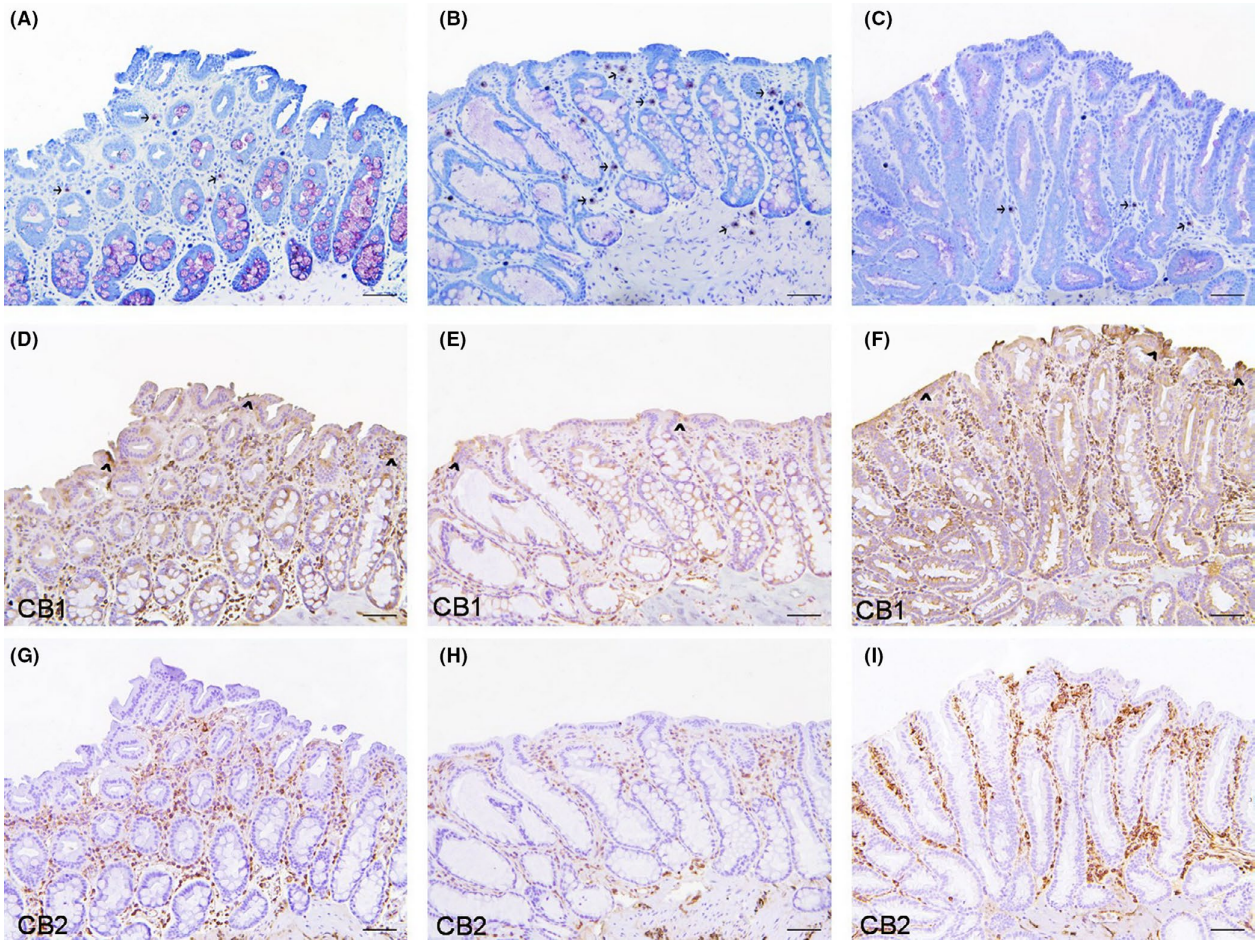

FIGURE 2 Histology of intestinal mucosa of dogs with IBS after treatment with probiotics (B,C,E,F,H,I) and CG (A,D,G). A residual inflammatory infiltrate with numerous, toluidine blue–stained MCs (arrows) is evident before the therapy in IBS affected dogs (B); the concentration of MC drops significantly compared to T0, although it remains higher than in control (A) after the 90-day treatment (C) (toluidine blue stain, 40X). In histological sections belonging to post-treated dogs (F and I), a similar concentration, but a different patterns of distribution of CB1 + and CB2 + cells is observed. In particular, a different concentration of positive cells is observed in the epithelial layer (arrow heads—D,E, and F). Note the lower concentration of CB1+ and CB2+ cells in dogs before treatment (E, and H), if compared with the concentration of the same cells in a control dog (D, and G). (IHC, ABC method, Harris hematoxylin nuclear counterstain, 40X)

observed in negative controls, and no primer-dimer formations were observed in the control templates. The data obtained were analyzed using the CFX Manager Software version 3.1 (Bio-Rad), including GeneEx Macro Conversion and GeneEx Macro files. The primer sequences used are reported in Table 2.

2.10 | Statistical methods

Data were statistically analyzed using a commercial software (MedCalc® Statistical Software version 16.4.3). Determination of data distribution was established using the Shapiro-Wilk test. The Wilcoxon test was utilized to evaluate differences in CCECAI, histology, MCs+, CB1+, and CB2+ cell expression in SG group before and after treatment, and the Mann-Whitney test to evaluate differences in histology, MCs+, CB1+, and CB2+ cell expression in SG vs CG groups.

The mRNA abundance of CB1 and CB2 results was performed with the one-way ANOVA followed by Tukey multiple comparison tests. Results are presented as mean ± standard deviation. A significance level of $P < .05$ was used for all analyses.

3 | RESULTS

Table 1 summarizes characteristics of enrolled dogs. Table 3 and Figure 1 summarize changes in CCECAI in SG group before and after treatment, and histology scores, MCs+, CB1+, and CB2+ cell expression in both SG and CG groups.

3.1 | Histology scores

The histological analysis showed an intact epithelial barrier with a columnar uniform epithelial and regular apical brush border without signs of damage in the colonic biopsies of both SG and CG dogs. Although there was a minimal inflammatory infiltrate present in some colonic section of dogs from SG (Figure 2), no significant difference was observed in histology scores (P = .8125) evaluated in biopsies, sampled before (T0) and after (T1) the treatment; similar results were obtained also comparing the histological scores of SG and those of CG (Table 3 and Figure 1). There were no significant differences in the magnitude of inflammatory infiltrate between groups.

3.2 | CCECAI scores

The severity of clinical presentation, as indicated by the clinical index, was significantly higher at T0 in the SG group (median 8.15, range 4-13) compared to the T1 value (median 0.8, range 0-3); clinical scores were significantly lower after treatment (P < .0001). However, the improvement did not occur in a very short time, as referred by the owners; the median time of clinical remission of the most relevant clinical sign (ie, diarrhea or tenesmus and colic spasms) resulted in 23.4 days (range: 17 to 28 days of interval).

3.3 | Mast cells+

Mast cells, interspersed throughout the colonic mucosa, decreased significantly between T0 and T1, after the treatment (P = .0001). In addition, the MC concentration remained significantly higher in colonic's samples from dogs diagnosed with IBS, compared to dogs of the control group (CG) (P < .0001 C vs T0 and P = .0018 C vs T1) (Figure 1 and Figures 2,3A-B-C).

3.4 | CB1+ cells

The immunolocalization of CB1 receptor in colonic mucosa of healthy dogs was detected in cytoplasm of colonocytes. Strong CB1 immunoreactivity was detected in reserve epithelial cells of colonic crypts (ie, proliferative and poor differentiated colonocytes) and also in the cytoplasm of some goblet cells interspersed in the glandular epithelium. In *lamina propria* and in the submucosal areas, diffuse cytoplasmic staining was observed in perivascular cells with typical MC morphology, well stained in subsequent histological sections also with alcian blue and toluidine blue dyes. Cannabinoid receptor type 1 immunoreactivity was also detected in the cytoplasm of scattered fibroblast-like cells and, rarely, in endothelial cells. IHC analysis of gut biopsies from treated animals highlighted strong presence of CB1 also in the submucosal ganglia of the superficial portion of the circular muscular layer of the intestine wall. The number of CB1+ cells (colon epithelial cells) was significantly increased (P < .0001) in dogs with IBS (SG) at T1; after the probiotic treatment, the number of CB1 + cells remained lower in SG with respect to CG. In addition, after the probiotic supplementation therapy, there were significant differences in the magnitude of this reduction between both groups (P < .0001 C vs T0 and P < .005 C vs T1) (Figure 1 and Figures 2,3D-E-F).

3.5 | CB2 + cells

Cannabinoid receptor type 2 immunolocalization in colonic biopsies of healthy dogs was substantially different compared to CB1 localization; CB2 cytoplasmic positivity was detected in a very scarce number of epithelial cells of the mucosal lying layer (Figure 2), without any expression in goblet cell or in glandular epithelium of colonic crypts. In *lamina propria* and in the submucosal areas, a strong cytoplasmic immunolocalization was observed in perivascular leukocytes and, more pronounced, inside MCs. A strong cytoplasmic CB2 receptor immunoreactivity was also detected in diffusely located fibroblast-like cells principally located in the lamina propria and particularly in endothelial cells, but also in central and peripheral areas of Peyer patches, with a strong number of B lymphocytes stained (*data not shown*). Similarly to CB1+ expression, a significant increase in the number of CB2+ cells was observed in dogs in the SG (P = .0001) after probiotic treatment. While the number of CB2+ cells was lower in SG dogs versus CG dogs after probiotic therapy, there were

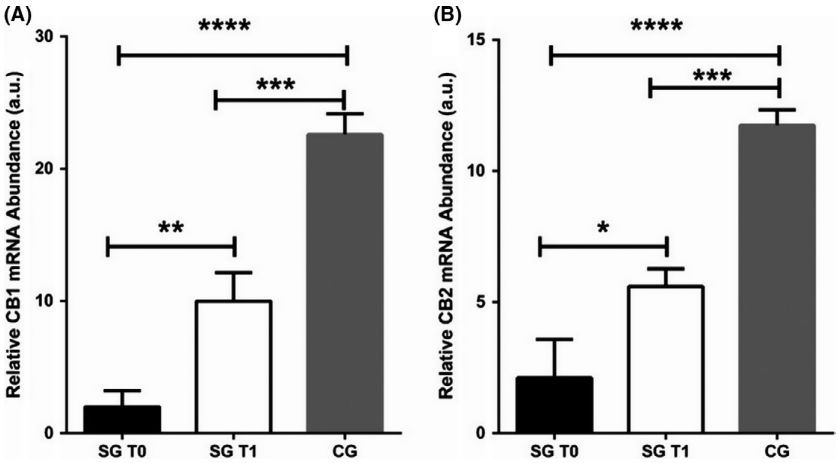

FIGURE 3 qRT-PCR analysis of (A) CB1 and (B) CB2 in control (CG), IBS suffering at time 0 (SG T0) and IBS suffering after 90-day probiotic treatment (SG T1). Values represent the mean ± Sd. Statistical significance was checked by two-way ANOVA followed by Tukey's multiple comparison test. *P < .05; ***P < .01; ***P < .001; ****P < .0001

significant differences in the magnitude of this reduction between both groups ($P < .0001$ C vs T0-T1) (Figure 1 and Figure 2G-H-I). Finally, immunolocalization of CB1 and CB2 was detected in perivascular cells with MC morphology, endothelial cells lining postcapillary venules of the intestine, and diffusely located in elongated fibroblastic cells. Results were consistent among tissue samples of each type (colonic samples from healthy dogs, hippocampal, and lymph node samples). Confirming the specificity of the anti-CB1 and anti-CB2 antibodies, negative control tissue in which primary antibodies were replaced with an unrelated antibody, or incubated with specific antibodies that was preabsorbed to the corresponding CB1 or CB2 blocking peptide, did not have immunoreactivity (data not shown).

3.6 | qPCR results

The quantitative measurements of CB1 and CB2 mRNA by real-time PCR are shown in Figure 3. A significant decrease of both endocannabinoid receptors was evident in SG animals before the probiotic treatment with respect to CG ones ($P < .0001$ CB1 and CB2). Anyway, among SG animals, a significant increase of both CB1 and CB2 was evidenced after 90 days of probiotic treatment ($P = .029$ CB1 and $P = .0121$ CB2).

4 | DISCUSSION

In the endocannabinoid system, there are many potential components that could be strictly related to the pathophysiology of IBS. It could be related to endocannabinoid insufficiency,[35] alterations of CB receptors, inadequate endocannabinoid synthesis, or breakdown due to enzymatic dysfunction. This complex of endocannabinoid activities may participate in the pathogenesis and clinical presentation of IBS, alone or in concert with MCs.[17] For some aspects relating to the pathophysiology of IBS (ie, dysmotility[1] or MC degranulation with production of pro-inflammatory/algic mediators), participation of the endocannabinoid system has been previously shown.[17] Therefore, a targeted pharmacological treatment directed toward the endocannabinoid system could lead to attenuation or remission of clinical signs. In spite of this, the participation of the endocannabinoid system in the pathophysiology of IBS has not been clearly defined. The endocannabinoid system also plays a part in the response to stressors in laboratory animals, but its role in emotional stress, that is recognized to aggravate clinical signs in some cases of IBS, is less understood.[36]

Endocannabinoid receptors in canine intestinal mucosa are mentioned and indirectly characterized, as described by Mechoulam et al, 1995.[37] Recently,[31] CB1 receptor immunoreactivity has been demonstrated in the enterocytes of healthy dogs, with CB1/CB2 receptor immunoreactivity observed in gut lamina propria, and CB2 expression shown in MC, immunocytes, blood vessels, and smooth muscle cells. Interestingly, weak CB2 receptor immunoreactivity was also found in neurons and glial cells of the submucosal plexus. On the basis of these earlier results, the authors hypothesized that the use of cannabinoid receptor agonists as therapy could mitigate dysmotility and visceral hypersensitivity in dogs suffering from acute or chronic enteropathies.

Our results indicate that dogs suffering from CILBD/IBS had decreased numbers of both CB1 and CB2 receptors in colonic mucosa, compared with control dogs. In addition, these same dogs showed increased numbers of MCs in their *lamina propria* versus control dogs. These results, obtained by a direct visual count of positive-stained cells in IHC tests, were confirmed by quantification and comparison of the total RNA of receptors. It is our belief that the significant reduction of CB1/CB2 receptors in colonic mucosa of dogs with dysmotility suggests that these receptors are involved in the motility/secretion and immunologic homeostasis of canine gut mucosa.

It has been widely demonstrated in human and in rodents that the stimulation of CB1 receptors corresponds to reduced motility of the small and large intestines.[38-40] In contrast, physiological CB2 receptor stimulation does not seem to evoke any relevant stimulation of intestinal motility; however, their stimulation seems to modify motility in pathophysiological states.[41,42] Interestingly, the administration of the CB1 antagonists *rimonabant* and *taranabant* has been linked to symptoms like diarrhea and other gastrointestinal motor side effects (ie, nausea and vomiting) which are transitory.[43-45] On the contrary, treatment with a CB2 receptor agonist ameliorates chronic colitis by lowering the numbers and suppressor functions of MCs and by inducing apoptosis in activated T cells, NK cells, and neutrophils at sites of inflammation.[46]

Finally, both CB1 and CB2 receptor agonists have been shown to decrease the visceromotor responses in rodents to gradual colorectal dilatation.[13] Two separate studies showed that during an hyperalgesia status, the endocannabinoid system is more susceptible and suggested that in these situations, similarly to IBS, patients may respond better to cannabinoid treatment.[13,47]

In the present study, dogs responded positively to a long-term (3 months) administration of a probiotic mixture, and the CCECAI significantly decreased post-treatment ($P < .001$). These results suggest potential interplay between the intestinal microbiota, endocannabinoid system, and mucosal MC activity. Previous data indicate that certain probiotics may reduce abdominal symptoms correlated to IBS in man,[48] and lessen visceral hypersensitivity in rats and mice.[48,49] It has been shown an association between gut microflora and the expression of CB2 receptors.[20] Their expression (and of mu opioid) was upregulated after chronic treatment (15 days) with *Lactobacillus acidophilus* in vivo.

By using the same probiotic mixture to that used in the present study, similar results of CB1 and CB2 receptor expression were obtained by Gioacchini et al,[50] in another animal model, *Danio rerio*. It is currently not known whether bacteria (probiotic or pathogens) could modify CB1 expression and whether acute or chronic modifications of the intestinal microbiota, as seen with postinfectious IBS,[51] could cause changes of CB2 expression modifying visceral sensitivity.

In dogs, physiological functions of intestinal MCs include control of blood flow and smooth muscle contraction and peristalsis and the production of acid, electrolytes, and mucus by epithelial cells.[52]

Interestingly, low-grade inflammation (infiltrates often rich in MCs) involving both the small and large bowel was reported in both dogs with GI signs[7] and in humans with IBS.[53]

Our results showed that the number of MCs was higher in dogs with colonic dysmotility with respect to control dogs ($P < .0001$ C vs T0 and $P = .0018$ C vs T1). The direct link of MCs to many important GI functions, such as epithelial secretion and permeability, neuroimmune interactions, visceral sensation, and peristalsis, leads researchers to carefully evaluate the important role played by MCs in the pathogenesis of IBS.[54] IBS patients have a higher number of MCs in colon and ileum as compared to healthy controls,[55] and the amount of mucosal MCs and increased gut permeability is positively correlated.[56] In addition, increased mucosal expression of tryptase[57] and release of tryptase into the intestinal lumen[55] have been reported in IBS patients. Of interest, the MC stabilizer *ketotifen* reduced the visceral hypersensitivity and diminished symptoms in IBS patients.[58]

Enteric nervous cellular components and functions are modulated by MCs by releasing pro-inflammatory mediators such as histamine, initiating that way innate defense mechanisms.[59] In rodents, it has been shown that MC proteases are directly accountable for increased epithelial paracellular permeability and altered expression of tight junctions during parasitosis and stress.[60] MCs act between innate and adaptive immune responses influencing tolerance against commensal flora and enhancing the response to pathogens to maintain tissue homeostasis.[61]

In our study, the observation that MC numbers were significantly reduced in colonic biopsies post-therapy indicates a key role for MCs in the *cross talk* between microbiota, endocannabinoid, and enteric nervous system. MCs identify pathogen-associated molecular patterns (PAMPs) due to the interaction with antibodies and via pattern-recognition receptors (PRRs).[59]

Probiotic bacteria, as well as certain commensal microbes, have also been shown to modulate intestinal epithelial barrier function also by modulating MCs degranulation, via peroxisome proliferator-activated receptor (PPAR) pathways.[62,63]

Different studies demonstrate that probiotic bacteria are able to interact with MCs to activate PPAR-γ, and to induce MC endogenous agonists, such as linoleic acid, which is known to be a PPAR-γ ligand.[64] Degradation of PPAR-γ occurs via the ubiquitin-proteasome system,[65] and probiotics have been shown to decrease cellular proteasome activity.[66,67] It is reasonable to assume, therefore, that probiotics reduce the degradation of PPAR-γ activity and reduce harmful MC degranulation in response to stress or diminished CB1/CB2 receptor expression.

At present in dogs, as in humans, the etiology of IBS is unknown. However, it is likely that more than one mechanism is involved as evidenced by its varied clinical presentations.[68] IBS symptoms cannot be entirely explained through structural/histological abnormalities, and to date, there are no laboratory tests or biomarkers are specific to this condition. Therefore, IBS is considered as a functional disorder being diagnosed primarily based on historical findings and clinical presentation.[69] In addition, routine histopathology does not identify relevant colonic mucosal anomalies in many IBS patients; however, as in humans, our study demonstrated that quantitative histological, immunohistochemical, and biomolecular analyses may suggest to the presence of subtle organic alterations in affected dog. This suggests that in both species, low-grade inflammation of the colonic mucosa may be present and contribute to clinical presentation. Our observation that we could normalize CB1/CB2 expression in dogs treated with multi-strain probiotic and reduce the numbers of MCs in tissues of affected dogs suggests that probiotic administration is a possible therapy for canine colonic dysfunction. Possibly, these beneficial effects are, at least in part, attributable to probiotic modulation of the GI microbiota communication to the host, via multiple mechanisms including epithelial cell receptor-mediated signaling. Further studies are required to confirm this hypothesis and to verify whether probiotics are a suitable alternative therapy for canine IBS.

CONFLICT OF INTEREST

No competing interests declared.

AUTHOR CONTRIBUTIONS

GR designed the research study; GR, GG, GP, SS, SB, and LG performed the study; GR, GG, JSS, AEJ, KA, AG, GB, and MC analyzed the data and wrote the manuscript; and all authors revised the manuscript and approved the final version of the paper.

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