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Research article

APPLICATION OF CANINE-DERIVED *ENTEROCOCCUS FAECIUM* DSM 32820 IN DOGS WITH ACUTE IDIOPATHIC DIARRHOEA

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Modulation of the intestinal microbiota through the application of probiotic bacteria is currently one possible way to improve gastrointestinal health in dogs. Knowledge on the efficacy of lactic acid bacteria in a diarrhoeic disorder of dogs is still spreading; however, the used or commercialized strains are often not of canine origin. In this study, *E. faecium* DSM 32820 strain (a canine isolate selected in our laboratory based on safety and probiotic criteria) was fed to nine dogs suffering from acute non-haemorrhagic diarrhoea at a dose of $1x10^9$ CFU/ml for 7 days. Samples of feces and blood were taken on day 0 and 7. Evaluation of the CIBDAI (Canine Intestinal Bowel Disease activity index) score showed significantly lower vomiting frequency, stool frequency, stool consistency and weight loss at day 7 compared to day 0 ($P<0.05$). The 16S rRNA gene analysis revealed Firmicutes as the predominant phylum on both sampling days (72.0% vs. 67.9%, day 0 and 7) followed by Proteobacteria (13.4% vs. 6.0%), Actinobacteria (10.0% vs 13.5%), Fusobacteria (4.2% vs. 2.3%) and Bacteroidetes (0.4% vs. 10.4%). The abundance of family Erysipelotrichiaceae was higher on day 7 compared to the initial levels ($P<0.05$). Among 19 detected fecal enzymatic activities, five (α-chymotrypsin, β-glucuronidase, α-fucosidase, β-galactosidase, N-acetyl-glucosaminidase) were changed ($P<0.05$). After the application of the DSM 32820 strain, mean fecal dry matter was significantly higher on day 7 compared to baseline ($P< 0.05$). Although hematological and biochemical parameters in the blood were not significantly different on average, individual values of certain parameters in several dogs were improved.

Keywords: dog, diarrhea, *Enterococcus faecium*, microbiota, probiotic

INTRODUCTION

Diseases of the gastrointestinal tract are very common in dogs, which is also confirmed by some statistics determining leading gastropathies and enteropathies [1]. The main

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clinical symptom of these diseases is diarrhea, which is defined as an increase in fecal water content, usually leading to changes in fecal volume, fluidity and frequency of defecation [2]. There are many causes of diarrhea – dietary (change of diet, spoiled feed, hypersensitivity or dietary intolerance), bacterial pathogens (*Escherichia coli*, *Clostridium* sp.), parasites (*Isospora* spp., *Giardia* spp.) and also medicines (mainly antibiotics). Considering the duration of diarrhea, we recognize acute (lasts for less than 14 days) and chronic (persists for more than 14 days). Four pathophysiologic mechanisms that can result in diarrhea have been described; however, diarrhea can also be caused by more than one mechanism simultaneously [3]. These mechanisms may manifest as 1. osmotic diarrhea, caused by unusually large amounts of poorly absorbable osmotically active solutes in the intestinal lumen (these active particles could arise as result of changes in intestinal microbiota and fermentation of carbohydrates); 2. secretory diarrhea, caused by abnormal ion transport in intestinal epithelial cells, for example, diarrhea incurred by enteropathogenic bacteria; 3. diarrhea caused by increased mucosal permeability resulting in the loss of fluids, electrolytes, proteins etc. into the intestinal lumen (for example, in inflammatory conditions); 4. abnormal motility, mainly ileal and colonic, can contribute to clinical manifestation of inflammatory bowel disease (IBD). Of course, the method of therapy depends on the cause of the diarrhea, for example, dietary treatment, the addition of fluids, administration of motility modifiers, corticosteroids or also use of antimicrobials, which is not recommended as the main therapy in the management of noninfectious or uncompleted diarrhea (because of their effects on normal microbiota and the formation of dysbiosis or the possibility of promoting resistant strains). On the other hand, a novel alternative therapeutic method could be the administration of probiotics. Many researchers have observed several specific effects. Specific probiotic strains can normalize symbiosis or reduce harmful, potentially pathogenic bacteria [4,5], modulate immune functions [6,7], normalize hematological and biochemical parameters [8] and/or show anti-inflammatory effects [9].

The potential probiotic strain used in this canine experiment, *E. faecium* DSM 32820, was selected in our laboratory (Centre of Biosciences, Institute of Animal Physiology) from a group of 160 enterococci isolated from the feces of 105 dogs based on the results of its safety and probiotic criteria [10,11]. After *in vitro* testing, the *E. faecium* strain was also applied *in vivo* in order to obtain information on how clinically healthy dogs tolerate the daily administration of this canine-derived strain [12]. The results showed good tolerability of the strain from the preventive viewpoint, sufficient colonization, solid fecal consistency, improvement of cellular immunity and no side effects throughout the study.

After testing the *E. faecium* DSM 32820 strain properties *in vitro* and its effects in clinically healthy dogs after oral administration, we decided to acquire information on the effects of its application in diseased dogs with clinical symptoms of gastrointestinal disease, mainly acute diarrhea.

MATERIAL AND METHODS

Strain *E. faecium* DSM 32820 for experimental application

The rifampicin-resistant variant of the *E. faecium* DSM 32820 strain was prepared by repeated cultivation on M-*Enterococcus* agar plates (Merck) supplemented with a high concentration of rifampicin (100 μg/mL, Rifasynt, Medochemie Ltd., Cyprus) to differentiate the applied strain from the other fecal enterococci in the canine experiment. The rifampicin-resistant strain was then cultivated in MRS broth (Merck) at 37 ºC for 24 h; cells were harvested after centrifugation for 10 min at 2000 rpm, and the culture sediment was resuspended in Ringer solution (Merck, pH 7.0) to a concentration of 10^9 CFU/mL.

Animals

The selected *E. faecium* DSM 32820 strain was applied to nine dogs (eight adult dogs, one two-month-old puppy; different breeds: Doberman, Yorkshire terrier, Maltese terrier, French bulldog, German shepherd, Bull terrier, Cross-breed dog). Dogs for this experiment were selected in the Small Animal Clinic (University of Veterinary Medicine and Pharmacy in Košice, Slovakia) on the basis of clinical symptoms indicating digestive problems (acute gastroenteritis/enteritis). The main common symptoms for all dogs were an increased frequency of defecation and changed stool consistency (watery, liquid stool). We excluded dogs having blood in the stool and/or fever. None of the dogs had other medical conditions or took any antibiotics or other remedies. They were kept indoors (in the flat or house of their owners), had free access to fresh water at all times and received their fixed daily rations of maintenance commercial diet for the duration of the study. In our experiment, the dogs were administered the bacterial solution daily during the feeding time at a dose of 1 mL/dog/day for 7 days. Samples (feces, blood) were collected at days 0 and 7.

Clinical status of the dogs

The Canine Intestinal Bowel Disease activity index (CIBDAI) was used to describe the clinical status of the dogs. This activity index is a summation of the score of 6 different clinical signs: attitude/activity, appetite, vomiting, stool consistency, stool frequency and weight loss. All of the signs were scored from 0 to 3 (0 – normal, 1 – mild change, 2 – moderate change, 3 – severe change).

Analysis of fecal samples

Fresh fecal samples were collected in air-tight containers during individual walks. Fecal samples were firstly used for the measurement of pH values (pH Meter, Hanna Instruments, USA). The dry matter (DM) content was determined after drying to a constant weight at 105 ºC (Ecocell, BMT Medical Technology s.r.o, Czech Republic).

The population of the applied rifampicin-resistant strain *E. faecium* DSM 32820 in the fecal samples was determined according to the standard microbiological method using the M-*Enterococcus* agar supplemented with rifampicin (100 mg/mL). Plates were incubated aerobically at 37 ºC for 24 h.

The enzymatic activity of each of the canine fecal samples was established, according to the API ZYM Kit (bioMérieux, Marcy-l'Étoile, France) manufacturer's manual. The activities of the following enzymes were tested: alkaline phosphatase, esterase, esterase/lipase, lipase, leucine arylamidase, valine arylamidase, cysteine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase. Freshly collected fecal samples (0.1 g) were suspended in saline (2 mL) and centrifuged for 10 min at 2,000 rpm to remove debris. The solution thus formed was inoculated (65 µL into each cup) into an API-ZYM strip. Enzyme activity readings were taken after 4 h of incubation at 37 °C and after the addition of Zym A and Zym B reagents. Color intensity values from 0 to 5 and their relevant value in nanomoles were assigned for each reaction according to a color chart enclosed with the kit.

DNA isolation from samples

The microbial population in the canine feces was determined by sequencing the hypervariable V3-V4 region (341F-785R) of the bacterial 16S rRNA gene. Canine feces were diluted 5x with molecular grade water and homogenized by vortexing with Zirconia beads 2.3 mm (BioSpec, USA). Homogenized feces (260 µL) were used for DNA isolation performed using the DNeasy PowerLyzer PowerSoil Kit (QIAGEN, Germany) according to the manufacturer's instructions.

PCR amplification and Illumina library preparation

Isolated DNA was used as a template in a PCR reaction targeting the hypervariable V3-V4 region (341F-785R) of the bacterial 16S rRNA gene (16S Metagenomic sequencing Library Preparation protocol; Illumina, USA). Briefly, the PCR detection protocol was as follows: 95 °C (3 min); 95 °C (30 s), 55 °C (30 s), 72 °C (30 s), 25 cycles; 72 °C (5 min). A list of primer pairs is presented in Table 1. PCR clean-up was performed with Agencourt AMPure XP beads (Beckman Coulter Genomics), and the concentration was measured using the Qubit® dsDNA HS Assay Kit (Invitrogen™, USA). Based on the concentration values, samples with different inner tags were equimolarly pooled and used as a template for a second PCR with Nextera XT indexes (Illumina, USA). Pools containing different indexes were quantified using the Illumina – LightCycler 480 qPCR MasterMix (Roche, USA) and equimolarly pooled. The prepared library was checked with a High Sensitivity D5000 Screen tape (Agilent Technologies, USA) and with the Illumina – LightCycler 480 qPCR MasterMix (Roche, USA). The library was diluted to a final concentration of 8 pM, and 20% of PhiX DNA (Illumina, USA) was

added. Sequencing was performed with the MiSeq reagent kit V3 using a MiSeq 2000 instrument according to the manufacturer's instructions (Illumina, USA).

Table 1. List of used primers

EMP16S-1	F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGAGCCTTCGTCGCGTGTGYCAGCMGCCGCGGTAA
	R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCCTAACGGTCCACCGGACTACNVGGGTWTCTAAT
EMP16S-2	F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTCCATACCGGAAGTGTGYCAGCMGCCGCGGTAA
	R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCGCGCCTTAAACCCGGACTACNVGGGTWTCTAAT
EMP16S-3	F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGAGCCCTGCTACAGTGTGYCAGCMGCCGCGGTAA
	R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTATGGTACCCAGCCGGACTACNVGGGTWTCTAAT
EMP16S-4	F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTGAGACCCTACAGTGTGYCAGCMGCCGCGGTAA
	R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGCCTCTACGTCGCCGGACTACNVGGGTWTCTAAT
EMP16S-5	F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGACTTGGTGTAAGGTGTGYCAGCMGCCGCGGTAA
	R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGACTACTGAGGATCCGGACTACNVGGGTWTCTAAT
EMP16S-6	F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGATTACGTATCATGTGTGYCAGCMGCCGCGGTAA
	R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGAATTCACCTCCTCCGGACTACNVGGGTWTCTAAT
EMP16S-7	F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCACGCAGTCTACGTGTGYCAGCMGCCGCGGTAA
	R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCGTATAAATGCGCCGGACTACNVGGGTWTCTAAT
EMP16S-8	F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTGTGCACGCCATGTGTGYCAGCMGCCGCGGTAA
	R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGATGCTGCAACACCCGGACTACNVGGGTWTCTAAT
EMP16S-9	F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCGGACAAGAAGGTGTGYCAGCMGCCGCGGTAA
	R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGACTCGCTCGCTGCCGGACTACNVGGGTWTCTAAT
EMP16S-10	F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTTGCTGGACGCTGTGTGYCAGCMGCCGCGGTAA
	R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTTCCTTAGTAGTCCGGACTACNVGGGTWTCTAAT
EMP16S-11	F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTACTAACGCGGTGTGTGYCAGCMGCCGCGGTAA
	R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCGTCCGTATGAACCGGACTACNVGGGTWTCTAAT
EMP16S-12	F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGCGATCACACCTGTGTGYCAGCMGCCGCGGTAA
	R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGACGTGAGGAACGCCGGACTACNVGGGTWTCTAAT
EMP16S-13	F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCAAACGCACTAAGTGTGYCAGCMGCCGCGGTAA
	R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGTTGCCCTGTACCGGACTACNVGGGTWTCTAAT
EMP16S-14	F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGAAGAGGGTTGAGTGTGYCAGCMGCCGCGGTAA
	R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCATATAGCCCGACCGGACTACNVGGGTWTCTAAT
EMP16S-15	F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTGAGTGGTCTGTGTGTGYCAGCMGCCGCGGTAA
	R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGCCTATGAGATCCCGGACTACNVGGGTWTCTAAT

16S Metagenomic sequencing Library Preparation protocol; Illumina, USA (EMP 515-806)

Bioinformatic and statistical analysis

Sequencing data were analyzed using the fastq-join method within the join_pair_ends.py command in QIIME 1.9.1. Data were demultiplexed, and barcodes and primers were trimmed using the package Biostrings in R. Operational taxonomic units (OTUs) were constructed by binding sequences into clusters of greater than 97% sequence similarity using QIIME. Chimeras were detected on the set of representative sequences of each OTU with UCHIME in USEARCH v6.1.544 and excluded from the analysis.

Taxonomy was assigned to each OTU based on the SILVA 123 reference database. The analyses were performed on each of the three taxonomy levels (Phylum, Family, Genus) and OTUs, separately, and the resulting p-values were adjusted for hypothesis testing using the Benjamini-Hochberg procedure. Results were considered significant at FDR=10%.

Analysis of blood samples

Blood samples were collected into plastic tubes containing 10 IU heparin (20 μL/mL of blood) for analysis of hematological parameters and tubes without anticoagulant for analysis of biochemical parameters (blood from *vena cephalica antebrachii*). The hematological parameters were analyzed using the Cell-Dyn 3700 (Abbott Laboratories, USA), and the biochemical parameters in blood serum were determined using colorimetric methods (Spectrophotometer UV-2550 Shimadzu, Japan) with kits (Randox Laboratories Ltd., UK) for the following parameters: total protein (TP245), albumin (AB 362), urea (UR 107), triglyceride (TR 210), cholesterol (CH 200), glucose (GL 2623), alanine aminotransferase (AL 100), calcium (CA 590) and inorganic phosphorus (PH 1016).

Statistical analysis

The results are expressed as the mean and standard deviation. Statistical analyses were performed using GraphPad Prism software (version 6.0). A paired t-test with the level of significance set at $P<0.05$ was used to evaluate data obtained before and after probiotic treatment.

Ethical approval

The study involved dogs visiting the Small Animal Clinic at the University of Veterinary Medicine and Pharmacy in Košice (SK). All handling of animals was in accordance with standard veterinary practices according to Slovak legislation (no. 377/2012 and 436/2012).

RESULTS

Clinical status of the dogs

The clinical status of the dogs expressed by the CIBDAI score showed several significant changes ($P<0.05$, Table 2). Despite the fact that the exact diagnoses and major causes were not determined, this scoring system was used for a specific description of the clinical conditions of the dogs. Criteria, such as vomiting frequency, stool frequency, stool consistency and also weight loss, were significantly lower on day 7 compared to day 0. The results showed a decrease in the score, which indicates a normal clinical condition. This fact was also confirmed by the dogs' owners, who

described an obvious retreat of symptoms and improvement in their clinical status. No significant differences in the mean activity and appetite values were observed.

Table 2 Canine inflammatory bowel disease activity index (CIBDAI) in dogs on the day before (0 day) and after treatment (7 day)

CIBDAI criteria	day 0	day 7	P-value
Attitude/activity	0.2 ± 0.4	0.0 ± 0.0	0.3632
Appetite	0.2 ± 0.4	0.0 ± 0.0	0.3632
Vomiting	1.3 ± 0.8	0.0 ± 0.0	0.0103
Stool consistency	2.2 ± 1.0	0.8 ± 1.0	0.0250
Stool frequency	1.7 ± 0.8	0.5 ± 0.5	0.0335
Weight loss	0.8 ± 0.8	0.0 ± 0.0	0.0422

Scored 0-3: 0- normal, 1- mild change, 2- moderate change, 3- severe change

Analysis of fecal samples

Different analyses of fecal samples were tested in this study. The population of the applied rifampicin-resistant strain *E. faecium* DSM 32820 was determined according to the standard microbiological method using a selective medium supplemented with rifampicin. The strain was detected in the canine fecal samples after 7 days of strain addition, with a mean fecal level of 3.89±2.53 log CFU (colony forming unit/g). The microbial population in the feces of dogs, determined by sequencing, showed just one significant difference. The abundance of the family Erysipelotrichiaceae was significantly higher at day 7 compared to the baseline ($P<0.05$). The results also showed a trend for higher abundance in populations of the phylum Actinobacteria ($P=0.06$) and the genus Turicibacter ($P=0.08$). The detailed bacterial composition, expressed in the relative abundance (mean) of detected major phyla, family and genera in the feces of dogs before (day 0) and after treatment (day 7) is shown in the figures (Fig. 1, 2, 3). The phylum Firmicutes was detected as predominant (72.0%), followed by Proteobacteria (13.4%), Actinobacteria (10.0%), Fusobacteria (4.2%) and Bacteroidetes (0.4%) on day 0. After the treatment, we observed some nuances in the percentage representation of the bacterial phyla. The phylum Firmicutes was also predominant (67.9%) at day 7, followed by Actinobacteria (13.5%), Bacteroidetes (10.4%), Proteobacteria (6.0%) and Fusobacteria (2.3%). The predominant genera identified in fecal bacterial microbiota accounted for > 72% at day 0 and 66% at day 7: *Peptoclostridium* (mean [%] day 0 – 14.2 / day 7 – 7.8), *Escherichia-Shigella* (12.8 / 4.2), *Lactobacillus* (12.4 / 11.0), *Blautia* (12.0 / 9.4), *Lachnoclostridium* (11.3 / 10.2), *Collinsella* (9.5 / 11.7), *Bacteroides* (0.1 / 7.7) and *Turicibacter* (0.01 / 4.9).

Another performed test of fecal samples was the determination of enzymatic activity. Such activity was tested using a semiquantitative micro method (Table 3). We observed significant differences in 5 enzymes on day 7 compared to day 0. Production of the enzymes α-chymotrypsin, β-glucuronidase, α-fucosidase and also of the desirable enzyme β-galactosidase was significantly increased, while the activity of N-acetyl-

glucosaminidase associated with intestinal disease was decreased. No significant changes in the production of all others enzymes were detected.

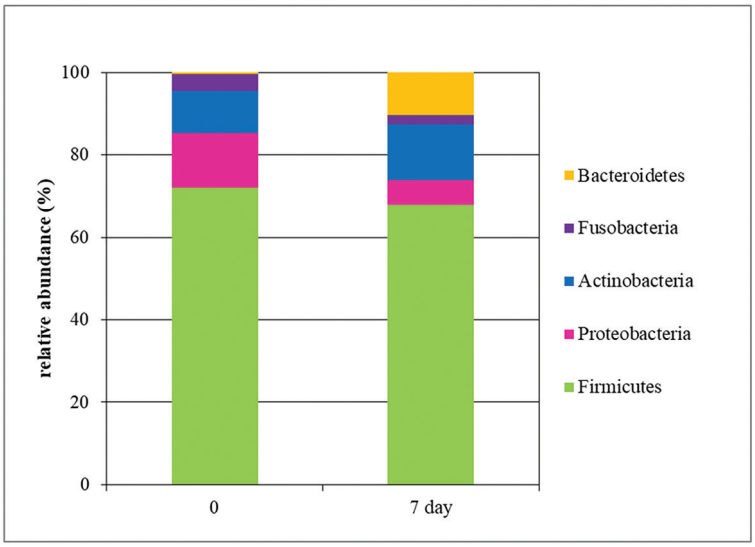

Figure 1. Bar charts showing the relative abundance (mean) of major phyla in feces of dogs before (day 0) and after treatment (day 7)

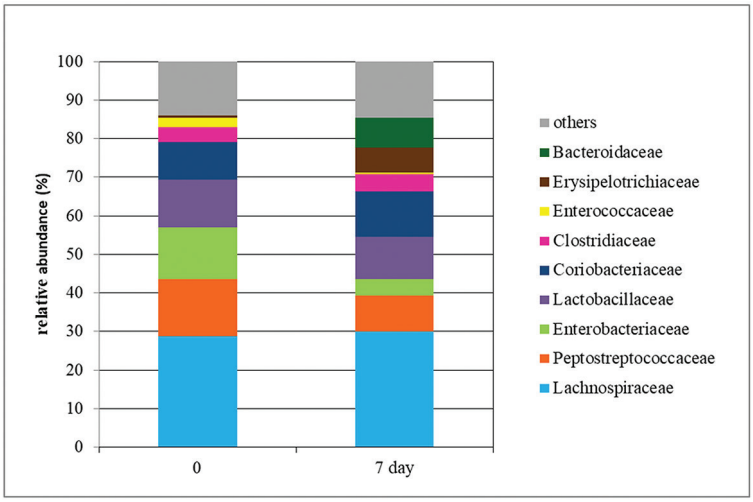

Figure 2. Bar charts showing the relative abundance (mean %) of family in feces of dogs before (day 0) and after treatment (day 7)

After the application of DSM 32820 strain, the mean fecal dry matter was significantly higher on day 7 compared to baseline ($P< 0.05$), and the mean fecal pH value was not significantly changed (day 0 – 6.08±0.64, day 7 – 6.02±0.89, $P=0.70$).

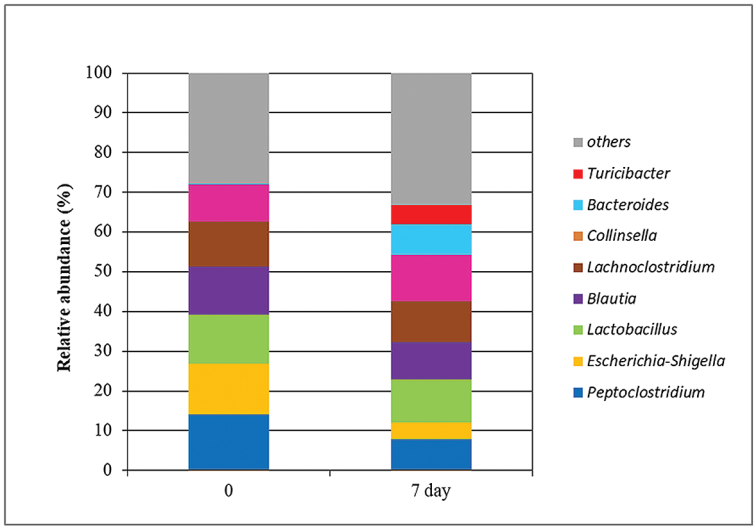

Figure 3. Bar charts showing relative abundance (mean %) of detected genera in feces of dogs before (day 0) and after treatment (day 7)

Table 3. Enzyme activity in the feces of dogs (nmol/4h) before (day 0) and after treatment (day 7)

Enzyme	Day 0	Day 7	P-value
Alkaline phosphatase	25.0 ± 5.5	23.3 ± 13.7	0.7711
Esterase (C4)	6.7 ± 2.6	13.3 ± 9.8	0.2065
Esterase Lipase (C8)	6.7 ± 2.6	13.3 ± 9.8	0.2213
Lipase (C14)	7.5 ± 2.7	14.2 ± 9.2	0.1576
Leucine arylamidase	33.3 ± 5.2	30.0 ± 8.9	0.4650
Valine arylamidase	28.3 ± 9.8	26.7 ± 10.3	0.8090
Cystine arylamidase	7.5 ± 2.7	11.7 ± 4.1	0.1412
Trypsin	16.7 ± 8.2	16.7 ± 8.2	1.000
α-chymotrypsin	7.5 ± 2.7	18.3 ± 4.1	0.0062
Acid phosphatase	28.3 ± 7.5	26.7 ± 8.2	0.7412
Naphthol-AS-BI-phosphohydrolase	31.7 ± 7.5	30.0 ± 11.0	0.8220
α-galactosidase	16.7 ± 8.2	20.0 ± 8.9	0.5761
β-galactosidase	20.0 ± 8.9	33.3 ± 5.2	0.0103
β-glucuronidase	25.0 ± 5.5	35.0 ± 8.4	0.0117
α-glucosidase	35.0 ± 5.5	33.3 ± 8.2	0.6109
β-glucosidase	26.7 ± 8.2	21.7 ± 9.8	0.4560
N-acetyl-glucosaminidase	28.3 ± 7.5	18.3 ± 7.5	0.0117
α-mannosidase	8.3 ± 2.6	10.0 ± 0.0	0.1747
α-fucosidase	5.0 ± 3.2	18.3 ± 7.5	0.0171

Analysis of blood samples

We observed no significant differences in the hematological parameters in dogs (Table 4). Nevertheless, there were some individual changes in a few parameters from initially higher values to the reference range. For example, leukocytes, erythrocytes, MCH and MCHC were adjusted to reference values in one dog. Moreover, the level of leukocytes was decreased to the reference range in another dog.

Table 4 Hematological parameters in dogs before (day 0) and after treatment (day 7)

Parameter	Unit	Reference range	Day 0	Day 7	P-value
Erythrocytes	T/L	5.5 - 8.5	6.77 ± 0.94	6.70 ± 0.82	0.7023
Hemoglobin	g/L	150 - 190	165.8 ± 19.2	153.3 ± 18.3	0.1708
Hematocrit	L/L	0.440 - 0.520	0.47 ± 0.07	0.46 ± 0.06	0.4166
Leukocytes	G/L	6.0 - 12.0	9.89 ± 3.16	9.74 ± 2.32	0.7899
Neutrophils	G/L	3.60 - 12.50	6.70 ± 3.05	6.60 ± 1.78	0.9005
Lymphocytes	G/L	0.70 - 6.00	1.94 ± 0.93	2.05 ± 0.63	0.5938
Monocytes	G/L	0.10 - 1.70	0.72 ± 0.34	0.67 ± 0.25	0.6155
Eosinophils	G/L	0.10 - 1.80	0.41 ± 0.72	0.33 ± 0.36	0.6829
Basophils	G/L	0.00 - 0.100	0.13 ± 0.09	0.09 ± 0.08	0.2625
MCV	fL	60.0 - 70.0	69.6 ± 1.7	69.0 ± 2.4	0.2786
MCH	pg	17.0 - 23.0	25.0 ± 6.6	22.3 ± 0.7	0.3491
MCHC	g/L	330 - 360	358.5 ± 95.1	323.6 ± 5.5	0.3593

Table 5. Biochemical parameters in the blood serum of dogs before (day 0) and after treatment (day 7)

Parameter	Unit	Reference range	Day 0	Day 7	P-value
Total protein	g/L	57.00 - 75.00	60.6 ± 4.7	58.7 ± 3.8	0.2836
Albumin	g/L	28.00 - 43.00	34.8 ± 2.1	34.4 ± 2.1	0.3594
Urea	mmol/L	3.33 - 6.99	7.93 ± 1.76	8.32 ± 1.53	0.3302
Glucose	mmol/L	4.00 - 6.00	4.89 ± 0.71	4.69 ± 0.71	0.5665
Cholesterol	mmol/L	3.25 - 6.25	6.12 ± 1.35	5.87 ± 0.88	0.5113
Alanine aminotransferase	μkat/L	up to 0.333	0.263 ± 0.175	0.227 ± 0.078	0.6161
Aspartate aminotransferase	μkat/L	0.103 - 0.333	0.429 ± 0.146	0.464 ± 0.148	0.4967
Calcium	mmol/L	2.25 - 2.99	2.46 ± 0.14	2.38 ± 0.16	0.0976
Phosphorus	mmol/L	1.29 - 2.91	1.98 ± 0.55	1.71 ± 0.30	0.2879

Similarly, no statistically significant differences in serum biochemical parameters were detected ($P>0.05$; Table 5). However, evaluation of some parameters showed a change in the results from out of the reference range to the desired values. In detail, the concentration of cholesterol, which in two dogs (dog 6 and 8) was over the reference range (over 6.5 mmol/L) on day 0, was decreased in one dog to the reference range

after 7 days of application of the DSM 32820 strain. Similarly, the activity of alanine aminotransferase, initially over the range (over 0.333 μkat/L) in one dog (dog 4), was within the reference range on day 7. The serum mineral profile was stable, except for higher phosphorus concentrations in two dogs (dog 1 and dog 4) on day 0, which were decreased to the reference level on day 7.

DISCUSSION

In this study, the potentially probiotic strain *E. faecium* DSM 32820 was applied to nine adult dogs with acute idiopathic non-hemorrhagic diarrhea. This strain of canine origin was selected from 160 enterococci isolated from 105 healthy dogs. Our previous studies described its safety, potential probiotic properties and effect *in vitro* as well as *in vivo* [11,12]. As required, the selected strain meets the criteria proposed by EFSA for *E. faecium* species intended for use in animal nutrition [13]. The *E. faecium* DSM 32820 strain is sensitive to ampicillin and other antimicrobials of veterinary importance; it does not possess virulence determinants, such as *IS16*, *hyl*-like and the *Esp* gene, and it shows no hemolytic and harmful enzymatic activity. Moreover, application to clinically healthy dogs showed no negative effects [12]. In detail, dogs of the DSM 32820 group had optimal fecal consistency throughout the experiment and significantly stimulated phagocytic activity, metabolic burst activity of leukocytes and lowered serum glucose concentration at the end of the experiment. The mean fecal levels of the strain DSM 32820 during the application period reached 10^6 CFU/g.

In this study, many significant differences related to the clinical conditions were observed. According to the CIBDAI scoring system, parameters such as stool consistency, stool frequency and vomiting significantly improved after the application of *E. faecium* DSM 32820. Similarly, Sauter et al. [14] observed a significant decrease of the same parameters involved in the CIBDAI scoring system. In their study, a probiotic cocktail of three different lyophilized *Lactobacillus* spp. strains: two *L. acidophilus* (NCC2628, NCC2766) and one *L. johnsonii* (NCC2767) was applied to dogs (n=21) with food-responsive diarrhea being treated with an elimination diet. In our study, consistency according to a visual scoring system but also fecal dry matter results (22 vs. 28%) showed significant improvement after application of the DSM 32820 strain. The possible mechanisms of this effect include enhancement of intestinal barrier functions (e.g. via alteration tight junction protein expression, enhancing the electrical resistance of tight junctions, increased production of cytoprotective molecules), increased absorption of water and sodium from the colon stimulated by the increased amount of short-chain fatty acids (SCFA), reduction of intestinal motility, an anti-inflammatory effect and antimicrobial activity of lactic acid or other metabolites [15,16].

Based on 16S rRNA gene analysis, the predominant phylum in all experimental dogs was Firmicutes (52 – 92%, mean 72%) on day 0 as well as on day 7 (42 – 93%, mean 68%). The second most abundant phylum was Proteobacteria (0.2 – 47%, mean 13.4%) on day 0, with Actinobacteria on day 7 (0.1 – 46%, mean 13.5%). Phylum

Bacteroidetes, which was negligible on day 0 (0.4%) was increased by 10% on day 7. According to the study by Kim et al. [17], the proportion of core gut microbiota in dogs also depends on the type of diet. In their study, Firmicutes constituted 64% of the microbiota in healthy dogs fed a natural diet and 73% in dogs fed commercial feed. Initially, after application of the DSM 32820 strain, the lower amount of Bacteroidetes in diarrheic dogs was increased almost to levels commonly detected in healthy dogs (17–20%, [17]). Bacteroidetes is a very diverse bacterial phylum and is increasingly regarded as a specialist for the degradation of high molecular weight organic matter, i.e., proteins and carbohydrates. Gut Bacteroidetes generally produce butyrate, an end product of colonic fermentation, which is thought to have antineoplastic properties and thus play a role in maintaining a healthy gut [18]. They are also involved in bile acid metabolism and the transformation of toxic and/or mutagenic compounds [19]. Similarly, Suchodolski et al. [20] observed a lower proportion of phylum Bacteriodetes in dogs with acute non-hemorrhagic diarrhea. SCFA, the main fermentation products of Bacteroidetes, also play an important role in the absorption of water and electrolytes in the intestine [21]. The abundance of the morphologically and metabolically diverse phylum Proteobacteria contributing to homeostasis of the anaerobic environment of the gastrointestinal tract and the stability of the strictly anaerobic microbiota was lower on day 7 by 7% compared to day 0. However, this change did not reach a significant difference. In general, the abundance of Proteobacteria in dogs comprised from 0 to 22% of 16S rRNA reads of the fecal microbiomes reported in dogs [22,23]. Within phylum Firmicutes, the family Erysipelotrichaceae showed increases after 7-day application of the DSM 32820 strain (from 0.6 to 6.6%, $P<0.05$). A study by Suchodolski et al. [20] observed a similar difference in the abundance of this family between healthy dogs (mean, 7.8%) and dogs with acute diarrhea (0.7%). The trend for a higher *Turicibacter* spp. population – a producer of butyric acid belonging to the family Erysipelotrichiceae – was detected at the end of our experiment. Detection of supplemented strain DSM 32820 by the cultivation of fecal samples revealed a lower mean count (3.9 $\log_{10}$ CFU/g) compared to counts detected in healthy dogs (6.7 $\log_{10}$ CFU/g, [12]).

Semiquantitative determination of enzyme activities in fecal samples revealed significant differences in 5 out of 19 enzymes (α-chymotrypsin, β-glucuronidase, α-fucosidase, β-galactosidase and N-acetyl-glucosaminidase). Researchers have mainly observed changes in fecal enzyme activities affected by diet and related changes in the microbial population [24,25]. Moreover, probiotic strains also showed enzymatic activities which play a role in their biological effects. In our case, the diet and also the appetite of dogs with acute diarrhea was not significantly changed. Moreover, the supplemented strain *E. faecium* DSM 32820 did not produce any of these five mentioned enzymes; therefore, enzymatic differences could be associated with the application of the *E. faecium* DSM 32820 strain only indirectly, e.g. via changes in the microbial community. Specifically, we observed a significant increase in bacterial

enzyme β-galactosidase, which catalyzes the first step of lactose fermentation in the colon, thus improving symptoms of lactose intolerance – a very frequent condition in older adults with a global prevalence of 68% [26]. Intestinal bacterial β-glucuronidase hydrolyses glucuronidated metabolites to their toxic forms in the intestines, resulting in intestinal damage. Despite this, some probiotic strains can decrease β-glucuronidase activity (e.g. *L. casei* Shirota and *B. breve*, [27]), and in our study it was significantly increased. Monosaccharide fucose is metabolized by an enzyme called α-fucosidase, which was increased in our experiment. This enzyme has been used as a tumor marker in the diagnosis of hepatic carcinoma and colorectal cancer [28]. On the other hand, a significant decrease in N-acetyl-β-glucosaminidase (the enzyme involved in the degradation of gut mucus gel) could be considered as a beneficial effect of *E. faecium* DSM 32820 application.

Although the determination of hematological parameters after *E. faecium* DSM 32820 application showed no significant changes of mean values, the individual values of several parameters were modified to the reference range. Concretely, dogs 4 and 6 with an initially higher leukocyte count (over the reference range of 12 G/L) showed a lower count (within the reference range) after 7 days of application of the DSM 32820 strain. Moreover, the count of erythrocytes, MCH (mean corpuscular hemoglobin) and MCHC (mean corpuscular hemoglobin concentration) was also modified in dog 6. Other studies describing hematological parameters in dogs with an acute disorder with diarrhea have shown predominantly low leukocyte levels in viral infection and high levels due to primary or secondary bacterial infection. Terzungwe [29] tested the hematological parameters of dogs infected with canine parvovirus enteritis in his study and observed the occurrence leukocytosis in just 27.6% (8/29) of diarrheic dogs. Likewise, Sharma et al. [30] observed leukocytosis in 31 dogs (from a group of 40 dogs) with hemorrhagic gastroenteritis with the manifestation of diarrhea.

Biochemical mean values detected in the blood serum of dogs were also not significantly changed. Almost all individual biochemical results were within physiological levels, except higher cholesterol concentration in dog 6 (7.7 vs. 5.8 mmol/L, day 0 vs. 7), the activity of alanine aminotransferase in dog 4 (0.584 vs. 0.221 μkat/L) and the phosphorus concentration in dogs 1 and 4 (3.6, 4.8 vs. 1.9, 1.5 mmol/L). A hypocholesterolemic effect is commonly observed after application of many enterococcal probiotic strains (*in vitro*, in humans, in animals) [31-33], which is attributed to enzymatic deconjugation of bile acids by bile-salt hydrolase in probiotics, to the ability to bind cholesterol onto cellular surface, to incorporation into cellular membranes or to the conversion of cholesterol to coprostanol [34]. Optimisation of biochemical parameters, such as cholesterol and ALT, have also been detected after application of the probiotic bacteria *Lactobacillus fermentum* CCM7421, which was applied to different groups of dogs (healthy or suffering from gastrointestinal disorders, [35]).

CONCLUSION

In conclusion, the 7-day application of *E. faecium* DSM 32820 to dogs with acute idiopathic diarrhea led to improvement of some parameters of the CIBDAI score (stool consistency, stool frequency and vomiting), some microbiological community and fecal enzyme activity changes, as well as to the individual modification of biochemical or hematological parameters.

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Authors' contributions

IK drafted the manuscript; IK, LŠ, DB and VS performed blood and fecal analyses; LM performed sequencing of fecal samples; AM provided samples from dogs; VS supervised the project and contributed to the final version of the manuscript.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Statement of Informed Consent

the owner understood procedure and agrees that results related to investigation or treatment of their companion animals, could be published in Scientific Journal Acta Veterinaria-Beograd.

REFERENCES

1. Nationwide 2018. http://nationwidedvm.com/common-medical-conditions-for-dogs-and-cats/.
2. Beasley SS, Manninen TJK, Saris, PEJ: Lactic acid bacteria isolated from canine faeces. J Appl Microbiol 2006, 101:131-138.
3. Marks, SL: Chapter 11 – Diarrhea, in: Washabau, RJ, Day MJ (Eds.), Canine and Feline Gastroenterology. W.B. Saunders, 2013, pp. 99-108.

4. Rossi, G., Pengo, G., Caldin, M., Piccionello AP, Steiner JM, Cohen ND, Jergens AE, Suchodolski JS: Comparison of microbiological, histological, and immunomodulatory parameters in response to treatment with either combination therapy with prednisone and metronidazole or probiotic VSL#3 strains in dogs with idiopathic inflammatory bowel disease. Plos One 2014, 9:e94699.
5. Gomez-Gallego C, Junnila J, Mannikko S, Hameenoja P, Valtonen E, Salminen S, Beasley S: 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.
6. Kim EJ, Kang YI, Bang TI, Lee M, Lee SW, Choi I, Song C-S, Lee J-B, Park S-Y: Characterization of *Lactobacillus reuteri* BCLR-42 and *Lactobacillus plantarum* BCLP-51 as novel dog probiotics with innate immune enhancing properties. Korean J Vet Res 2016, 56:75–84.
7. Kumar S, Pattanaik AK, Sharma S, Jadhav SE, Dutta N, Kumar A: Probiotic potential of a *Lactobacillus* bacterium of canine faecalorigin and its impact on select gut health indices and immune response of dogs. Prob Antimicrob Prot. 2017, 9:262–277.
8. Kumar S, Pattanaik AK, Sharma S, Jadhav SE: Species-specific probiotic *Lactobacillus johnsonii* CPN23 supplementation modulates blood biochemical profile and erythrocytic antioxidant indices in Labrador dogs, Indian J. Anim Sci 2016, 86:918–924.
9. Kainulainen V, Tang YR, Spillmann T, Kilpinen S, Reunanen J, Saris PEJ, Satokari R: The canine isolate *Lactobacillus acidophilus* LAB20 adheres to intestinal epithelium and attenuates LPS-induced IL-8 secretion of enterocytes *in vitro*. BMC Microbiol 2015, 15:4.
10. Kubašová I, Strompfová V, Lauková A: Safety assessment of commensal enterococci from dogs. Folia Microbiol 2017, 62:491–498.
11. Kubašová I, Lauková A, Hamarová I', Pristaš P, Strompfová V: Evaluation of enterococci for potential probiotic utilization in dog., Folia Microbiol 2018, 64:177–187.
12. Strompfová V, Kubašová I, Ščerbová J, Maďari A, Gancarčíková S, Mudroňová D, Miltko R, Belzecki G, Lauková A: Oral administration of bacteriocin-production and non-producing strains of *Enterococcus faecium* in dog. Appl Microbiol Biotechnol 2019, 103:4953-4965.
13. European Food Safety Authority Guidance on the safety assesment of *Enterococcus faecium* in animal nutrition. EFSA J 2012a, 10:2682. https://doi.org/10.2903/j.efsa.2012.2682
14. Sauter SN, Benyacoub J, Allenspach K, Gaschen F, Ontsouka E, Reuteler G, Blum JW: Effects of probiotic bacteria in dogs with food responsive diarrhoea treated with an elimination diet. J Anim Physiol Anim Nutrition 2006, 90:269–277.
15. Madsen KL: Enhancement of Epithelial Barrier Function by Probiotics. J Epithelial Biol Pharmacol 2012, 5:55-59.
16. Cristoferi F, Dargenio VN, Dargenio C, Miniello VL, Barone M, Francavilla R: Anti-inflammatory and immunomodulatory effects of probiotics in gut inflammation: A door to the body. Front Immunol 2021, 12:578386.
17. Kim J, An JU, Kim W, Lee S, Cho S: Differences in the gut microbiota of dogs (*Canis lupus familiaris*) fed a natural diet or a commercial feed revealed by the Illumina MiSeq platform. Gut Pathog. 2017, 9:68.
18. Kim YS, Milner JA: Dietary modulation of colon cancer risk. J Nutr 2007, 137:2576–2579.
19. Smith CJ, Rocha ER, Paster BJ: The medically important *Bacteroides* spp. in health and disease. *Prokaryotes* 2006, 7:381–427.
20. Suchodolski JS, Markel ME, Garcia-Mazcorro JF, Unterer S, Heilmann RM, Dowd SE, Kachroo P, Ivanov I, Minamoto Y, Dillman EM, Steiner JM, Cook AK, Toresson L: The

fecal microbiome in dogs with acute diarrhea and idiopathic inflammatory bowel disease. Plos One 2012, 7:e51907.

21. Vlieg JEVH, Veiga P, Zhang C, Derrien M, Zhao L: Impact of microbial transformation of food on health - from fermented foods to fermentation in the gastro-intestinal tract. Curr Opin Biotechnol 2011, 22:211-219.
22. Handl S, Dowd SE, Garcia-Mazcorro JF, Steiner JM, Suchodolski JS: Massive parallel 16S rRNA gene pyrosequencing reveals highly diverse fecal bacterial and fungal communities in healthy dogs and cats. FEMS Microbiol Ecol 2011, 76:301–310.
23. Garcia-Mazcorro JF, Dowd SE, Poulsen J, Steiner JM, Suchodolski JS: Abundance and short-term temporal variability of fecal microbiota in healthy dogs. Microbiology Open 2012, 1:340–347.
24. Martineau B, Laflamme DP: Effect of diet on markers of intestinal health in dogs. Res Vet Sci 2002, 72:223-227.
25. Simpson JM, Martineau B, Jones WE, Ballam JM, MacKie RI, Characterization of fecal bacterial populations in canines: effects of age, breed and dietary fiber. Microb Ecol 2002, 44:186-197.
26. Storhaug CL, Fosse SK, Fadnes LT: Country, regional and global estimates for lactose malabsorption in adults: a systematic review and meta-analysis. Lancet Gastroenterol Hepatol 2017, 2:738-746. https://doi.org/10.1016/S2468-1253(17)30154-1
27. De Preter V, Raemen H, Cloetens L, Houben E, Rutgeerts P, Verbeke K: Effect of dietary intervention with different pre- and probiotics on intestinal bacterial enzyme activities. Eur J Clin Nutr 2008, 62:225-231.
28. Gan Y, Liang Q, Song X: Diagnostic value of alpha-L-fucosidase for hepatocellular carcinoma: a meta-analysis. Tumor Biol 2014, 35:3953-3960.
29. Terzungwe TM: Hematological parameters of dogs infected with canine parvovirus enteritis in sumy Ukraine. World J Innov Res 2018, 5:01-05.
30. Sharma R, Kumar A, Goel P, Kumar R: Clinical haemathology in canine haemorrhagic gastroenteritis. Haryana Vet 2008, 47:35-38.
31. Nami Y, Bakhshayesh RV, Jalaly HM, Lotfi H, Eslami S, Hejazi MA: Probiotic properties of *Enterococcus* isolated from artisanal dairy products. Front Microbiol 2019, 10:300.
32. Sivamaruthi BS, Kesika P, Chaiyasut C: A mini-review of human studies on cholesterol-lowering properties of probiotics. Sci Pharm 2019, 87:26.
33. Saeidi E, Yarahmadi HM, Fakhraei J, Mojahedi S: Effect of feeding fructooligosaccharides and *Enterococcus faecium* and their interaction on digestibility, blood, and immune parameters of adult horses. J Equine Vet Sci 2021, 99:103410.
34. Ooi LG, Liong MT: Cholesterol-lowering effects of probiotics and prebiotics: a review of *in vivo* and *in vitro* findings. Int J Mol Sci 2010, 11:2499-2522.
35. Strompfová V, Kubašová I., Lauková A: Health benefits observed after probiotic *Lactobacillus fermentum* CCM 7421 application in dogs. Appl Microbiol Biotechnol 2017, 101:6309-6319.

PRIMENA *ENTEROCOCCUS FAECIUM* DSM 32820 POREKLOM OD PASA KOD PASA SA AKUTNOM IDIOPATSKOM DIJAREJOM

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Modulacija intestinalne mikrobiote primenom probiotskih bakterija je jedan o mogućih načina poboljšavanja zdravlja gastrointestinalnog trakta pasa. Sve je bolje poznavanje efikasnosti bakterija mlečne kiseline u slučajevima prisustva dijareje kod pasa; međutim često se dešava da sojevi bakterija koji se komercijalno nalaze nisu poreklom iz pasa. U ovoj studiji upotrebljen je soj *E. faecium* DSM 32820 (soj bakterija koje su izolovane iz pasa u našoj laboratoji i koji je proveren u odnosu na bezbednost i probiotske kriterijume), koji je davan u hrani za devet pasa koji su imali simptome akutne nehemoragične dijareje, u dozi od $1x10^9$ CFU/ml, u trajanju od sedam dana. Uzorci fecesa i krvi su uzimani nultog i sedmog dana ogleda. Evaluacija CIBDAI (*Canine Intestinal Bowel Disease activity index*) skora, pokazala je značajno manju učestalost vomitusa, broja defekacija, konzistencije fecesa i gubitka telesne mase sedmog dana u poređenju sa nultim danom ogleda ($P<0,05$). Analiza 16SrRNK gena ukazala je na *Firmicutes* kao dominantan rod bakterija i to oba dana kada su obavljana uzorkovanja (72,0% nultog dana i 67,9% sedmog dana). Usledila je izolacija *Proteobacteria* (13,4% prema 6,0%), *Actinobacteria* (10,0% prema 13,5%), *Fusobacteria* (4,2% prema 2,3%) i *Bacterioides* (0,4% prema 10,4%). Značajo prisustvo *Erysipelotrichiacae* bilo je uočljivo u većoj meri sedog dana, u poređenju sa početnim vrednostima ($P<0,05$). Od ukupno 19 aktivnosti fekalnih enzima, bilo je promenjemo ($P<0,05$) pet (α-himotripsin, β-glukuronidaza, α-fukozidaza, β-galaktozidaza i N-acetil-glukosaminidaza). Posle primene DSM 32820 soja bakterije, srednja vrednost suve materija fecesa, značajno se povećala sedmog dana, u poređenju sa osnovnim vrednostima ($P<0,05$). Uprkos tome što se srednje vrednosti hematoloških i biohemijskih parametara nisu značano razlikovale, rezultati nekih pojedinačnih parametara kod nekoliko pasa su bili poboljšani.