Article

A multi-strain probiotic promoted recovery of puppies from gastroenteritis in a randomized, double-blind, placebo-controlled study

Rosa A. Molina, Marcela D'Urso Villar, María H. Miranda, Natalia C. Maldonado, Graciela M. Vignolo, María E.F. Nader-Macías

Abstract

Objective and animals

Acute diarrhea is among the most common causes of veterinary consultations for dogs. A double-blind, placebo-controlled intervention trial was done with 120 puppies with gastroenteritis. These dogs were 1 to 4 mo old, male and female, of various breeds and sizes.

Procedure

Dogs were randomly allocated into 2 groups: Those in the treated group (TG) received a multi-strain probiotic with *Lactobacillus johnsonii* CRL1693, *Ligilactobacillus murinus* CRL1695, *Limosilactobacillus mucosae* CRL1696, and *Ligilactobacillus salivarius* CRL1702 (1×10^9 CFU/mL) daily for 7 d, whereas those in the control group (CG) received a placebo. All puppies received intravenous fluids, an antiparasitic, amoxicillin PO, and enrofloxacin SC.

Results

At the start of the trial, the 2 groups were similar. Probiotic administration for 7 d normalized fecal consistency, with 69, 50, and 80% of small, medium, and large puppies in the TG achieving a fecal score of 1 (separate hard lumps) at 7 d, significantly better than puppies in the CG. After 7 d of treatment, most puppies (70%) in the TG had an excellent recovery, whereas in the CG, recoveries were 35.7% "bad" and 30.4% "fair." Therefore, treatment with probiotics hastened recovery ($P < 0.0001$). At the end of the trial, there was a significant increase of cultivable lactobacilli in the feces of TG puppies, but no significant differences between the 2 groups in numbers of total mesophylls, enterobacteria, or Gram-positive cocci. Total mortality was 5.8%, including 4 puppies from the CG and 3 from the TG.

Conclusion

In a randomized, double-blind, placebo-controlled study, puppies with gastroenteritis symptoms receiving a multi-strain probiotic had rapid improvement, implying beneficial effects on the microbiota and its functionality.

Résumé

Un probiotique multi-souches a favorisé la guérison des chiots de la gastro-entérite dans une étude randomisée, en double aveugle et vérifiée par placebo

Objectif et animaux

La diarrhée aiguë fait partie des causes les plus fréquentes de consultations vétérinaires pour les chiens. Un essai d'intervention en double aveugle et vérifié par placebo a été réalisé avec 120 chiots atteints de gastro-entérite. Ces chiens étaient âgés de 1 à 4 mois, mâles et femelles, de différentes races et tailles.

Centro de Referencia para Lactobacilos (CERELA), CONICET, Chacabuco 145, (T4000) Tucumán, Argentina (Molina, Miranda, Maldonado, Vignolo, Nader-Macías); Facultad de Agronomía y Zootecnia (Molina) and Cátedra de Bioestadística, Facultad de Medicina (D'Urso Villar), Universidad Nacional de Tucumán (UNT), (T4000) Tucumán, Argentina.

Address all correspondence to Dr. María Elena Fátima Nader-Macías (ORCID: 0000-0001-7526-1860); email: fnader@cerela.org.ar or fatynader@gmail.com

Funding: PICT start-up 2018-0473 from MINCyT, Argentina.

Unpublished supplementary tables are available online from: www.canadianveterinarians.net

Procédure
Les chiens ont été répartis au hasard en 2 groupes : ceux du groupe traité (TG) ont reçu un probiotique multi-souches contenant *Lactobacillus johnsonii* CRL1693, *Ligilactobacillus murinus* CRL1695, *Limosilactobacillus mucosae* CRL1696 et *Ligilactobacillus salivarius* CRL1702 (1×10^9 UFC/mL) quotidiennement pendant 7 j, tandis que ceux du groupe témoin (CG) ont reçu un placebo. Tous les chiots ont reçu des liquides intraveineux, un antiparasitaire, de l'amoxicilline PO et de l'enrofloxacine SC.

Résultats
Au début de l'essai, les 2 groupes étaient similaires. L'administration de probiotiques pour une durée de 7 j a normalisé la consistance fécale, avec 69, 50 et 80 % des chiots petits, moyens et grands dans le TG obtenant un score fécal de 1 (morceaux durs séparés) à 7 jours, ce qui était significativement meilleur que les chiots dans le CG. Après 7 jours de traitement, la plupart des chiots (70 %) dans le TG ont eu une excellente récupération, alors que dans le CG, les récupérations étaient de 35,7 % « mauvaises » et 30,4 % « passables ». Par conséquent, le traitement avec des probiotiques a accéléré la récupération ($P < 0{,}0001$). À la fin de l'essai, il y avait une augmentation significative des lactobacilles cultivables dans les fèces des chiots TG, mais aucune différence significative entre les 2 groupes en nombre de mésophylles totaux, d'entérobactéries ou de coques à Gram positif. La mortalité totale était de 5,8 %, dont 4 chiots du CG et 3 du TG.

Conclusion
Dans une étude randomisée, en double aveugle et vérifiée par placebo, des chiots présentant des symptômes de gastro-entérite recevant un probiotique multi-souches ont présenté une amélioration rapide, impliquant des effets bénéfiques sur le microbiote et sa fonctionnalité.

(Traduit par Dr Serge Messier)

Can Vet J 2023;64:666–673

Introduction

All animals have a complex diversity of microorganisms in their gastrointestinal tract (GIT); the equilibrium of this system and its interactions with the host affect general health and wellbeing. The gut microbiome contributes to host metabolism, protects against pathogens, and modulates the immune system, thereby either directly or indirectly influencing most physiologic functions of the host. Effects of the GIT microbiome on health and disease as well as regulation of immunity in dogs and cats have been reported (1,2). Age, diet, and many other environmental factors affect maintenance of a healthy microbiome. Alterations in gut microbial population lead to GIT dysfunctions, which are related to functional changes in the microbial transcriptome, proteome, or metabolome (3,4). The gut microbiome was altered during acute or chronic diarrhea, with various agents, including parasites, bacteria *(Enterobacteriaceae* and *Clostridiaceae)* and viruses (parvovirus, coronavirus, and rotavirus) having the highest incidences (5–7). Microbial imbalances have been manipulated using several approaches, including diet, prebiotics, probiotics, symbiotics, and antibiotics. However, antimicrobials induce a rapid and relevant decrease in taxonomic richness, diversity and evenness, with serious consequences for intestinal microbiota. Consequently, they should be evaluated for appropriateness on a case-by-case basis, rather than being used as a standard treatment for gastrointestinal disease (3).

Due to the well-known consequences of antibiotic use, there is renewed interest in probiotics, defined as "live microorganisms, which confer physiological health effects to the host if administered in sufficient amounts" (8). Based on their beneficial effects on gut health and other aspects of wellness, probiotics are increasingly included in human and animal diets. Probiotics are proposed to exert their beneficial effects through various mechanisms, involving production of antimicrobial metabolites-peptides, enhanced growth of favorable endogenous microorganisms, competition for epithelial colonization sites, and immunomodulatory functions (9). To date, several bacterial strains or products have been examined by the European Food Safety Authority (EFSA) for their safety and efficacy as probiotics or feed additives in dogs. Among them, species from the genera *Enterococcus, Lactobacillus,* and *Bifidobacterium* were approved (10). Clinical effects of probiotics in prevention or treatment of GIT diseases in dogs were recently reviewed (9–12). Various forms of acute gastroenteritis in dogs for which probiotics were administered include stress-associated, antibiotic-induced, and idiopathic diarrhea (5); results were variable depending on the probiotic strain and the dog population under evaluation.

We conducted a randomized, double-blind study with 120 puppies of various sizes and breeds, aged 1 to 4 mo, with GIT symptoms (diarrhea and/or fever and/or vomiting). Puppies received gelatin capsules containing either a probiotic (lactobacilli multi-strain probiotic previously characterized and selected due to beneficial properties) or a placebo (as a control).

Materials and methods

Puppies and treatments

The *in vivo* evaluations were conducted according to recommendations of the Argentine Association for the Science and Technology of Laboratory Animals, which are based on the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the Federation of European Associations of Laboratory Animal Sciences (NIH Publication Nos. 8023, 1978). The protocol was evaluated and approved by the CERELA (Centro de Referencia para Lactobacilos) Ethics Committee and CICUAL (Institutional Committee for the

Care and Use of Laboratory Animals, Resolutions 12/2018 and 04/2019). All owners signed an informed consent document. An analytical, prospective, randomized, double-blind case-control protocol was applied. The trial, conducted in a private veterinary clinic (Tucuman, Argentina), used 120 puppies: 60 male and 60 female. The puppies were 1 to 4 mo of age, of various breeds, and with GIT symptoms such as diarrhea and/or fever and/or vomiting (excluding those with diarrhea due to ingestion of foreign bodies or toxic products).

Puppies were randomly assigned to 2 groups: a control group (CG; n = 60) that received standard treatments (rehydration salts, antibiotics, antiparasitics) plus placebo capsules during the trial; and a probiotic lactobacilli-treated group (TG; n = 60) that received the same standard treatments plus a freeze-dried probiotic lactobacilli mixture in gelatin capsules. The standard treatment (given to all puppies) was as follows: i) rehydration: intravenous Ringer solution with B-Lactate (B. Braun, Buenos Aires, Argentina); ii) antiparasitic: praziquantel (Cestodan; Konig Lab, Berlin, Germany), 5 mg/kg, SC plus 1% ivermectin (Immunovet Lab, Argentina), 200 μL/kg; iii) antibiotics: 20% amoxicillin 0.3 mL/kg, IM or SC, q24h for 5 d plus enrofloxacin (Zoovet Lab, Santa Fe, Argentina), 5 mg/kg, SC, q24h for 5 d; and iv) antiemetic: metoclopramide 0.5%, when required. All puppies were treated at the clinic; those with mild symptoms were sent home and brought back to the clinic daily for assessment and treatment, whereas those that were more severely affected were hospitalized until their conditions improved.

Microorganisms and culture conditions

A multi-strain probiotic formula was used. The microorganisms selected were *Lactobacillus (L.) johnsonii* CRL1693, *Ligilactobacillus (L.) murinus* CRL1695, *Limosilactobacillus (L.) mucosae* CRL1696, and *Ligilactobacillus (L.) salivarius* CRL1702 (13). These microorganisms share some complementary beneficial and functional characteristics and are known to be safe in animals. These strains were isolated from young calves, previously evaluated (14,15) and included in the CERELA culture collection (Table 1). They were stored in milk yeast extract (13% skimmed milk: Milkaut, Santa Fe, Argentina; 0.5% yeast extract: Britania, Los Patos, Argentina; and 1% glucose) at −20°C until use.

Bacterial propagation, freeze-drying, and capsule preparation

Lactobacilli strains were subcultured 3× in MRS broth (Biokar Diagnostics, Germany) (16) at 37°C. The last subculture (900 mL and 9 h incubation) was carried out with shaking (WiseStir SMHS-3 Multi Hotplate Stirrer; Witeg, Wertheim, Germany), and then centrifuged (10 min, 7000 × g) under refrigeration and washed twice in sterile saline solution (0.85% NaCl). The pellet was resuspended in 40 mL of sterile cryoprotective solution (10% lactose) (15). For each strain, the concentration of viable cells was 10^{10} colony-forming units (CFU)/mL, as determined by successive dilution and subsequent plating on MRS. Cell pellets were transferred into Petri dishes and frozen at −20°C for 24 h, then freeze-dried (Lyovac GT2; Leybold, Cologne, Germany) for 16 h at 0.3 mbar. Freeze-dried lactobacilli were combined in equal proportions and the mixture was distributed into gelatin capsules (size no. 1; ~10^9 CFU/capsule). The bacterial mixture (0.2 g) was added to each capsule using a semiautomatic encapsulator (Maclen, Buenos Aires, Argentina) under aseptic conditions. Placebo capsules were prepared with 10% lactose (cryoprotectant) as described in this report. All capsules were kept in hermetically sealed plastic containers under refrigerated conditions (4°C) and then apportioned into small, polyethylene bags containing capsules to be administered to each puppy. Every 15 d, capsule contents were rehydrated in 1 mL of physiological solution for 15 min at room temperature, and viable bacteria were enumerated (15).

Treatment of puppies and data collection

Seven gelatin capsules containing freeze-dried lactobacilli mixture (10^9 CFU/capsule) or placebo were administered to each puppy, 1 capsule q24h for 7 d. When a puppy arrived at the veterinary clinic, a comprehensive examination was done, with a record of the following:

i) general characteristics, including breed; sex; size/weight (small: 893.9 to 1706.4 g, medium: 1685.5 to 3789.2 g, or large: 2739.7 to 6482.5 g); age; previous vaccinations (parvovirus and a pentavalent that protects against 5 diseases: diphtheria, kennel cough, tetanus, influenza type b, and hepatitis B); clinical history; symptoms of gastrointestinal or respiratory diseases (duration/number of episodes); previous antibiotic treatments;
ii) type of diet (homemade/balanced/mixed), quality of feed (good/fair/bad), previous indigestion/excess of feed; and
iii) clinical and general conditions by early inspection and posture of the animal, sensorium condition (depressed/alert), current symptoms (fever/vomiting/diarrhea/anorexia/respiratory signs), and body weight. Body weight and body temperature of each puppy were determined by the same person at the same time (before treatments), using the same instruments (mechanical scale and digital thermometer).

Also recorded were appearance of mucous membranes; digestive signs; and other relevant data (conjunctivitis, skin infections, or other concomitants); degree of dehydration by palpation (skin fold); and previous specific symptoms related to the condition, such as abdominal pain, vomiting, and diarrhea (bloody or not). In addition, whether these were accompanied by clinical signs related to the respiratory tract (*e.g.,* dyspnea, nasal secretions, cough, retching) or ocular manifestations was also noted.

Evaluation of feces

The appearance and consistency of the puppies' feces were inspected and scored using the 7-point comparative Bristol Stool Scale (BSS) classification (17), as follows: 1, separate hard lumps; 2, lumpy sausage-shaped; 3, like a sausage with cracks on its surface; 4, like a soft and smooth sausage/snake; 5, soft blobs with clear-cut edges; 6, fluffy pieces/mushy stool; and 7, watery with no solid pieces. The presence of blood and mucus were also recorded. In addition, fecal samples were collected, and the following analyses performed:

Table 1. Probiotic bacteria used in the trial and their properties.

Probiotic strains (Zheng *et al,* 2020)	Surface characteristics		Production of antagonistic compounds			
	Autoaggregation	Hydrophobicity	Pathogen inhibition	H_2O_2	Lactic acid (g/L)	EPS
Lactobacillus johnsonii CRL1693	High	High	*Escherichia coli* 3511AD *Salmonella* Dublin MP/07 *Staphylococcus aureus* MP/0	Low	10.56	+
Ligilactobacillus murinus CRL1695	High	Low	*Salmonella* Typhimurium MP/08	Low	6.38	−
Limosilactobacillus mucosae CRL1696	Low	Low	Not determined	High	5.70	−
Ligilactobacillus salivarium CRL1702	Low	Medium	*Salmonella* Typhimurium MP/08	Low	8.12	−

EPS — Extracellular polymeric substances.

Table 2. Evaluation of feces from puppies in the control group (CG) and treated group (TG) at the start (T0) and end (T7) of the trial.

	T0		T7	
Bristol Stool Scale score	CG, number (%)	TG, number (%)	CG, number (%)	TG, number (%)
1			47 (84)	36 (63)
2		2 (3)	9 (16)	20 (35)
3	1 (1.7)	2 (3)		1 (2)
4	12 (20)	6 (10)		
7	12 (20)	6 (10)		
6	19 (31.7)	15 (25)		
7	16 (26.6)	29 (48)		
Fisher's exact test	$P = 0.048$		$P = 0.024$	

i) ***Direct parasitological examination.*** An aliquot of the stool sample was suspended in saline solution and placed between slide and coverslip for direct observation with a 40× objective (Zeiss Optical Microscope); parasite eggs, red blood cells, and white blood cells were identified. Then, a flotation technique was done by transferring 1 g of feces to a beaker flask containing 10 mL of saturated NaCl solution, filtering through gauze, and collecting in a test tube. A coverslip was placed on the edge of the tube to contact the liquid. After 5 to 10 min, floating cysts or eggs were attached to the surface of the coverslip, carefully removed, placed on a slide, and examined by light microscopy (40× objective).

ii) ***Microbiological examination by quantification of cultivable microorganisms.*** Fecal or rectal samples were taken with sterile swabs, placed in transport medium (LAPT broth), and refrigerated until analyzed. The number of cultivable microorganisms was determined by successive dilutions (in 0.5% peptone water) and subsequent plating in agar media. Total mesophilic bacteria (Plate Count Agar; PCA), lactic acid bacteria (MRS and LBS agar), fecal enterococci (SF agar), and *Enterobacteriaceae* (MacConkey agar) were enumerated. The plates were incubated at 37°C for 24 to 48 h and the number of CFU/g was determined.

Canine nutritional analysis and recovery

Monitoring puppy growth and recovery included determining body weight with a mechanical scale at the start and at the end of the trial, with mean weight gain calculated as Day 7 weight minus Day 0 weight. A puppy was considered to be in recovery based on clinical conditions, including absence of fever, vomiting, and diarrhea; recovery of appetite; and sensory alertness. Based on the number of d in which an individual re-established the aforementioned conditions, recovery was classified as excellent (2 d), good (3 d), fair (5 d), or bad (≥ 7 d). Mortality was also recorded.

Statistical analyses

Data were recorded on a clinical sheet at the start of the trial (T0) and after 7 to 10 d (T7 to T10). Qualitative variables were compared between the TG and CG with the Fisher's exact test and the Marascuilo multiple comparisons test. For quantitative variables, an unpaired Student's *t*-test was used to compare the 2 groups, and a paired Student's *t*-test was used to compare T0 *versus* T7 within each group. The significance level considered was 5%. Data analysis was performed using Minitab and Stata 15 IC software.

Results

There were no apparent side effects of probiotic treatments for puppies from the experimental group during 7 d of treatments. All puppies assigned to each group were fasted and received conventional broad-spectrum antiparasitic treatment, antibiotics, and oral or parenteral hydration as required. Most puppies completed the treatment, indicating adherence to the experimental protocol. The high degree of owner compliance facilitated evaluation of all puppies.

Canine evaluation at the start of the trial (T0)

The mean age of the puppies at T0 was 63.3 d (95% CI: 58.9 to 67.8 d) in the control group (CG) and 70.1 d (95% CI: 65.1 to

Table 3. Body weight (BW) and body weight gain (BWG) for puppies in the control group (CG) and treated group (TG).

	Control group (CG)	Treated group (TG)	Paired *t*-test comparison of means between T0 and T7	
Puppy size	Mean, g (95% CI)	Mean, g (95% CI)	CG	TG
BW at T0				
Small	1156.7 (893.9 to 1419.4)	1382.1 (1057.9 to 1706.4)	***P* < 0.0001**	***P* < 0.0001**
	Unpaired *t*-test *P* = 0.2701			
Medium	2057.9 (1685.5 to 2430.3)	3040.9 (2292.6 to 3789.2)	***P* < 0.0001**	***P* < 0.0001**
	Unpaired *t*-test ***P* = 0.0249**			
Large	5090.9 (3699.3 to 6482.5)	4500.0 (2739.7 to 6260.3)	***P* = 0.0065**	***P* = 0.0003**
	Unpaired *t*-test *P* = 0.5574			
BW at T7				
Small	1513.3 (1216.7 to 1809.9)	1700.0 (1315.6 to 2084.4)		
	Unpaired *t*-test *P* = 0.4259			
Medium	2447.1 (1981.8 to 2912.3)	3627.3 (2827.2 to 4427.4)		
	Unpaired *t*-test ***P* = 0.0186**			
Large	6288.9 (4080.2 to 8497.5)	5430.0 (3751 to 7109)		
	Unpaired *t*-test *P* = 0.4833			
BWG (T0 to T7)	Mean (g)	Mean (g)	Unpaired *t*-test	
Small	356.6	317.9	*P* = 0.4944	
Medium	389.2	586.4	*P* = 0.1293	
Large	1198.0	930.0	*P* = 0.68	

T0 — Start of the trial; T7 — End of the trial; 95% CI — 95% confidence interval.

75.1 d) in the treatment group (TG). At the beginning of the study, both groups were homogeneous in terms of general characteristics, current clinical conditions, current clinical signs, and feeding, with no significant differences between CG and TG (see Table S1, available online from: www.canadianveterinarians.net). In initial evaluations of stool samples (both macroscopic and microscopic), there were no significant differences between groups regarding presence of blood or mucus or the proportion of parasitized puppies evaluated through either direct or enrichment diagnostic methods (see Table S2, available online from: www.canadianveterinarians.net). However, when fecal scores were evaluated using the BSS, there were lower proportions of puppies with fecal consistency scores of 6 (31.7%) or 7 (26.6%) in the CG, whereas 73% of TG puppies had fecal scores of 6 and 7 at T0 (Table 2). Therefore, at the start of the trial, puppies in the CG had more formed stools, whereas those in the TG had waterier stools. Differences between the 2 groups in stool consistency arose from random assignment as part of the experimental design.

Evaluation at the end of the trial (T7) and recovery

Changes in fecal scores and body weights were assessed. No puppy had a fecal score higher than 3, and there were 2 TG puppies with a score of 1. In multiple comparisons with the Marascuilo test, these differences were in score 1 (higher for TG) and score 3 (higher for CG), which highlights the beneficial effects of probiotic treatment (Table 2). Average body weight of medium-sized puppies was significantly greater in TG than in CG at both T0 and T7, but not significantly different between the 2 groups for small and large puppies (Table 3). Furthermore, there were highly significant increases in body weight between T0 and T7 for puppies within a specific size group and treatment group. At T7, recovery was judged as excellent, good, fair, or bad in 70.2, 10.5, 14.0, and 5.3% of TG puppies, respectively; and in 16.1, 17.9, 30.4, and 35.7% of CG puppies, respectively. Therefore, treatment with probiotics hastened recovery ($P < 0.0001$).

Overall mortality was 7%, including 4 puppies from CG and 3 from TG. The puppies from CG that died (2 medium- and 2 large-sized puppies) had severe clinical signs on Day 0, none was vaccinated, and they had a survival time of 3 d. Similarly, the 3 small-sized TG puppies that died also had serious clinical signs on Day 0 and only 1 was vaccinated against parvovirus. These puppies died after 6 d of probiotic treatment (data not shown).

Microbiological analysis of canine feces

Cultivable microorganisms were enumerated in canine feces from both CG and TG at T0 and T7 (Table 4). At T7, mean total mesophilic bacteria was significantly higher in the CG *versus* the TG, whereas mean lactobacilli was significantly lower in the CG *versus* the TG. When comparing samples between T0 and T7, there were only significant differences for enterococci in the control group ($P = 0.0214$).

Table 4. Microbiological analysis (CFU/g) of feces from puppies in the control group (CG) and treated group (TG) at the start of the trial (T0) and 7 d later (T7).

	Mean value	95% confidence interval	Unpaired *t*-test (*P*-value)	Paired *t*-test (T0 *versus* T7: *P*-value)
Total mesophilic bacteria (TMB)				
T0				
CG	1.23×10^9	$(8.19 \times 10^8$ to $1.64 \times 10^9)$	0.3829	CG 0.3060
TG	1.31×10^9	$(9.42 \times 10^8$ to $1.68 \times 10^9)$		
T7				
CG	7.54×10^8	$(4.51 \times 10^8$ to $1.06 \times 10^9)$	**0.0445**	TG 0.7297
TG	1.58×10^9	$(6.72 \times 10^8$ to $2.49 \times 10^9)$		
Lactobacilli				
T0				
CG	6.22×10^8	$(3.85 \times 10^8$ to $8.59 \times 10^8)$	0.6684	CG 0.2390
TG	5.55×10^8	$(3.62 \times 10^8$ to $7.49 \times 10^8)$		
T7				
CG	2.72×10^8	$(1.70 \times 10^8$ to $3.74 \times 10^8)$	**0.0006**	TG 0.3279
TG	8.14×10^8	$(5.09 \times 10^8$ to $1.12 \times 10^9)$		
Enterobacteria				
T0				
CG	1.47×10^8	$(89.81 \times 10^7$ to $1.97 \times 10^8)$	0.1954	CG 0.8047
TG	1.85×10^8	$(1.13 \times 10^8$ to $2.57 \times 10^8)$		
T7				
CG	1.10×10^8	$(2.19 \times 10^7$ to $1.99 \times 10^8)$	0.2919	TG 0.3222
TG	1.50×10^8	$(3.52 \times 10^7$ to $2.66 \times 10^8)$		
Enterococci				
T0				
CG	3.23×10^7	$(1.31 \times 10^7$ to $5.14 \times 10^7)$	0.0631	CG **0.0214**
TG	1.66×10^7	$(9.00 \times 10^6$ to $2.41 \times 10^7)$		
T7				
CG	1.08×10^7	$(4.13 \times 10^6$ to $1.75 \times 10^7)$	0.4350	TG 0.3186
TG	1.01×10^7	$(4.83 \times 10^6$ to $1.54 \times 10^7)$		

Discussion

Gastroenteritis is among the most common reasons puppies are presented to a veterinarian (18,19). In dogs and cats, diarrhea is common and can result from various factors, including stress; diet change; and a wide variety of bacterial, viral, and parasitic agents (7,18,19). Regardless of the cause, diarrhea in dogs or cats can be a source of zoonotic infections for humans, can delay pet adoption, and, in extreme cases, can result in euthanasia (18,20). In dogs, intestinal inflammation, whether chronic or acute, alters the composition of the intestinal microbiota and commonly affects metabolite production, including short-chain fatty acids and amino acids; *e.g.,* tryptophan and its catabolites (3).

In this study, a lactobacilli consortium involving *L. johnsonii* CRL1693, *L. murinus* CRL1695, *L. mucosae* CRL1696, and *L. salivarius* CRL1702 was used as feed additive for puppies with gastroenteritis. These probiotic strains already had good evidence of efficacy, particularly for newborn calves, with improved gut health and calf performance plus safety, and no concerns regarding antimicrobial resistance or virulence determinants (15). Results obtained in weaned calves laid the foundation for trialing this multi-strain probiotic in puppies with no apparent adverse effects. In addition, recommendations from reference organisms and information reported in numerous studies indicated that experimental assays with microorganisms and probiotic formulas should be done using double-blind and randomized protocols (8,21). As gastroenteritis has higher morbidity and mortality in younger puppies, probiotics and prebiotics have been explored as strategies to promote gut health and decrease diarrhea in young animals. The transition from a predominantly milk diet to a solid diet during a relatively short interval may also provide an opportunity to use microbial-based products. The probiotic lactobacilli were administered to puppies at 1 to 4 mo of age for 7 d; these conditions were chosen based on clinical experience (critical age for puppy gastroenteritis) and the animal owners' non-acceptance of prolonged treatments. All dogs received antibiotic treatment, as this was the standard of care and withholding it would have been unethical for dogs in the CG (as they were clinical cases) and a serious confounding factor for dogs in the TG.

Preliminary determinations on puppies assured relative homogeneity in the 2 groups. Age, sex, breed, diet, and environmental factors can affect the GIT and fecal microbiome of dogs (2,22–24). Indeed, there were significant differences between groups in fecal characteristics: more CG than TG puppies had solid feces (the latter had more individuals with fecal scores of 6 or 7). However, after 7 d, both CG and TG puppies had considerable reductions in fecal moisture content. Orally administered probiotics in TG were regarded as critical in achieving fecal score changes to 1 and 2 at T7, despite this group having the highest proportion of puppies with score 7 at T0. Nonetheless, a high proportion of puppies given placebo (CG)

also achieved fecal scores of 1 or 2 at T7, although this might be attributed to fewer puppies with scores of 6 or 7 at T0, as well as the recovery treatment received by animals in the trial.

Fecal score is a relevant indicator of gut functionality and can be altered from normal values depending mainly on the type and quality of the diet and occurrence of GIT diseases or intestinal dysbiosis. A strong association between stool consistency and gut microbiota richness and composition, enterotypes, and bacterial growth rates was reported using the Bristol Stool Scale (BSS) classification, which reflects fecal water content and activity and is considered a proxy measure for intestinal colonic transit time/rate (25). Each consistency category reflects differences in moisture content of fecal material, with decreased water activity (associated with prolonged intestinal transit) limiting microbial growth through reduction of nutrient mobility and hampered enzymatic activity. Species richness declines with higher BSS scores, reaching its minimum in individuals with loose stool. Therefore, BSS categorization summarizes the effects of 2 major and selective forces shaping the gut ecosystem: rate of intestinal transit and water activity. In this study, improvements in stool consistency were significantly better in the group treated with probiotics (TG). Similar to these results, a double-blind study conducted in dogs with acute diarrhea demonstrated that the administration of acidic milk containing a probiotic lactobacilli mixture (2×10^9 CFU/mL) normalized fecal consistency in addition to maintaining appetite and reducing vomiting (26). However, reports using *Lactobacillus acidophilus* strains had variable results that ranged from unchanged to good effects on fecal scores of healthy cats and dogs (27,28).

Nutritional condition of TG puppies assessed by weight changes and body weight gain during the 7-day protocol had different weight increases for each size category. Although various canine breeds have different growth patterns, dog growth can be described using size categories rather than curves for specific breeds (29). Small- and large-sized dog breeds have considerably different growth patterns, with a shorter duration of a period of faster growth in small breeds (30). Weight gain improvement in this study agreed with that reported when a probiotic compound containing lactobacilli and bifidobacteria was orally administered to Belgium shepherds (31). An age-related response was described after oral administration of *L. casei* Zhang, *L. plantarum* P-8, and *Bifidobacterium animalis* subsp. *lactis* V9, improving canine feed intake, weight gain, immunity, and intestinal microbiota (31). In addition, health benefits involving the normalization/duration of bowel diseases agree with the intestinal physiology and immunity modulation observed after the probiotic *L. fermentum* CCM 7421 was given to dogs (32). Similarly, giving *L. acidophilus* D2/CSL improved the nutritional status and fecal parameters of boxer dogs (27).

At the end of the trial (T7), 67% of puppies given probiotics (TG) had an excellent recovery, a much better response compared to puppies that received placebo (CG). Most probiotic administration protocols applied to dogs had multiple beneficial effects attributed to their abilities to improve dysbiosis by reduction of inflammatory cell infiltrates or inflammatory receptor expression and motility modulation that led to resolution of clinical signs (33). Indeed, after 7 d of probiotic administration, not only was a clinical improvement observed in puppies (evidenced by the normalization of fecal score), but the greatest recovery of puppies from the TG categorized as excellent was attributed to the effective reduction of intestinal inflammation and restoration of a healthy gut microbiome eliminating dysbiosis, as recently reported (34). Conversely, even though most puppies from the placebo (CG) group reached fecal scores of 1 or 2 at T7 after oral or parenteral rehydration, antibiotics, or anthelmintics, their recovery was mostly categorized as "fair" or "bad," needing more than 2 d to be re-established. In addition to more puppies having a fecal score ≤ 5 from the CG ($n = 21$) compared to the TG ($n = 15$) at T0, during acute diarrhea episodes and when antibiotics are used, a strong effect on the canine microbiota composition is produced (35). An increased abundance of *Clostridium* and *Enterobacteriaceae* was reported to alter the overall metabolic profile of the host; thus, reversion of dysbiosis and recovery of the initial microbiota is rarely fully achieved, or may occur after a longer interval (3,36).

Although puppies were randomly assigned to groups, 7 puppies that entered with severe clinical conditions died during the trial. At the end of the trial, 3 small-sized puppies treated with probiotic (TG) died after several days of treatment, whereas 4 medium- or large-sized puppies from the CG died. Based on experience, large- and medium-sized puppies usually suffer more severely from viral diseases due to their greater body volumes and high electrolytes losses; thus, their clinical condition deteriorates more quickly.

Microbiological evaluation of the feces revealed a significant increase in the abundance of beneficial lactic acid bacteria in puppies treated with probiotics (TG) at T7 compared to T0 and dogs from the CG involved in the trial. Similarly, dietary supplementation with the probiotic mixture Slab51 (contains streptococci, bifidobacteria, and lactobacilli strains) as well as *L. acidophilus* D2/CSL given to healthy dogs increased the presence of *Bifidobacterium/Lactobacillus* and *Lactobacillus* in dog feces after 28 d and 8.5 d, respectively (27,34). However, similar abundances of facultative anaerobic bacteria from the *Enterobacteriaceae* family and Gram-positive cocci (enterococci) as common markers of dysbiosis were present in canine feces between T0 and T7 when comparing TG and CG in this study. When *L. acidophilus* D2/CSL was administered to healthy boxer dogs or cats for 28 d or 35 d, respectively, significant reductions in total *Escherichia coli* counts compared to control were reported (27,28). In this study, the short-interval probiotics treatment (7 d) may explain the lack of reductions of *Enterobacteria* and enterococci. Similar to a previous report (27), the formula of the probiotic used herein contained a high concentration of bacteria compared to several other probiotic products, which may have promoted the normalizing effects.

In conclusion, we inferred that the probiotic mixture used in this study was associated with increased beneficial bacteria and a corresponding reduction in potentially pathogenic bacteria. This effect was confirmed by changes in the BSS scores of fecal samples and the puppies' rapid recovery. Due to the short duration of the trial, further studies with a longer duration of testing in dogs with GIT disorders are necessary to confirm the probiotic effects. CVJ

References

1. Tizard IR, Jones SW. The microbiota regulates immunity and immunologic diseases in dogs and cats. Vet Clin North Am Small Anim Pract 2018;48:307–322.
2. Wernimont SM, Radosevich J, Jackson M, *et al.* The effects of nutrition on the gastrointestinal microbiome of cats and dogs: Impact on health and disease. Front Microbiol 2020;11:1266.
3. Pilla R, Suchodolski JS. The role of the canine gut microbiome and metabolome in health and gastrointestinal disease. Front Vet Sci 2020; 6:498.
4. Zeng Y, Inohara N, Nunez G. Mechanisms of inflammation-driven bacterial dysbiosis in the gut. Mucosal Immunol 2017;10:18–26.
5. Zobba R, Visco S, Sotgiu F, Pinna Parpaglia ML, Pittau M, Alberti A. Molecular survey of parvovirus, astrovirus, coronavirus, and calicivirus in symptomatic dogs. Vet Res Commun 2021;45:31–40.
6. Marks SL, Kather EJ. Antimicrobial susceptibilities of canine *Clostridium difficile* and *Clostridium perfringens* isolates to commonly utilized antimicrobial drugs. Vet Microbiol 2003;94:39–45.
7. Raza A, Rand J, Ghaffar Qamar A, Jabbar A, Kopp S. Gastrointestinal parasites in shelter dogs: Occurrence, pathology, treatment and risk to shelter workers. Animals 2018;8:108.
8. Hill C, Guarner F, Reid G, *et al.* The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. Nat Rev Gastroenterol Hepatol 2014;11:506–514.
9. Schmitz SS, Suchodolski JS. Understanding the canine intestinal microbiota and its modification by pro-pre- and symbiotics — What is the evidence? Vet Med Sci 2016;2:1–94.
10. Jensen AP, Bjørnvad CR. Clinical effect of probiotics in prevention or treatment of gastrointestinal disease in dogs: A systematic review. J Vet Int Med 2019;33:1849–1864.
11. Schmitz SS. Value of probiotics in canine and feline gastroenterology. Vet Clin North Am Small Anim Pract 2021;51:171–217.
12. Suchodolski JS, Jergens AE. Recent advances and understanding of using probiotic-based interventions to restore homeostasis of the microbiome for the prevention/therapy of bacterial diseases. Microbiol Spectr 2016;4. doi: 10.1128/microbiolspec.VMBF-0025-2015.
13. Zheng J, Wittouck S, Salvetti E, *et al.* A taxonomic note on the genus *Lactobacillus:* Description of 23 novel genera, emended description of the genus *Lactobacillus* Beijerinck 1901, and union of *Lactobacillaceae* and *Leuconostocaceae.* Int J Syst Evol Microbiol 2020;70:2782–2858.
14. Maldonado NC, Silva de Ruiz C, Otero MC, Sesma F, Nader-Macías ME. Lactic acid bacteria isolated from young calves — Characterization and potential as probiotics. Res Vet Sci 2012;92:342–349.
15. Maldonado NC, Nader-Macías MEF. Production of fermented milk with autochthonous lactobacilli for newborn calves and resistance to the dairy farm conditions. J Bioprocess Biotech 2016;6:1000278.
16. De Man JC, Rogosa M, Sharpe EM. A medium for the cultivation of lactobacilli. J Appl Bacteriol 1960;23:130–135.
17. Blake MR, Raker JR, Whelan K. Validity and reliability of the Bristol Stool Form Scale in healthy adults and patients with diarrhoea-predominant irritable bowel syndrome. Aliment Pharmacol Ther 2016; 44:693–703.
18. Pugh CA, Bronsvoort BM, Handel IG, *et al.* Incidence rates and risk factor analyses for owner reported vomiting and diarrhea in Labrador retrievers — Findings from the Dogslife Cohort. Prev Vet Med 2017;140:19–29.
19. Sævik BK, Skancke EM, Trangerud C. A longitudinal study on diarrhea and vomiting in young dogs of four large breeds. Acta Vet Scand 2012;54:8.
20. Stull JW, Brophy J, Weese JS. Reducing the risk of pet-associated zoonotic infections. CMAJ 2015;187:736–743.
21. Allenspach K. Diagnosis of small intestinal disorders in dogs and cats. Clin Lab Med 2015;35:521–534.
22. Calalang J, Cheung H, Lichimo K, So B. Identifying breed, dietary, and reproductive factors affecting the gut microbiome of dogs with inflammatory bowel disease. Undergrad J Exp Microbiol Immunol 2021;26:1–13.
23. Schmidt M, Unterer S, Suchodolski JN, *et al.* The fecal microbiome and metabolome differs between dogs fed bones and raw food (BARF) diets and dogs fed commercial diets. PLoS ONE 2018;13:e0201279.
24. Reddy KE, Kim H-R, Jeong JY, *et al.* Impact of breed on the fecal microbiome of dogs under the same dietary condition. J Microbiol Biotechnol 2019;29:1947–1956.
25. Vandeputte D, Falony G, Vieira-Silva S, Tito RY, Joossens M, Raes J. Stool consistency is strongly associated with gut microbiota richness and composition, enterotypes and bacterial growth rates. Gut 2016;65: 57–62.
26. Gómez-Gallego C, Junilla J, Männikkö S, *et al.* A canine-specific probiotic product in treating acute or intermittent diarrhea in dogs: A double-blind placebo-controlled efficacy study. Vet Microbiol 2016;197: 122–128.
27. Marelli SP, Fusi E, Giardini A, *et al.* Effects of probiotic *Lactobacillus acidophilus* D2/CSL (CECT 4529) on the nutritional and health status of boxer dogs. Vet Record 2020;187:e28.
28. Fusi E, Rizzi R, Polli M, *et al.* Effects of *Lactobacillus acidophilus* D2/CSL (CECT 4529) supplementation on healthy cat performance. Vet Rec Open 2019;6:e000368.
29. Salt C, Morris PJ, German AJ, *et al.* Growth standard charts for monitoring bodyweight in dogs of different sizes. PLoS ONE 2017;12: e0182064.
30. Posada Ochoa S, Gomez L, Rosero Noguera R. Application of the logistic model to describe the growth curve in dogs of different breeds. Revista MVZ Córdoba 2014;19:4015–4022.
31. Xu H, Huang W, Hou Q, *et al.* Oral administration of compound probiotics improved canine feed intake, weight gain, immunity and intestinal microbiota. Front Immunol 2019;10:666.
32. Strompfovà V, Kubašová I, Lauková A. Health benefits observed after probiotic *Lactobacillus fermentum* CCM 7421 application in dogs. Appl Microbiol Biotechnol 2017;101:6309–6319.
33. Rossi G, Pengo G, Galosi L, *et al.* Effects of the probiotic mixture Slab51® (SivoMixx®) as food supplement in healthy dogs: Evaluation of fecal microbiota, clinical parameters and immune function. Front Vet Sci 2020;7:613.
34. Rossi G, Cerquetella M, Gavazza A, *et al.* Rapid resolution of large bowel diarrhea after the administration of a combination of a high-fiber diet and a probiotic mixture in 30 dogs. Vet Sci 2020;7:21.
35. Reese AT, Cho EH, Klitzman B, *et al.* Antibiotic-induced changes in the microbiota disrupts redox dynamics in the gut. Elife 2018;7:e35987.
36. Ziese A-L, Suchodolski JS, Hartmann K, *et al.* Effect of probiotic treatment on the clinical course, intestinal microbiome, and toxigenic *Clostridium perfringens* in dogs with acute hemorrhagic diarrhea. PLoS ONE 2018;13:e0204691.