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Effects of a probiotic (SLAB51™) on clinical and histologic variables and microbiota of cats with chronic constipation/megacolon: a pilot study

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RESEARCH ARTICLE

Abstract

Chronic constipation (CC) and idiopathic megacolon (IMC) occur frequently in cats. The aim of the study was to investigate the effects of a multi-strain probiotic (SLAB51™) in constipated cats (n=7) and in patients with megacolon and constipation (n=3). Ten pet cats with a diagnosis of chronic constipation, non-responsive to medical management received orally 2×10^{11} bacteria daily for 90 days. For microbiota analysis, selected bacterial groups were analysed by qPCR. Histological samples in megacolons were evaluated for interstitial cells of Cajal (ICC), enteric neurons, and neuronal apoptosis. Biopsies were compared at baseline (T0) and after the end of treatment (T1), and with those obtained from healthy control tissues (archived material from five healthy cats). Constipated cats displayed significantly lower ICC, and cats with idiopathic megacolon had significantly more apoptotic enteric neurons than controls. After treatment with SLAB51™, significant decreases were observed for feline chronic enteropathy activity index (FCEAI) (P=0.006), faecal consistency score, and mucosal histology scores (P<0.001). In contrast, a significant increase of ICC was observed after probiotic therapy. *Lactobacillus* spp. and *Bacteroidetes* were increased significantly after treatment (comparing constipated cats before and after treatment, and control healthy cats to constipated cats after treatment), but no other differences in microbiota were found between healthy controls and constipated cats. Treatment with SLAB51™ in cats with chronic constipation and idiopathic megacolon showed significant clinical improvement after treatment, and histological parameters suggest a potential anti-inflammatory effect of SLAB51™, associated with a reduction of mucosal infiltration, and restoration of the number of interstitial cells of Cajal.

Keywords: cats, constipation/megacolon, enteric nervous system, microbiota, probiotics

1. Introduction

The pathogenesis of chronic constipation (CC) and idiopathic megacolon (IMC) is poorly understood in humans and animals, even though some studies in humans revealed consistent abnormalities of the enteric nervous system (Bassotti *et al.*, 2006a,b,c). In humans, constipation is defined by the various associations of reduced evacuatory frequency, straining at stools, presence of hard stools, sensation of incomplete evacuation, and manual manoeuvres to help defecation, according to the Rome criteria (Mearin *et al.*, 2016). In cats, constipation is defined as absent, infrequent, or difficult defecation associated with the retention of faeces within the colon and rectum (Jones, 2000). There are many recognised causes of constipation (Jones, 2000; Washabau and Hasler, 1997; Yam, 1997) and management of animals with this condition depends on the clinician's ability to recognise and understand the aetiology in each case.

In some animals with constipation, megacolon may also be seen, and should be considered as a disorder characterised by recurrent constipation and/or obstipation associated with colonic dilatation and hypomotility. Megacolon is a

descriptive term only, and its use imparts no information regarding specific aetiology or pathophysiology, and its aetiology may be considered congenital or acquired. Congenital or idiopathic megacolon is well recognised in humans (Bassotti *et al.*, 1995; De Giorgio *et al.*, 2000), and it has been also described in cats (Rosin, 1993; Rosin *et al.*, 1988). Megacolon in cats may be due to aganglionosis of a colonic tract (Rosin, 1993) resulting in the persistent muscle contraction within the affected segment and the subsequent proximal colonic dilatation, to a generalised smooth muscle dysfunction of the large bowel (Washabau and Stalis, 1996), or to secondary conditions such as active colonic inflammation, disautonomia and metabolic disorders including hypokalaemia and hypothyroidism, nutritional *osteodystrophia fibrosa* or pelvic fractures (Palmer, 1968; Rosin, 1993; Rosin *et al.*, 1988). However, most cases are idiopathic, orthopaedic or neurologic in origin (Washabau and Hasler, 1997).

There is a growing interest in human medicine concerning the use of probiotics (live microorganisms that when administered in an adequate amount provide benefits to host health (Reid, 2016) in gastrointestinal disorders (Bron *et al.*, 2017; Sebastián Domingo, 2017). An imbalance in the colonic microflora has been suggested to play a role in some human gastro-intestinal diseases, such as constipation (Picard *et al.*, 2005; Zhao and Yu, 2016). Some probiotics (e.g. *Bifidobacterium* and *Lactobacillus* strains) lower colonic pH enhancing colonic peristalsis and decreasing colonic transit time (Marteau *et al.*, 2002; Picard *et al.*, 2005), and there is evidence that probiotic treatments may improve constipation symptoms (Dimidi *et al.*, 2014; Kim *et al.*, 2015; Magro *et al.*, 2014; Yeun and Lee, 2015). Regardless of whether differences in gut microbiota are a cause or an effect of constipation, some studies indicate that patients with constipation exhibit different compositions of gut microbiota. However, the precise differences in composition are poorly characterised and conflicting data have been found. Thus, no definitive association between constipation and the abundance or lack of certain bacterial taxa in the gut microbiome has been yet established.

Although probiotic supplementations have been successful in animal models in the prevention and treatment of acute gastroenteritis (Lei *et al.*, 2016), treatment of inflammatory bowel diseases (Yao *et al.*, 2017), and prevention of allergy (Neau *et al.*, 2016), no data are available on their effects on constipation in cats (Grześkowiak *et al.*, 2015). We hypothesised that a combination of several strains of *Bifidobacterium* and *Lactobacillus* might be effective in the treatment of constipation in cats. In this pilot study, we aimed to determine the therapeutic effect of a combination of probiotics mixture in cats with constipation/megacolon.

2. Material and methods

Animals

Ten privately owned pet cats (Table 1) of different breeds and ages referred for evaluation of chronic constipation, non-responsive to medical management, were selected on the basis of recurrence of clinical signs (see below). The study was approved by the Camerino University Institutional Animal Care and Use Committee protocol and all cats' owners gave written informed consent before enrolment. In all animals enrolled in the study, the colon was found to be impacted with faeces. Three of these 10 cats were diagnosed with IMC. The minimum diagnostic evaluation in all cats included a complete blood count, serum biochemistry, urinalysis, direct (wet mount) and indirect (flotation) examination of faeces for endoparasites, and survey abdominal radiographs. The measurement of serum concentrations of folate and cobalamin, plus an abdominal ultrasonography were performed in all cats; in the three cases of IMC, a contrast radiography was also done. The diagnosis of megacolon was made on the basis of a ratio of maximal diameter of the colon to L5 length >1.48 (Trevail *et al.*, 2011). Constipation was defined as having at least 2 out of 7 of the following signs: dyschezia, loss of appetite/weight, abdominal discomfort, faecal incontinence >2 times/week, large amounts of stools once in 10 days, painful defecation, and palpable abdominal or rectal mass on physical examination. Inclusion criteria comprised recurrence of clinical signs and absence of any immunomodulating drug therapy (e.g. corticosteroids, metronidazole, and sulfasalazine) within a month before referral. Exclusion criteria were the use of any oral laxative or antibiotic <4 weeks before intake, metabolic disease, functional non-retentive incontinence, history of spinal cord injuries and neurologic diseases, and a history of orthopaedic problems involving the pelvic canal, as deformity associated with a displaced malunion pelvic fracture.

Ten pet cats (Table 1), living in home environments and free of gastrointestinal signs for at least four months, were enrolled as control group (healthy cats; HC) for comparison of faecal microbiota to cats with CC and IMC.

Study design

The trial was a 90 day evaluation of the effects of SLAB51™ strains on clinical, histological, and microbiological parameters of the ten constipated cats described above. SLAB51™ (Sivoy®, Mendes SA, Lugano, Switzerland) is a probiotic blend containing 2×10^{11} lactic acid bacteria composed of the 8 following strains: *Streptococcus thermophilus* DSM32245, *Lactobacillus acidophilus* DSM32241, *Lactobacillus plantarum* DSM32244, *Lactobacillus casei* DSM32243, *Lactobacillus helveticus*

Table 1. Summary characteristics of enrolled cats.[1]

	Treatment group (n=10)	Healthy control (n=10)
	European (4), Russian Blue, Persian, Sphynks, Certosino, Scootish, Burmese	European (4), Burmese, Maine Coon, Birman, Bengal (2), Norwegian
Sex	m=3, mn=1, f=2, fs=4	m=2, mn=3, f=1, fs=4
Median age (range) in years	9.7 (6-14)	2.3 (0.8-5)
Body weight (range) in kg	4.14 (3.2-5.4)	4.8 (3.5-6)
Median (range) time to faecal normalisation (days)	38.6 (18-56)	n/a

[1] m = male; mn = neutered male; f = female; fs = spayed female; n/a = not applicable.

DSM32242, *Lactobacillus brevis* DSM27961, *Bifidobacterium lactis* DSM32246, and *B. lactis* DSM32247.

Starting seven days prior to baseline assessment and during the entire treatment period, all owners recorded frequency of bowel movements, the number of faecal incontinence episodes, stool consistency (using the faecal consistency score (FCS)), and signs of abdominal pain during defecation (yes/no) as well as other symptoms, such as vomiting and diarrhoea (yes/no and number of episodes), in a standardised bowel daily diary. All cats remained under the same diet they were fed before the study; similarly, also the exercise/activity level remained the same, nevertheless due to the difficulty to assess it, this last aspect was not measured by the owners. Before starting the probiotic treatment, all cats received a water rectal enema once daily for 3 days to accomplish rectal disimpaction. After disimpaction, cats received orally, daily for 90 consecutive days, 2×10^{11} lyophilised bacteria per 5 kg body weight. Cats were assessed clinically with the feline chronic enteropathy activity index (FCEAI) score, based on 6 criteria, each scored on a 0-3 scale: attitude/activity, appetite, vomiting, stool consistency (this parameter was evaluated also separately, by a specific score), stool frequency, and weight loss (Jergens *et al.*, 2010), and also histologically and microbiologically before and after the therapy. Faecal samples were also evaluated daily by the owners, using a 6-point visual scale to assign a FCS ranging from 1 (hard and dry faeces) to 5 (liquid diarrhoea) (while score 2: normal; score 3: moist beginning to lose form; score 4: moist not formed) (Moxham, 2001). Each faecal sample was scored including also the intermediate 1.5 score, because in our series there were many cats that had faeces classifiable in an intermediate degree of consistency between scores one and two. A faecal sample taken from each cat at T0 (the time of enrolment) and the last day of the therapy (after 90 days – T1), was immediately stored at -80 °C, until microbiota analysis. Samples of faeces from controls were also collected and stored in the same manner.

Data analysis

After summation, the FCEAI score was defined as: clinically insignificant (score 0-3), mild (score 4-5), moderate (score 6-8) or severe (score 9 or greater) (Jergens *et al.*, 2010). Owners subjectively evaluated faecal consistency at the time of collection (Table 2). A score of 2 represented a well-formed stool that was easy to collect but was not too dry; this was considered optimum. A score of grade 4 or 5 was considered consistent with diarrhoea (Moxham, 2001). The average value of the FCS, per cat, obtained from the data collected by owners seven days prior to T0, represented the baseline assessment, while the average value of the FCS obtained the last week of the treatment was assumed as the final, T1, faecal consistency score for each cat of the study.

Tissue sampling

After enrolment (time point T0) and after 90 days (T1), multiple (10-15 specimens) mucosal biopsy specimens were sampled from the large intestine of all cats enrolled in the study (n=10); from cats suffering of IMC (n=3), full thickness biopsies were sampled surgically at T0 for histology for diagnostic purposes, to have a general morphological evaluation of the large intestinal wall and ganglia, otherwise not evaluable with endoscopic biopsies. Biopsy specimens

Table 2. Faecal consistency scores recorded in cats before and after treatment.

Faecal consistency score	1	1.5	2	3	4	5
Before treatment (T0)	7	3	0	0	0	0
After treatment (T1)	1	2	5	2	0	0

were obtained directly from mucosal lesions that showed abnormalities such as increased granularity or erosions, as well as from areas of normal-appearing mucosa. Tissues were placed in 10% neutral buffered formalin, then paraffin embedded and serial 3 µm thick sections were prepared. Histopathology was performed by a single pathologist, who was blinded regarding history, clinical signs, or endoscopic observations. A severity score was assigned for each cat, by using a standardised and previously described histologic grading system, based on the extent of architectural disruption and mucosal epithelial changes (German *et al.*, 2000; Jergens *et al.*, 2003), as recently proposed by the World Small Animal Veterinary Association (WSAVA) for diagnosis of gastrointestinal inflammation (Day *et al.*, 2008). To obtain control tissue from healthy cats to compare with IMC, archived formalin-fixed and paraffin-embedded colonic tissues from five cats (two males and three females) without history of intestinal disease were retrieved from the University of Camerino Veterinary Pathology Unit archives. These samples were obtained immediately post mortem from cats that were presented for euthanasia (euthanised cats, EC) for severe injury due to car accidents and histopathological examination of full-thickness colonic biopsies was normal in all cases. Histological samples were evaluated for the immunohistochemical expression of CD117+ interstitial cells of Cajal, enteric neurons, and intra-gangliar cell apoptosis (these two latter parameters were evaluated only in biopsies obtained at T0 from cats with IMC and in biopsies from control cats). ICC count was evaluated before and after therapy, and the data obtained were compared with the values expressed by control tissues from healthy cats; mast cells, which are known to express the same antigen, were differentiated by staining with Alcian blue.

Immunohistochemical evaluation

Paraffin sections were rehydrated and neutralised for endogenous peroxidases with 3% hydrogen peroxide for 5 min, followed by rinsing for 5 min in distilled water. For antigen retrieval, slides were incubated in antigen retrieval solution in a steamer (Black & Decker, Towson, MD) for 20 min. Non-specific immunoglobulin binding was blocked by incubation of slides for 10 min with a protein-blocking agent (Dako, Carpinteria, CA, USA) before application of the primary antibody. Then, the slides were incubated overnight in a moist-chamber with the following primary antibodies: rabbit anti-human CD117 (c-kit) antibody (Dako), used at dilutions of 1:50, and rabbit polyclonal anti-neuron specific enolase (NSE), ready to use (Source BioScience, Santa Fe Springs, CA, USA). A streptavidin-immunoperoxidase staining procedure (Dako) was used for immunolabelling. The immunoreaction was observed with 3,3'-diaminobenzidine as substrate (Vector, Burlingame, UK). Sections were counterstained with Mayer's haematoxylin. Positive immunohistochemical controls included known mast cell tumour (MCTs) for CD117 positive stain, and bovine brain tissue for NSE stain, respectively. Negative immunohistochemical controls were known MCTs or bovine brain sections, treated identically as routine sections, with 20 min antigen retrieval and 10 min protein blocking, except that the overnight incubation with primary antibodies was replaced by an overnight incubation with buffer.

For scoring of intestinal ICC, and NSE+ cells, these cells were quantified in select compartments of the large intestine (apical crypt area, basal crypt area). All cellular types were evaluated using a light microscope (Carl Zeiss, Jena, Germany), 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 intestinal 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 each cat, the total biopsy area was calculated as the sum of the areas of all fields in all six biopsy cross sections on one slide. ICC and NSE positive cells were counted per section, and stained cell densities were expressed as the number of cells per square millimetre of analysed biopsy area (Engel and Arahata, 1986).

In addition, to evaluate dystrophic or suffering tissues and neuronal death in colonic sections of IMC cats, pro-apoptotic effect highlighted through a terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) colorimetric staining (DeadEnd; Promega, Madison, WI, USA) was performed according to the manufacturer's instructions. To enumerate the TUNEL positive nuclei, ten appropriate fields were chosen and examined under a dry-×40 objective for each compartment and arithmetic means were calculated for colonic sub-mucosal and muscular layers. Results were expressed as TUNEL positive gangliar cells per 62,500 µm^2. As above, cells on the margins of the tissue sections were not considered for evaluation to avoid inflation of positive cell numbers.

Microbiota analysis

Total DNA from 100 mg (wet weight) of each faecal sample was extracted by a bead-beating method using the ZR Faecal DNA Kit™ (Zymo Research Corporation, Irvine, CA, USA) following the manufacturer's instructions.

To measure abundances of selected bacterial taxa, quantitative real-time PCR (qPCR) assays were performed for bacteria on various phylogenetic levels, which have been shown previously to be altered in intestinal diseases

in dogs and cats: total bacteria, *Bacteroidetes*, *Firmicutes*, *Fusobacteria*; *Ruminococcaceae*; *Bifidobacterium* spp., *Blautia* spp., *Faecalibacterium* spp., *Lactobacillus* spp., *Streptococcus* spp. and *Turicibacter* spp. (Suchodolski *et al.*, 2015; Vázquez-Baeza *et al.*, 2016). The qPCR cycling, oligonucleotide sequences of primers and probe, and respective annealing temperatures were described previously (Minamoto *et al.*, 2014).

Statistical analysis

To evaluate differences at baseline as well as post-treatment in constipated cats, and between constipated and healthy control cats, a combined statistical analysis model was used. This model takes into account the effects of time (i.e. T0 or T1), treatment, and their 2-way interaction on the various outcome parameters were measured (histology, FCEAI, FCS, ICC+, NSE+, and TUNEL+ cells). Cat was modelled as a random effect to account for repeated measures (before and after treatment) for individual cats; time, treatment, and their 2-way interaction were modelled as fixed, categorical variables. Confidence intervals (CIs) were estimated using maximum likelihood methods. The correlation structure for mixed-effects modelling was that of compound symmetry. A significance level of $P<0.05$ was used for all analyses (S-PLUS, Version 8.2; TIBCO Inc., Palo Alto, CA, USA).

The number of ICC between T0 and T1, and that of neurons, as well as the apoptotic scores, between constipated cats and controls were compared by Wilcoxon matched pair test.

3. Results

All cats completed the study. Baseline characteristics are summarised in Table 1. The average age was 9.7 years and in 85% of them clinical signs of constipation/obstipation were present for at least two years. The severity of clinical signs was significantly higher at baseline of CC (median 7.7, range 7-10) compared to the value at the end of the treatment (median 1.8, range 1-4; $P<0.0001$) as demonstrated by the FCEAI score (Table 3).

After starting the probiotic treatment at T0, the median time of faecal normalisation was 38.6 (18-56) days. As reported in Table 2, in the 10 cats all presenting with ≤1.5 point of FCS before the treatment, FCS improved significantly at the end of the period of probiotic treatment, with 5 cats (50%) shifting at score 2 ('normal' aspect of the faeces), and 2 cats (20%) shifting at score 3 (moist beginning to lose form, leaving a define mark when picked up). Thus, marked improvement was seen in 70% of cats, and only one of them maintained the same score at T0 and T1 (FCS = 1) with resistant hard faeces.

In addition, indication of abdominal pain decreased significantly as demonstrated by FCEAI score (Table 3). There were no side effects such as vomiting, bloating and increased flatulence during the study period. Table 3 summarises the changes in mucosal histology scores, FCEAI, and ICCs number in cats before and after probiotic treatment. Although there was a residual inflammatory infiltrate present, mucosal histology scores were significantly ($P<0.0001$) reduced at T1 relative to T0 in treated cats.

Concerning the enteric nervous system the number of ICCs was compared in biopsy samples taken from colon of cats before and after the treatment and compared with the number found in EC (Figure 1A). The number of ICCs in IMC was significantly lower compared to cats in the EC group (average 117.3±51.36 vs 1,133.4±80.74 cells, $P<0.01$, Figure 2A and B), but increased significantly in treated cats between T0 and T1 (an average of 117.3±51.36 vs

Table 3. Summary statistics for evaluated markers.[1]

	Treated		Control[3]	
	CC/IMC (n=10 cats)		EC (n=5 cats)	
	T0	T1	–	
Histology score	10.8 (8-13)	3.5 (3-8)	–	$P<0.0001$*
FCEAI score	7.7 (3-10)	1.8 (1-4)	–	$P<0.0001$*
ICC[2]	117.3±51.36	973.9±81.78	1,133.4±80.74	$P<0.001$**
				$P<0.01$***

[1] CC = chronic constipation; EC = euthanised cats; FCEAI = feline chronic enteropathy activity index; ICC = interstitial cells of Cajal; IMC = idiopathic megacolon.

[2] Cells per 62,500 µm^2.

[3] * Significant differences between CC/IMC before and after treatment; ** Significant difference between CC/IMC (T0) and EC; *** Significant difference between CC/IMC (T1) and EC.

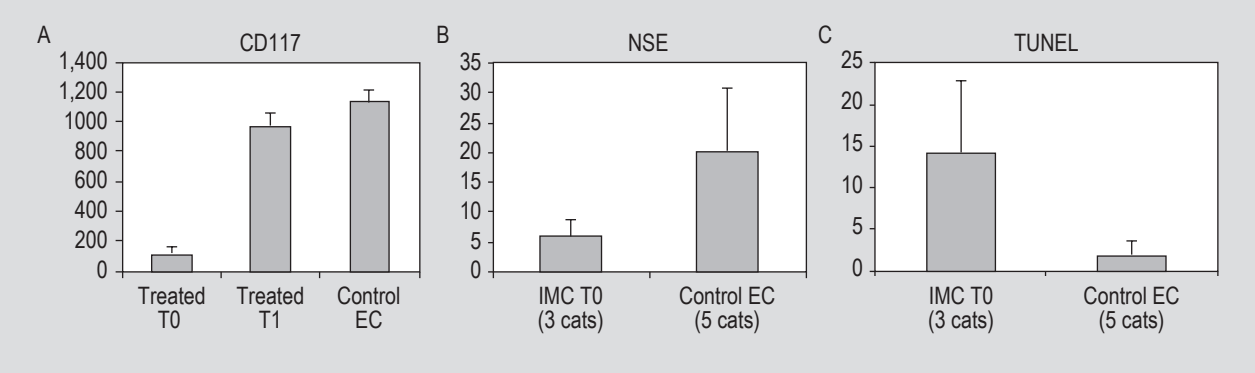

Figure 1. (A) Number of CD117 + cells (ICCs) in cats with megacolon before (T0) and after (T1) treatment, compared with controls (euthanized cats (EC)). (B) Number of enteric neurons in cats with megacolon compared with controls (EC). (C) Number of TUNEL positive enteric neurons in cats with megacolon compared with controls (EC).

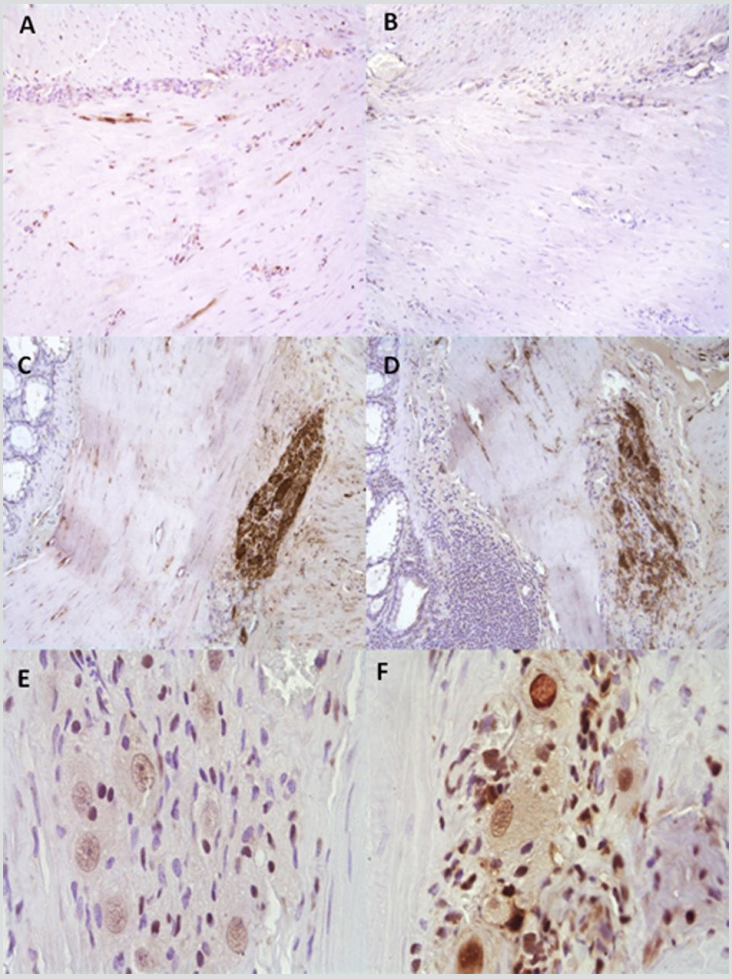

Figure 2. Representative images of interstitial cells of Cajal in (A) a control cat and (B) a cat with megacolon (CD 117, original magnification ×10); (C) A normal myenteric ganglion, and (D) a dystrophic ganglion in a cat with megacolon (anti-neuron specific enolase; original magnification ×10); (E) gangliar neurons in a control; (F) an increased neuronal apoptosis shown in a cat with megacolon (TUNEL; original magnification ×40).

973.9±81.78 cells, $P<0.001$); however, the number of ICCs in the CC group after treatment (T1) remained significantly lower compared to controls (Table 3).

The total number of NSE+ neurons in the myenteric plexus were significantly decreased in IMC cats compared with EC controls cats, (an average of 6±2.86 vs 20.26±10.56 cells, $P<0.001$) (Table 4, Figure 1B). In addition, the neurons within the ganglia often appeared degenerated (Figure 2, C and D). Expression of TUNEL positive neuron nuclei was significantly increased in IMC cats compared with controls (an average of 14.22±8.70 positive neuron nuclei vs 1.93±1.76 nuclei, $P<0.001$) (Figure 1C, Table 4, and Figure 2, E and F).

There were no significant differences in any of the 9 analysed bacterial taxa between healthy cats and diseased cats at baseline ($P>0.05$ for all). After treatment with the probiotic, there was a significant increase in the diseased cats between baseline (T0) and after the end of treatment (T1) in *Streptococcus* ($P=0.003$) and *Lactobacillus* ($P=0.03$), and there was a trend for an increase in *Bifidobacterium* ($P=0.09$) and *Bacteroidetes* ($P=0.06$).

4. Discussion and conclusions

The quantitative and/or qualitative changes of the gut microbiota in idiopathic constipation are still poorly understood in man (Dimidi *et al.*, 2014), and to date no study has been conducted in cats. It is worth noting that, with respect to the medical literature in humans, most data on bacterial interactions and intestinal function are inferred from studies investigating gut motor activity.

There are few doubts that gut microbiota is associated with gastrointestinal motor function (Barbara *et al.*, 2005). In a germ free rat model, intestinal bacteria promoted or suppressed the initiation and migration of the migrating myoelectric complex, depending on the species (Quigley, 2011). Gut microbiota can affect intestinal motor functions either directly or indirectly via mediators released by the gut immune response, the end-products of bacterial fermentation, or intestinal neuroendocrine factors (Uribe *et al.*, 1994; Wu *et al.*, 2013). A recent study using humanised mice (ex-germ-free mice colonised with human faecal microbiota), elegantly demonstrated that the composition of the gut microbiota is changed by altered gastrointestinal motility (Kashyap *et al.*, 2013).

Some probiotic bacteria have been shown to stimulate intestinal motility in both animal and human studies. For instance, *Escherichia coli* Nissle 1917 supernatants enhanced colonic contractility by direct stimulation of smooth muscle cells (Bär *et al.*, 2009). In addition, intestinal bacteria stimulated myoelectric activity in the rat small intestine by promoting cyclic initiation and aboral propagation of migrating myoelectric complex (Husebye *et al.*, 1994). A meta-analysis of 11 randomised controlled trials, recruiting a total of 464 subjects, concluded that probiotic supplementation decreased intestinal transit time (Miller and Ouwehand, 2013). Probiotics may accelerate intestinal transit by decreasing intestinal methanogens, since overproduction of methane has been shown to directly inhibit motor activity (Sahakian *et al.*, 2010), by stimulation of intestinal myoelectric activity (Husebye *et al.*, 1994), and by increasing intestinal fermentation (that enhances colonic peristalsis and decreases transit time). For instance, colonic fermentation of a physiologically relevant malabsorbed quantity of starch has been shown to significantly increase the number of high amplitude propagated contractions (Jouët *et al.*, 2011), the manometric correlation of mass movements (Narducci *et al.*, 1987). In addition, short chain fatty acids (SCFAs), a major end-product of bacterial fermentation, have been shown to increase parietal tone and stimulate ileal propulsive contractions when administered into the human terminal ileum (Coffin *et al.*, 1997; Kamath *et al.*, 1988).

A recent study (Kim *et al.*, 2015) demonstrated that *Bifidobacterium* and *Bacteroides* species were significantly less abundant in faeces from patients with CC compared with healthy controls. However, no significant differences

Table 4. Statistical analysis of immunohistochemistry for NSE and TUNEL in IMC in cats.[1]

	No. cases	IMC T0 (3 cats)			EC (5 cats)			*P*-value[2]
		Mean	Median	SD	Mean	Median	SD	
NSE[3]	n=8	6	15.32	±2.86	20.26	87.06	±10.56	<0.001*
TUNEL[2]	n=8	14.22	95.88	±8.70	1.93	59.84	±1.76	<0.001*

[1] EC = euthanized cats (control); IMC = idiopathic megacolon; NSE = anti-neuron specific enolase; TUNEL = terminal deoxynucleotidyl transferase dUTP nick end labelling; SD = standard deviation.

[2] * Significant differences between IMC and EC.

[3] Cells per 62,500 μm^2.

were observed regarding the proportion of *Lactobacillus* spp., *Escherichia coli* and *Clostridium* species. These results are partially consistent with previous studies that have analysed the faecal microbiota in patients with CC (Chassard *et al.*, 2012; Khalif *et al.*, 2005), and are in accordance with our observation in constipated cats of the present study. In this study, we did not observe any significant changes in the here analysed bacterial groups between healthy cats and affected cats at baseline (T0).

Although for ethical reasons we were able to investigate the enteric nervous system in only 3 cats with constipation and megacolon, on the basis of previous observations (G Rossi and G Bassotti, unpublished data), we speculated that the motor and anatomical modifications might have been due to a decrease, within the myenteric plexus, of ICCs and neurons, the latter related to an increase of neuronal apoptosis. Therefore, reduction or loss of ICC function might decrease or eliminate colonic electrical slow wave activity (Huizinga *et al.*, 1995; Ward *et al.*, 1994), thereby reducing the contractile response and resulting in delayed transit in affected patients. The abnormal colonic motility leading to constipation in these animals was probably strengthened by the loss of enteric neurons, due to an increase in programmed cell death, representing an apoptotic phenomenon especially involving the myenteric neurons. Of interest, we have described similar abnormalities involving ICC and enteric neurons in patients with severe constipation and in those with megacolon (Bassotti *et al.*, 2006a; Iantorno *et al.*, 2007). Thus, neuronal depletion might support a role for the damaged enteric nervous system also in the pathophysiology of feline constipation and, probably, in most cases of feline idiopathic megacolon.

It is worth noting that a residual number of dystrophic neurons and ICC was nevertheless observed in cats with severe megacolon, and this may explain why residual colonic motor activity may be still detected also in very severely constipated cats. In this panorama, a very interesting result of this study was the effect of probiotic administration in terms of ICC increase after treatment, increase that we are at present unable to explain.

The fact that probiotics can ameliorate the clinical signs of constipated patients, as well as observed in cats of this study, may be related with some of the activities previously recorded in other animal models (Kunze *et al.*, 2009).

Bifidobacterium and *Lactobacillus* were shown to be significantly less abundant in adult patients with constipation (Khalif *et al.*, 2005). The fact that in our study these two families of bacteria significantly increased after the therapy, concomitantly with an improvement of clinical signs, suggest a possible direct involvement of these species in the mechanisms of ICC/neuron stimulation. This hypothesis is indirectly supported by some studies in which microvesicles derived from *Bacteroides fragilis* increased excitability of myenteric intrinsic primary afferent neurons when applied to the mucosal surface, but had no effect when applied directly to the myenteric plexus neurons, indicating that *Bacteroidetes* or their components may communicate with local neurons indirectly, through signals generated in the epithelium (Al-Nedawi *et al.*, 2014). On the other hand, lactobacilli also exert an important influence on neuronal excitability and motility in animal models; for instance, administration of *Lactobacillus rhamnosus* increases enteric neuron excitability and modulates colonic motility in rodents (Kunze *et al.*, 2009).

In conclusion, in this pilot study we demonstrated the beneficial effect of a 90 day treatment with SLAB51™ in cats with CC, using objective parameters such as FCEAI, stool consistency and some morphological parameters, although this study did not show a generalised view of the diverse faecal microbiota and was not a placebo-controlled trial. These findings are cautiously promising with respect to the use of probiotic species in the management of CC. The authors believe that additional clinical studies are needed to determine the true efficacy of probiotics in constipation, and to better elucidate their mechanisms of action. These may include by-products of bacterial metabolism (such as gas and SCFA), and can stimulate the activity of residual myenteric neurons population, by directly or indirectly increasing ICC population and function, possibly by regulating some bacterial species related to the function of these pacemaker cells.

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