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Research article

Study of the effects of new *Lactobacillus* strains on gut and oral microbiota in healthy dogs

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Abstract

Probiotics, particularly *Lactobacillus* spp., play a key role in supporting host health by modulating the gut and oral microbiota, enhancing immune responses, and maintaining microbial balance. While microbial diversity and composition are crucial for gastrointestinal and oral health, the impact of new *Lactobacillus* strains on healthy dogs has not been fully explored. This study evaluated the impact of new *Lactobacillus* strains on the gut and oral microbiota of healthy dogs. A sample of 35 adult dogs was divided into seven groups. Group 1 received a basal diet, while Groups 2–6 received individual *Lactobacillus* strain supplements: *L. plantarum* CM20-8 (TISTR 2676), *L. acidophilus* Im10 (TISTR 2734), *L. rhamnosus* L12-2 (TISTR 2716), *L. paracasei* KT-5 (TISTR 2688), and *L. fermentum* CM14-8 (TISTR 2720), respectively. Group 7 received a mixed probiotic supplement containing all five strains. Each dog received the respective *L.* strain at 10^9 CFU/day. Fecal and oral microbiota were analyzed using 16S rRNA sequencing. Based on the results, probiotic supplementation did not significantly change the overall microbial diversity. Dogs supplemented with *L. fermentum* CM14-8 (TISTR 2720) had a significantly (p=0.02) higher abundance (1.97%) of the beneficial genus *Faecalibacterium* in fecal samples than the control group (0.64%). In oral microbiota, compared to the control group, there were significant (p=0.03) reductions in the abundance of the Desulfobacterota in dogs receiving *L. acidophilus* Im10 (2.19%), *L. rhamnosus* L12-2 (2.32%), *L. paracasei* KT-5 (2.59%), *L. fermentum* CM14-8 (2.11%), and the mixed probiotic (2.69%). These findings highlighted the potential of specific probiotic strains, especially *L. fermentum* CM14-8, to modulate oral and gut microbiota in healthy dogs. Further studies are essential in clinical populations and functional assessments to define their preventive and therapeutic applications.

Keywords: Lactobacillus, Microbiota, Probiotics, 16S rRNA

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INTRODUCTION

Various microorganism communities inhabit the gastrointestinal tract and play vital roles in animal host physiology (Morelli and Capurso, 2012). The commensal gut microbiota ferment dietary fibers are converted into short-chain fatty acids (SCFAs), modulating the mucosal immune system and preventing pathogen colonization (Brestoff and Artis, 2013; Flint et al., 2012; Hooper et al., 2012; Jandhyala et al., 2015). Furthermore, probiotics can enhance these functions by promoting beneficial microbial populations and strengthening gut barrier integrity (Lee et al., 2022). Metagenomic studies of feline and canine fecal microbiota have classified over 98% of sequencing reads as bacterial species, including *Peptoclostridium*, *Lactobacillus*, and *Romboutsia* (producers of SCFAs), *Turicibacter* and *Enterococcus* (stimulation of the immune system), and *Bifidobacterium*, *Enterococcus*, and *Lactobacillus* (prevention of pathogen colonization) (Swanson et al., 2011; Moon et al., 2018; Suchodolski, 2022). Although gut microbiota composition varies among individuals, a stable microbiome is essential for maintaining host health (Gryaznova et al., 2022). Alterations in the gut microbiome can influence diseases affecting the digestive system, heart, kidneys, liver, and nervous system, through increased intestinal permeability, systemic inflammation, and immune dysregulation. Dysbiosis contributes to the development of various conditions by disrupting the balance of microbial metabolites, such as a reduction in SCFAs and an increase in pro-inflammatory compounds. These changes have been linked to the progression of diseases (Marchesi et al., 2016; Barko et al., 2018; Ciaravolo et al., 2021). The composition of, and interactions within, the microbiome play a crucial role in overall health. For example, gut microbiota influence oral health through mechanisms such as the gut-oral axis, where microbial metabolites or inflammatory signals from the gut affect the oral cavity (Mahasneh and Mahasneh, 2017; Yamazaki, 2023). In healthy individuals, the oral microbiome maintains equilibrium; however, dysbiosis in the oral microbiome disrupts this balance, leading to diseases, dental caries, and oral candidiasis (Kozak and Pawlik, 2023).

Probiotics are live microorganisms that when administered in adequate amounts, confer a health benefit to the host, helping to maintain a balance in intestinal microbiota, enhancing immune function, and supporting digestive health. Common probiotics include strains of *Lactobacillus* and *Bifidobacterium*, with formulations having recommended probiotic concentrations in the range 10^8–10^9 CFU per dose (Bertazzoni et al., 2013; Hill et al., 2014). Using probiotics in dogs offers several benefits through various mechanisms, including modulation of the immune system, reduction of stress, prevention of illnesses caused by intestinal pathogens, promotion of growth performance, and the maintenance of gut microbial balance (Sarowska et al., 2013; Hill et al., 2014). Probiotics enhance the intestinal immune function by stimulating B cells to produce IgA. Probiotic bacteria activate cytokine production, which activates T regulatory (Treg) cells to maintain immune homeostasis in the intestinal mucosa; Treg cells are effective suppressors and play a crucial role in regulating and controlling the immune response (Bertazzoni et al., 2013). Probiotics influence the gut-brain axis by producing neuroactive compounds, such as γ-aminobutyric acid (GABA), SCFAs, and serotonin, which have been linked to stress reduction and improved behavioral responses (Ma et al., 2021; Merkouris et al., 2024). The important characteristics of an ideal probiotic include the ability to withstand the acidic environment of the stomach and tolerate high bile acid concentrations in the gastrointestinal tract (de Melo Pereira et al., 2018). *Lactobacillus* spp. are more acid-tolerant probiotics being resistant to bile acid and able to promote the growth of beneficial bacteria while inhibiting the proliferation of pathogenic microbes and helping to maintain the equilibrium of gut microflora (Collins and Gibson, 1999; Panja et al., 2023). *Lactobacillus rhamnosus* MP01 and *Lactobacillus plantarum* MP02 (10^9 CFU/day) significantly elevated the levels of *Lactobacillus* and *Faecalibacterium* in fecal

samples from 1-month-old puppies (Fernández et al., 2019), while dogs that received 10^7–10^9 CFU/day of *L. fermentum* CCM 7421 had an enhanced lactic acid bacteria community, while the clostridia population was reduced in canine GI disorder (Strompfová et al., 2017). The abundance of *Lactobacilli* and *Enterococci* increased in healthy dogs after treatment with *L. fermentum* AD1 at a concentration of 3×10^9 CFU (Strompfová et al., 2006). *L. acidophilus* DSM13241 at more than 10^9 CFU/day increased the numbers of fecal *Lactobacilli* and decreased the level of *Clostridium* spp in healthy dogs (Baillon et al., 2004). *L. paracasei* strains (KBL382 and KBL385) had anti-colitis activities and improved host intestinal microbiota in mice (Kim et al., 2019). Another study reported that multi-strain probiotics were more effective than single-strain probiotics (Chapman et al., 2011). A probiotic mixture (*Streptococcus thermophilus*, *Bifidobacterium lactis*, *B. lactis*, *L. acidophilus*, *L. helveticus*, *L. paracasei*, *L. plantarum*, and *L. brevis*) reduced the abundance of *Clostridium perfringens* and increased the levels of *Bifidobacterium* and *Lactobacillus* in fecal-healthy dogs (Rossi et al., 2020). *Lactobacillus acidophilus* strain MJCD175 at concentrations of 10^8-10^9 per day reduced *Porphyromonas gingivalis* in oral-healthy dogs (You et al., 2022).

The physiology, feeding habits, and pathological characteristics of household pet dogs are generally the same as in humans, with the canine gut microbiome more closely resembling humans than pigs and mice, which has been attributed to the phylum-level distribution of genes in the dog gut, which closely aligns with the human gut microbiome. In addition, the dog gut gene pool has the highest overlap with the human microbiome; thus, dogs can be used effectively as an animal model (Coelho et al., 2018; Hernandez et al., 2022). The host's health is closely linked to the gut microbiota, which can be supported by the consumption of probiotics that provide numerous benefits, including improving digestive health, modulating the immune function, and supporting gastrointestinal health (Xu et al., 2019). Studies have been limited on reporting the effects of probiotics on the gut and oral microbiome in healthy dogs in Thailand, focusing primarily on specific strains such as *L. rhamnosus* (Chaiyasut et al., 2024). Although the oral microbiome of dogs and cats in Thailand has been characterized using next-generation sequencing, particularly in calculus-associated microbial communities (Radeerom et al., 2018), probiotic intervention studies remain underexplored. Therefore, the current research investigated a broader range of probiotic strains, including a multi-strain formulation, to better understand their influence on canine gut microbiota.

However, while several probiotic strains have been tested in dogs, reported research remains limited regarding the newly identified *Lactobacillus* strains and oral microbiome in dogs. Furthermore, the impacts remain largely unexplored of new *Lactobacillus* strains on the gut and oral microbiome in healthy dogs. Generally, these strains are recognized as safe for animal consumption (Panja et al., 2023). Therefore, the current study investigated the effects of these new *Lactobacillus* strains on the gut and oral microbiome in healthy dogs.

MATERIALS AND METHODS

Preparation of probiotic powders

The probiotics were supplied by the Biodiversity Research Centre at the Thailand Institute of Scientific and Technological Research in Pathum Thani, Thailand, consisting of *Lactobacillus plantarum* CM20-8 (TISTR 2676), *L. acidophilus* Im10 (TISTR 2734), *L. rhamnosus* L12-2 (TISTR 2716), *L. paracasei* KT-5 (TISTR 2688), and *L. fermentum* CM14-8 (TISTR 2720). The probiotics were administered at a final concentration of 10^9 CFU/dog/day by adding on top of the food. *Lactobacillus* strains in the mixed probiotic group were evenly proportioned, with a final concentration of 10^9 CFU/dog/day. These strains were able to endure gastric juice at pH 2 and bile acid at pH 8 for 180 minutes in the digestive tract (Charteris et al., 1998; Lee SangKi et al., 2017), and limited the growth of *Salmonella*

enterica serovar Typhimurium, *Escherichia coli*, *S. aureus*, and *Salmonella enterica* serovar Enteritidis. The probiotics were lyophilized and kept as a powder (Panja et al., 2023).

Experimental design and study animals

Animal selection and housing

The experimental canines were sourced from private owners and the study was conducted under the approval of the Institutional Animal Care and Use Committee (IACUC) of Kasetsart University, Bangkok, Thailand (ACKU64-VET-046). The study was conducted over a 28-day period between July and August 2021. A veterinarian performed health check-ups, vaccinations, and deworming procedures at least 1 month before the study on 35 healthy adult mixed-breed dogs (17 males and 18 females) aged 2–4 years. The dogs used in the study had mean (± standard error of mean) values for body condition score of 4.47 ± 0.28 on a nine-point scale and for body weight of 17.73 ± 0.76 kg. (Laflamme, 1997; Freeman et al., 2011). The dogs were not administered prebiotics, probiotics, antimicrobials, or any other medications for at least 90 days before the experimental trial. All the dogs were housed under the same controlled environmental conditions and fed a uniform commercial diet for 2 months before the intervention, which was maintained throughout the experiment. The animals exhibited no dental abnormalities, and no dental cleaning was carried out during the experimentation period. The dogs were housed at the canine experimental unit in Nakhon Nayok, Thailand, in cages measuring 2.0 × 2.0 × 3.0 m.

Group assignments

A completely randomized design was used to assign the 35 healthy adult dogs into seven groups, with five dogs per group. Group 1 was given a basal diet, Groups 2–6 received separate diets supplemented with one of *L. plantarum* CM20-8 (TISTR 2676), *L. acidophilus* Im10 (TISTR 2734), *L. rhamnosus* L12-2 (TISTR 2716), *L. paracasei* KT-5 (TISTR 2688), *L. fermentum* CM14-8 (TISTR 2720), respectively, and Group 7 received mixed probiotics (*L. plantarum, L. acidophilus, L. rhamnosus, L. paracasei,* and *L. fermentum*) at the rate of in 10^9 CFU/dog/day. Fecal and oral samples were collected on days 0 and 28 for the analysis of microbiota.

Diet and environmental conditions

The nutritional profile of the commercial extruded dry pet food (Ole Dog Beef flavor®; Greatest Pet Care Co., Ltd.; Bangkok, Thailand) consisted of crude protein (19.6%), crude fat (10.9%), crude fiber (6.7%), and crude ash (6.3%) on a fresh matter basis. The feed amount for each dog was calculated according to the energy requirements of adult dogs (Murphy and Parker, 2022). The dogs were kept in environmental conditions during the experiment of temperature (22–26°C) and humidity (45–60%) within the standard range for canine care, following the Guidelines for Standards of Care in Animal Shelters, to ensure a stable environment. In addition, regular socialization and exercise were provided to minimize stress and its potential impact on gut microbiota composition.

DNA extraction and purification

Each fecal sample was resuspended in phosphate-buffered saline (pH 8.0) at 1:10 w/v using a hand-held glass homogenizer for 5 minutes. A 1 mL sample of fecal slurry was placed in a 1.5 mL centrifuge tube and stored at –80 °C. Fecal samples were extracted using a bead beating unit (FastPrep-24 Benchtop Homogenizer; MP Biomedicals; Santa Anna, CA, USA) at a speed of 6.5 m/s for two cycles, each consisting of 1 minute of beating followed by resting for 5 minutes on ice, after which the supernatant was centrifuged at 12,000×*g* for 2 minutes and subsequently processed using the QIAamp® DNA Stool Mini Kit (Qiagen; Hilden, Germany) according to the manufacturer's protocols (Sathitkowitchai et al., 2021).

Oral samples were collected from the upper teeth and gums using a sterilized swab by performing 10 strokes over for 10–15 seconds, to ensure consistent microbial collection. Each swab was placed in a 15 mL conical tube containing 2 mL of sterile TE buffer (10 mM Tris HCl, 1 mM EDTA; pH 8.0) with one swab per tube. Then, samples were kept at below 4°C for a maximum of 24 hours before being processed using an I-genomic DNA Extraction Mini Kt (Intron;, Seoul, Republic of Korea) following the manufacturer's protocols (Oh et al., 2015).

The extracted DNA was assessed for quality and quantity using a Nanodrop Spectrophotometer (Thermo Fisher Scientific; Waltham, MA, USA). DNA samples were stored at -20 °C until further processing for polymerase chain reaction (PCR). For 16S rRNA gene sequencing, the V3-V4 hypervariable region of bacterial 16S rRNA genes was PCR-amplified using the specific primers Imina-V3-V4-F (5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3′) and Imina-V3-V4-R (5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3′). The thermal cycling protocol consisted of: initial denaturation (94°C, 2 minutes); 25 amplification cycles of denaturation (94°C, 20 seconds), primer annealing (57°C, 30 seconds); and elongation (72°C, 30 sec); concluding with terminal elongation (72°C, 10 minutes). PCR amplicons were purified using the NucleoSpin® Gel and PCR Clean-up system (Macherey-Nagel Inc.; Allentown, USA) following the manufacturer's instructions (Sathitkowitchai et al., 2021; Kingkaw et al., 2022).

Data analysis and processing of 16S rRNA gene sequences

Bioinformatics tools were used to process the raw sequencing data. High-quality sequences were obtained, with an average of 168,047 ± 14,569 reads per sample.

The Fastp package was used to trim and quality-check the pair-end reads (Chen et al., 2018) (read quality > 15 at 3'). Any consequential read that was smaller than 210 base was eliminated with its pair. FLASH was used to combine the remaining high-quality, paired-end readings into single reads (Magoč and Salzberg, 2011). Then, these single reads were converted into amplicon sequence variants (ASVs) using script package DADA2 v.1.6 (Bolyen et al., 2018) in the R software version 4.1.2. (R Core Team, 2010) with the default parameters. Taxonomy assignment was performed using the QIIME2's Naïve Bayes classifier v 2021.8 (Quast et al., 2012) and the SILVA 99% OTU database v. 138 (Oksanen et al., 2007) with a 70% cut-off. Any singleton ASV and ASVs that could not be recognized at the phylum level were removed from the analysis.

Statistical analysis

Before the differential analysis of relative abundances among the groups, the data were normalized using a centered log-ratio (CLR) transformation on each sample. Data normality was assessed using the Shapiro-Wilk test. Differences in relative abundances of bacterial taxa among the groups were evaluated using a two-way analysis of variance (ANOVA) followed by Duncan's multiple range test for post-hoc comparisons. The vegan (v. 2.5.6) package was used to calculate alpha diversity indices: observed species richness Chao1, Shannon, and Simpson indices (Oksanen et al., 2013). Distance metrics and ordination methods were applied using principal coordinate analysis (PCoA) based on weighted UniFrac distance metrics. Permutational multivariate ANOVA (PERMANOVA) was performed on the ordination results using the adonis function within the vegan package. All statistical analyses were performed using R software, with $p < 0.05$ considered statistically significant.

RESULTS

High-quality sequences were obtained (168,047 ± 14,569 reads per sample). The fecal samples from the 35 healthy mixed-breed dogs aged 2-4 years with body condition scores of 4–5 were analyzed to characterize the bacterial microbiota composition. Predominant bacterial phyla in the fecal microbiome overall datasets were identified as the Firmicutes, Actinobacteria, Proteobacteria, Bacteroidetes, Fusobacteria, and Acidobacteria. The most abundant bacterial families were the Erysipelotrichaceae, Peptostreptococcaceae, Lactobacillaceae, Clostridiaceae, Lachnospiraceae, and Bifidobacteriaceae (Table 1), with the dominant genera being *Turicibacter*, *Peptoclostridium*, *Lactobacillus*, *Romboutsia*, *Allobaculum*, *Clostridium*, *Bifidobacterium*, *Faecalibacterium*, *Enterococcus*, and *Fusobacterium.* There were no significant differences in the bacterial communities among the groups at the phylum or family levels (Table1). The relative abundance of the *Faecalibacterium* genus differed significantly among groups. *L. fermentum* CM14-8 (Group 6) had a significantly greater abundance of *Faecalibacterium* than the control sample (Group 1) and the *L. paracasei* KT-5 sample (Group 5; $p = 0.02$). Despite daily supplementation of *Lactobacillus*, its relative abundance in the gut did not increase significantly.

Table 1 Relative abundance of gut microbiota

Parameters	Groups[1]							SEM	P-value Group
	1	2	3	4	5	6	7		
Phylum									
Firmicutes	87.49	87.40	85.40	89.25	85.75	89.08	89.65	0.795	0.13
Actinobacteria	6.40	5.73	6.98	3.95	7.30	4.64	5.08	0.476	0.39
Proteobacteria	2.26	2.26	2.36	2.71	2.11	2.06	2.33	0.182	0.09
Bacteroidetes	2.26	2.26	2.66	2.36	2.71	2.11	2.06	0.317	0.08
Fusobacteria	1.44	0.95	1.03	1.42	0.96	0.96	0.98	0.123	0.88
Acidobacteria	0.37	0.33	0.37	0.37	0.33	0.33	0.25	0.059	0.50
Family									
Erysipelotrichaceae	33.75	31.91	30.84	32.67	29.64	32.43	27.05	1.107	0.77
Peptostreptococcaceae	22.01	24.12	22.31	24.72	22.79	19.95	21.74	0.772	0.45
Lactobacillaceae	8.48	9.59	8.98	8.05	8.07	12.19	14.27	0.907	0.31
Clostridiaceae	7.04	7.07	6.54	8.52	8.33	8.87	10.36	0.552	0.61
Lachnospiraceae	8.20	7.26	8.31	7.39	7.22	7.55	7.43	0.423	0.91
Bifidobacteriaceae	3.72	3.20	3.07	1.37	1.98	2.63	1.64	0.352	0.30
Genus									
Turicibacter	15.17	14.37	14.39	16.16	14.64	12.64	14.78	0.675	0.93
Peptoclostridium	14.66	15.89	14.56	15.48	12.82	13.48	13.70	0.636	0.72
Lactobacillus	9.63	11.08	10.08	9.09	8.96	14.13	15.91	1.388	0.75
Romboutsia	9.04	10.62	9.38	11.04	11.58	8.33	9.32	0.381	0.27
Allobaculum	12.00	10.17	11.22	10.06	9.70	11.36	6.29	1.013	0.70
Clostridium	7.25	7.34	6.82	8.72	8.76	8.25	8.12	0.406	0.59
Bifidobacterium	4.27	3.90	3.49	1.59	2.23	3.17	1.90	0.416	0.27
Faecalibacterium	0.64^{a}	0.99^{ab}	1.10^{ab}	1.48^{ab}	0.66^{a}	1.97^{b}	0.97^{ab}	0.155	0.02
Enterococcus	0.39	0.31	0.37	0.81	0.66	0.39	0.63	0.791	0.47
Fusobacterium	1.57	1.01	1.03	1.40	1.05	1.03	0.80	0.133	0.83

[1]Group 1=basal diet (control group), Group 2= *Lactobacillus plantarum* CM20-8 (TISTR 2676), Group 3= *Lactobacillus acidophilus* Im10 (TISTR 2734), Group 4= *Lactobacillus rhamnosus* L12-2 (TISTR 2716), Group 5=*Lactobacillus paracasei* KT-5 (TISTR 2688), Group 6= *Lactobacillus fermentum* CM14-8 (TISTR 2720), Group 7= mixed probiotics. Data are expressed as mean ± standard error of the mean (SEM). Different superscripts between groups represent statistically significant differences ($p < 0.05$). Units of measurement are provided in the corresponding table columns.

The effects of probiotic administration on the oral microbiome in dogs were assessed. At the phylum level, the Proteobacteria, Bacteroidetes, Firmicutes, Fusobacteria, Actinobacteria, and Desulfobacterota had the highest relative abundance levels. The most abundant bacterial families were the Porphyromonadaceae, Pasteurellaceae, Fusobacteriaceae, Moraxellaceae, Comamonadaceae, and Neisseriaceae, while the dominant genera were *Porphyromonas*, *Moraxella*, *Fusobacterium*, *Frederiksenia*, *Bergeyella*, *Conchiformibius, Actinomyces, Staphylococcus, Desulfomicrobium*, and *Prevotella* (Table 2). The probiotic-treated samples (Groups 3–7) had significantly reduced relative abundance of the phylum Desulfobacterota compared to the control (Group 1; p = 0.03). *L. acidophilus* Im10 (TISTR 2734) in Group 3, *L. rhamnosus* L12-2 (TISTR 2716) in Group 4, and *L. fermentum* CM14-8 (TISTR 2720) in Group 6 had significantly reduced relative abundance of the phylum Desulfobacterota compared to *L. plantarum* CM20-8 (TISTR 2676) in Group 2 (p=0.03). No significant differences were detected among the groups at the family level. At the genus level, the relative abundance of *Actinomyces* differed significantly among groups, with the mixed probiotics Group 7 having significantly lower abundance than the *L. paracasei* KT-5 (TISTR 2688) in Group 5 (p = 0.04).

Table 2 Relative abundance of oral microbiota

Parameters	Groups[1]							SEM	P-value Group
	1	2	3	4	5	6	7		
Phylum									
Proteobacteria	29.10	33.16	31.52	34.00	29.52	34.05	33.30	0.991	0.70
Bacteroidetes	28.81	27.61	27.23	30.91	30.42	29.56	31.38	0.718	0.67
Firmicutes	20.49	21.00	19.82	18.52	20.19	18.96	18.18	0.574	0.44
Fusobacteria	7.44	5.62	10.56	5.37	6.07	7.43	6.49	0.522	0.06
Actinobacteria	4.13	4.70	3.85	4.18	5.04	3.96	4.14	0.176	0.55
Desulfobacterota	3.97^{c}	3.74^{bc}	2.19^{a}	2.32^{a}	2.59^{ab}	2.11^{a}	2.69^{ab}	0.162	0.03
Family									
Porphyromonadaceae	17.30	14.70	16.34	15.41	16.37	15.69	17.84	0.660	0.88
Pasteurellaceae	5.29	8.95	10.10	8.07	6.15	8.02	6.07	0.668	0.40
Fusobacteriaceae	6.64	4.95	9.55	4.72	5.24	6.54	5.64	0.505	0.05
Moraxellaceae	8.22	7.76	5.67	7.94	7.19	8.16	9.57	0.565	0.07
Comamonadaceae	4.21	4.21	3.97	4.59	4.15	5.43	4.04	0.207	0.43
Neisseriaceae	4.01	4.83	4.94	4.85	4.12	4.39	5.01	0.301	0.25
Genus									
Porphyromonas	19.67	16.91	18.40	18.41	18.67	18.42	20.40	0.726	0.91
Moraxella	7.65	8.68	5.99	9.23	7.87	9.42	10.18	0.618	0.12
Fusobacterium	7.39	5.68	10.71	5.61	5.95	7.68	6.34	0.554	0.05
Frederiksenia	3.27	2.80	7.86	5.17	4.43	5.04	4.30	0.550	0.34
Bergeyella	2.99	2.67	3.65	6.09	3.44	4.39	3.55	0.367	0.13
Conchiformibius	2.67	3.51	3.73	3.70	2.55	3.32	3.62	0.300	0.13
Actinomyces	2.04^{ab}	2.29^{ab}	2.01^{ab}	1.89^{ab}	2.50^{b}	1.94^{ab}	1.62^{a}	0.095	0.04
Staphylococcus	0.17	0.36	0.17	0.23	0.40	0.29	0.46	0.053	0.54
Desulfomicrobium	0.26	0.16	0.17	0.20	0.18	0.14	0.13	0.026	0.49
Prevotella	0.03	0.03	0.01	0.02	0.03	0.07	0.03	0.008	0.30

[1]Group 1=*basal diet (control group),* Group 2= *Lactobacillus plantarum CM20-8 (TISTR 2676),* Group 3= *Lactobacillus acidophilus Im10 (TISTR 2734),* Group 4= *Lactobacillus rhamnosus L12-2 (TISTR 2716),* Group 5=*Lactobacillus paracasei KT-5 (TISTR 2688),* Group 6= *Lactobacillus fermentum CM14-8 (TISTR 2720),* Group 7= mixed probiotics. Data are expressed as mean ± standard error of the mean (SEM). Different superscripts between groups represent statistically significant differences ($p < 0.05$). Units of measurement are provided in the corresponding table columns.

The alpha diversity values for the fecal and oral microbiota are presented in Table 3, as assessed using the Chao1, Simpson, and Shannon indices. There were no significant differences among the groups, based on two-way ANOVA. Based on the results, it seemed that probiotic supplementation did not substantially alter the overall microbial richness or evenness in the fecal or oral microbiota under the conditions of this study. Consistent values for the Chao1, Simpson, and Shannon indices across the groups indicated a stable and diverse microbial community structure. Beta diversity was evaluated using the weighted UniFrac metric, with statistical comparisons performed using PERMANOVA. The PCoA revealed a clear separation of samples based on the experimental time point (day 0 versus day 28), while there was no evident clustering among treatment groups (Figure 1). The PERMANOVA results confirmed that the experimental time point significantly influenced the microbial community composition ($p < 0.05$), whereas no significant differences were detected among groups or group-by-time interactions for both fecal and oral microbiota (Tables 4 and 5). These findings suggested that the microbial profiles naturally shifted over time and probiotic supplementation did not substantially disrupt the overall structure of the microbiota.

Table 3 Alpha diversity using Chao1, Simpson and Shannon indices for fecal and oral microbiota in dogs.

Alpha diversity	Groups[1]							SEM	P-value
	1	2	3	4	5	6	7		Group
Chao1									
Fecal	873	868	918	894	885	857	874	30.78	0.99
Oral	1045	1033	1065	1033	1093	1054	1045	22.35	0.93
Simpson									
Fecal	0.05	0.05	0.05	0.05	0.05	0.05	0.06	0.02	0.51
Oral	0.03	0.02	0.03	0.02	0.03	0.03	0.03	0.01	0.49
Shannon									
Fecal	3.96	3.93	4.05	3.97	4.00	3.94	3.85	0.032	0.76
Oral	4.64	4.82	4.80	4.87	4.81	4.71	4.78	0.03	0.31

[1]Group 1= basal diet (control group), Group 2= *Lactobacillus plantarum* CM20-8 (TISTR 2676), Group 3= *Lactobacillus acidophilus* Im10 (TISTR 2734), Group 4= *Lactobacillus rhamnosus* L12-2 (TISTR 2716), Group 5= *Lactobacillus paracasei* KT-5 (TISTR 2688), Group 6= *Lactobacillus fermentum* CM14-8 (TISTR 2720), Group 7= mixed probiotics Data are expressed as mean ± standard error of the mean (SEM). Different superscripts between groups represent statistically significant differences ($p < 0.05$). Units of measurement are provided in the corresponding table columns.

Tables 4 Permutational multivariate analysis of variance of fecal microbiota

Factor	Df	Sums of squares	Mean squares	F.Model	R^2	p- value
Group	6	0.54	0.09	0.69	0.04	0.88
Time	1	6.34	6.34	49.11	0.44	<0.05
Group: time	6	0.50	0.08	0.64	0.03	0.92
Residuals	55	7.10	0.13		0.49	
Total	68	14.48			1.00	

Tables 5 Permutational multivariate analysis of variance of oral microbiota

Factor	Df	Sums of squares	Mean squares	F.Model	R^2	p-value
Group	6	0.42	0.07	0.62	0.05	1
Time	1	0.83	0.83	7.43	0.10	<0.05
Group: time	6	0.47	0.08	0.70	0.05	0.98
Residuals	56	6.27	0.11		0.78	
Total	69	8			1.00	

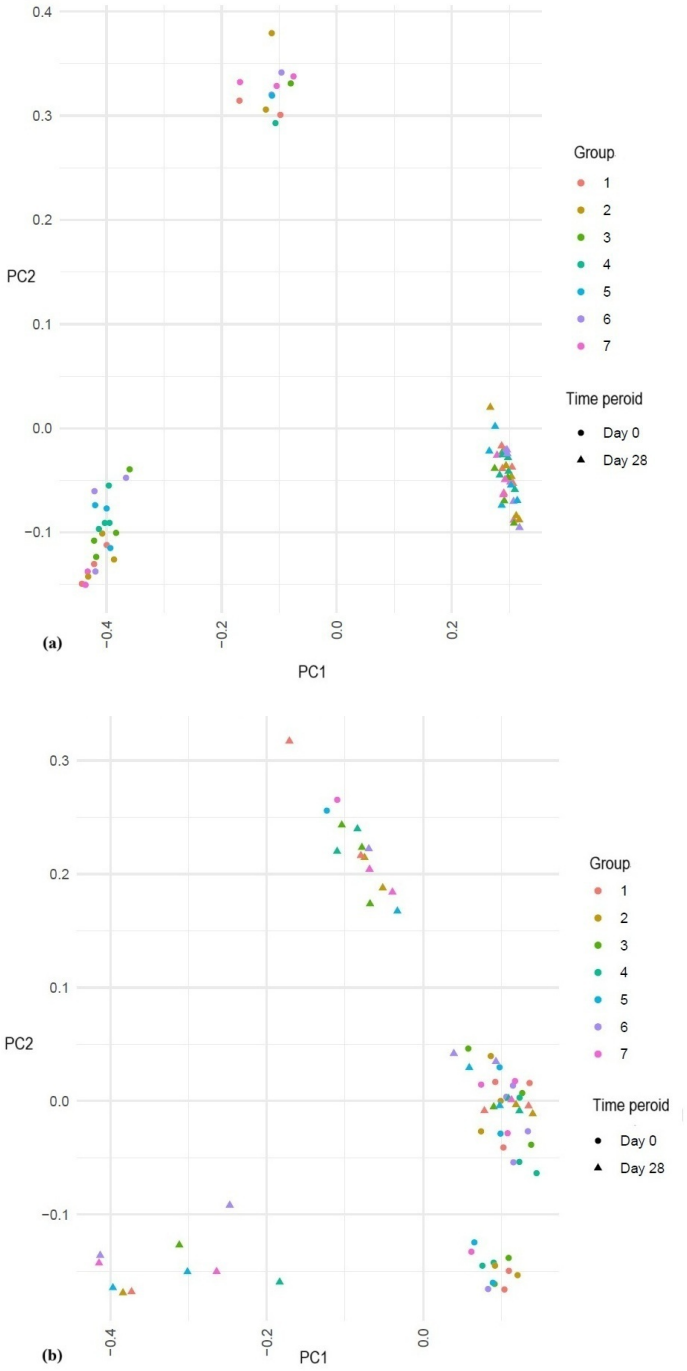

Figure 1 (a) Principal coordinate analysis (PCoA) using the weighted UniFrac metrics of the canine fecal microbiota among the seven treatments **(b)** Principal coordinate analysis (PCoA) using the weighted UniFrac metrics of the canine oral microbiota among the seven treatments, Group 1 =basal diet (control group), Group 2 = *Lactobacillus plantarum* CM20-8 (TISTR 2676), Group 3 = *Lactobacillus acidophilus* Im10 (TISTR 2734), Group 4= *Lactobacillus rhamnosus* L12-2 (TISTR 2716), Group 5=*Lactobacillus paracasei* KT-5 (TISTR 2688), Group 6= *Lactobacillus fermentum* CM14-8 (TISTR 2720), Group 7 = mixed probiotics. PC 1 and PC 2 represent the first and second principal coordinates. The axes are dimensionless and reflect differences in microbial profiles among samples. Each point corresponds to a single sample, and the distance between points indicates the degree of compositional dissimilarity.

DISCUSSION

The major bacterial phyla identified in the fecal samples were the Firmicutes, Actinobacteria, Proteobacteria, Bacteroidetes, and Fusobacteria. This composition concurred with other canine microbiome studies (Suchodolski et al., 2008; Handl et al., 2011; Hand et al., 2013; Lee et al., 2022). However, in the current study, the Actinobacteria had a relatively higher abundance than what has been reported in most other studies. The Firmicutes and Bacteroidetes contributed primarily to fiber fermentation and SCFA production (Suchodolski, 2011). The Actinobacteria had immunomodulatory functions (Noronha et al., 2024), while the Proteobacteria contributed to homeostasis of the anaerobic environment of the gastrointestinal tract and, hence, the stability of the strictly anaerobic microbiota (Moon et al., 2018). The Fusobacteria contributed to protein metabolism (Robinson et al., 2025).

The composition of the gut microbiota in healthy dogs is influenced by multiple factors including diet, stress, and geographic location (You and Kim, 2021). In Thailand, the Firmicutes and Actinobacteria are the most abundant phyla in healthy dogs (Areerat et al., 2023) and the current findings corroborated this trend. This similarity may be attributed to comparable climatic conditions, dietary habits, and environmental factors across regions in Thailand, which are known to influence the composition of the gut microbiota (Hasan and Yang, 2019; You and Kim, 2021)

The most prevalent bacterial families in the fecal microbiome were the Erysipelotrichaceae, Peptostreptococcaceae, Lactobacillaceae, Clostridiaceae, and Lachnospiraceae, as also reported by Gaspardo et al. (2020). These microorganisms play vital roles in metabolic functions. The Lactobacillaceae and Lachnospiraceae contribute to complex carbohydrate metabolic and butyrate production (Fusco et al., 2023), while the Clostridiaceae and Erysipelotrichaceae have dual functionalities, aiding protein catabolism under healthy conditions but potentially producing toxins during dysbiosis (Bermingham et al., 2017; Hong et al., 2023). The Peptostreptococcaceae may provide protection against diet-induced obesity through enhanced bile acid metabolism and signaling (Zhang et al., 2023).

No significant differences were detected at the phylum and family levels among the probiotic-treated groups. Other studies also reported that probiotics administration did not substantially affect the dominant bacterial phyla (Garcia-Mazcorro et al., 2011; Ciaravolo et al., 2021). There was a significant increase in the abundance of the genus *Faecalibacterium* in Group 6 dogs receiving *L. fermentum* CM14-8 (TISTR 2720), compared to the control (Group 1) and Group 5 (which received *L. paracasei* KT-5 (TISTR 2688)). This observation may hypothetically be related to the ability of *L. fermentum* CM14-8 (TISTR 2720) to tolerate harsh gastrointestinal conditions and adhere to intestinal epithelial cells. These characteristics could potentially enhance its persistence and probiotic effects in the host. However, the mechanism was not directly assessed in the present study and should be further investigated in the future. Additionally, It is possible that the increased abundance of *Faecalibacterium* in Group 6 resulted from the ability of *L. fermentum* CM14-8 (TISTR 2720) to suppress pathogenic bacteria and influence gut pH, which may contribute to a more favorable microenvironment for *Faecalibacterium* colonization and proliferation. However, these effects were not directly investigated in the present study, representing a limitation. This result supported the notion that probiotic effects are strain-specific or localized at the genus level, while potential confounding factors, such as individual variation among dogs, influenced these observed differences.

Faecalibacterium spp. serve as a critical marker of gut health; they are beneficial as they produce butyrate, which is known for its anti-inflammatory properties and its role in maintaining intestinal homeostasis, (Zheng et al., 2025). An increase in the abundance of this genus has been linked to improved gut barrier function and modulation of local immune responses (Quévrain et al., 2016). In dogs,

Faecalibacterium spp. are considered important indicators of gastrointestinal health due to their capacity to produce butyrate, an SCFA essential for colonocyte energy supply and maintenance of gut barrier integrity (Herstad et al., 2018). These findings aligned with other research that showed *L. rhamnosus* MP01 and *L. plantarum* MP02 increased *Faecalibacterium* counts in canine fecal samples (Fernández et al., 2019). Similarly, a study involving *L. rhamnosus*, *L. reuteri*, and *L. plantarum* reported enhancements in beneficial gut bacteria, including *Faecalibacterium*, along with increased concentrations of health-promoting metabolites, such as lactate, and concurrent reductions in potentially pathogenic genera (Asensio-Grau et al., 2023). The current study did not directly measure SCFA levels; however, other published research has demonstrated that increased *Faecalibacterium* abundance was associated with enhanced SCFA production, potentially contributing to the reduced incidence of gastroenteritis observed in probiotic-treated groups (Fernández et al., 2019). The increase in butyrate-producing bacteria, such as *Faecalibacterium*, was influenced by the metabolic activity of *Lactobacillus*, particularly its lactic acid production which likely promoted the growth of gut-associated anaerobic bacteria and boosted butyrate synthesis (Guard et al., 2015). Consistent trends in animal models have shown that *L. rhamnosus* GG supplementation led to increased butyrate levels in mice (Lin et al., 2020), *L. reuteri* ZLR003 improved lactic acid and butyric acid levels in piglets (Zhang et al., 2019) and *L. fermentum* CCM 7421 increased lactic, butyric, and succinic acids in dogs (Strompfová et al., 2017). *Faecalibacterium* abundance is markedly lower in dogs with acute diarrhea, colon tumors, and chronic inflammatory enteropathies than in healthy dogs, underscoring its role as a biomarker for gut health and gastrointestinal balance (Herstad et al., 2018; Fernández et al., 2019). The current study analyzed both oral and gut microbiota; notably, *Faecalibacterium* increased in the gut, while the Desulfobacterota decreased in the oral cavity following *L. fermentum* CM14-8 (TISTR 2720) supplementation. Although the underlying mechanisms were not directly investigated, it is possible that the increase in *Faecalibacterium*, a genus known to secrete anti-inflammatory peptides, contributed to systemic immune modulation (Huang et al., 2020), which may have influenced indirectly the oral microbial community via the gut-oral axis (Mahasneh and Mahasneh, 2017; Yamazaki, 2023). However, further mechanistic studies are needed to clarify this potential cross-effect between gut and oral microbiota (Xiang et al., 2024). Based on the current results, *L. fermentum* CM14-8 (TISTR 2720) might play a role in promoting gut health in dogs, potentially contributing to disease resistance.

The main phyla identified in the oral microbiota were the Proteobacteria, Bacteroidetes, Firmicutes, Fusobacteria, Actinobacteria, and Desulfobacterota, consistent with other findings (Flancman et al., 2018; Bell et al., 2020). The abundance of the phylum Desulfobacterota was less in the experimental groups receiving *L. acidophilus* lm10 (TISTR 2734), *L. rhamnosus* L12-2 (TISTR 2716), *L. paracasei* KT-5 (TISTR 2688), *L. fermentum* CM14-8 (TISTR 2720), and the mix of probiotics group than in the control group. Periodontitis, one of the most common disorders affecting the oral cavity, is associated with dysbiosis a microbial imbalance characterized by the replacement of beneficial bacteria with pathogenic species. This shift promotes biofilm formation in periodontal pockets, facilitating disease progression (Kushkevych et al., 2020). The Desulfobacterota belong to a group of sulfate-reducing bacteria (SRB; Song et al., 2021), which, although normally present in the oral microbiota, become pathogenic when their abundance increases under anaerobic conditions. SRB contribute to disease by producing hydrogen sulfide (H_2S), a cytotoxic and pro-inflammatory compound that damages host tissues and exacerbates inflammation (Costinar et al., 2010; Kushkevych et al., 2020). Compared to the healthy controls, increased abundance has been reported of the SRB genera *Desulfovibrio* and *Desulfobacter* in humans (Kushkevych et al., 2020) and in canines with periodontal disease (Costinar et al., 2010).

In the current study, the reduced levels of the Desulfobacterota in the experimental groups supplemented with *L. acidophilus* Im10 (TISTR 2734), *L. rhamnosus* L12-2 (TISTR 2716), *L. paracasei* KT-5 (TISTR 2688), *L. fermentum* CM14-8 (TISTR 2720), and the mixed probiotics group were observed in oral samples. This reduction may be explained by mechanisms similar to the reported in previous studies, which suggest that probiotics can exert indirect effects such as competitive exclusion, modulation of oral pH, or enhancement of host immunity (Melara et al., 2022; Beattie, 2024). However, these proposed mechanisms remain speculative, as the current study did not assess relevant parameters such as pH, immune markers, or biofilm formation. The reduced levels of the Desulfobacterota highlights the potential of these probiotics as candidates for future clinical studies on periodontitis. There were no significant differences among the groups at the family level. At the genus level, there was a significant reduction in the abundance of *Actinomyces* in the mixed probiotics group compared to the group receiving *L. paracasei* KT-5. The reduction in the abundance of *Actinomyces* in the mixed probiotics group was not significantly different from the control, suggesting that the combination of probiotics exerted a greater modulatory effect on the oral microbiota than a single-strain intervention. *Actinomyces* species, while considered part of the commensal oral microbiota, are also recognized as early colonizers in dental plaque formation and have been associated with the initiation of periodontal disease (Vielkind et al., 2015). Therefore, any reduction in the *Actinomyces* may indicate a shift toward a less plaque-promoting microbial environment, particularly in the context of early periodontitis or gingival inflammation. This could imply that although the mixed probiotics suppressed *Actinomyces* more effectively than *L. paracasei* KT-5 alone, the overall level of suppression did not exceed normal baseline levels in healthy conditions. Several studies have shown that probiotics modify the gut microbiota and exert beneficial effects. For example, *L. fermentum* CCM 7421, administered at a dose of 10^7–10^9 CFU/day, increased the population of lactic acid bacteria while reducing clostridia in dogs with gastrointestinal disorders (Strompfová et al., 2017). Lactic acid bacteria contribute to gut health by inhibiting pathogenic microorganisms and influencing immune regulation (Liu et al., 2024). In another study, *L. fermentum* AD1 (3×10^9 CFU) increased the abundances of lactobacilli and enterococci in healthy dogs (Strompfová et al., 2006). In contrast to the current results, which showed no significant difference between multistrain and non-probiotic supplementation, the study by Piyadeatsoontorn et al. (2018) demonstrated enhanced efficacy of multistrain probiotics in improving gut microbiota and growth performance in weaned pigs. This variation from the current findings may reflect differences in probiotic composition, host species, or environmental factors.

Alpha diversity, reflecting species richness and evenness within the microbial community and beta diversity, indicating ecological differences between communities, are widely used to assess microbiome health in dogs (Hullar et al., 2018). In the current study, the alpha diversity values of both fecal and oral microbiota were evaluated using the Chao1, Simpson and Shannon indices. There were no significant differences among the seven groups, suggesting that probiotic supplementation did not substantially affect species richness or evenness. The consistent Chao1, Simpson, and Shannon values across the groups implied that the microbial community structure remained relatively stable following the probiotic intervention. In addition, the beta diversity, assessed based on the weighted UniFrac distances and visualized using PCoA, indicated no distinct clustering among the groups on day 28. The PERMANOVA analysis detected significant shifts over time ($p < 0.05$), which were associated with natural temporal variations rather than treatment effects. No significant differences were detected in group or group-by-time interactions, indicating that the probiotics tested did not change the overall microbiota composition. The absence of significant diversity shifts may reflect the stability of the resident microbiota in healthy dogs (Bell et al., 2020), which may resist colonization or large-scale disruption by introduced probiotics. These

findings implied that probiotic supplementation in healthy individuals may negligibly alter the microbiota by modulating key taxa without causing dysbiosis or major ecological shifts. These findings aligned with other studies reporting no change in microbial diversity after probiotic administration (Bell et al., 2020; Ciaravolo et al., 2021). In the current study, the broad microbial diversity metrics remained stable, with the probiotics not exerting any localized or functional changes based on the alpha and beta diversity measurements. Future studies incorporating metagenomics or metabolomics functional profiling may help to reveal more subtle probiotic effects beyond compositional diversity.

In the current study, there were no significant changes in alpha and beta diversity following probiotic supplementation, though several probiotic strains influenced the relative abundance of specific beneficial and potentially pathogenic taxa. These findings concurred with other research examining multi-strain probiotics. For example, the combination of *L. casei*, *L. plantarum* P-8, and *Bifidobacterium animalis* administered to dogs with recurrent diarrhea significantly reduced the incidence of diarrhea and elevated the abundance of beneficial bacteria such as *L. johnsonii*, *L. reuteri*, *L. acidophilus*, and *B. pullicaecorum* (Xu, Zhao, et al., 2019). Similarly, a multi-strain probiotic combined with standard therapy increased the mucosal bacterial population, including *Lactobacillus* spp., in dogs with inflammatory bowel disease (Huang et al., 2020). The current study evaluated healthy dogs rather than clinical cases but parallels can be drawn. For example, probiotic administration, particularly with mixed formulations, reduced harmful taxa such as the Desulfobacterota in the oral microbiota and supported beneficial genera such as *Faecalibacterium* in the gut. Differences in outcomes among studies could be attributed to host health status (healthy versus diseased), the probiotic strains used, administration duration, dosage, and host-specific variables such as breed, age, and geographic location (Bell et al., 2020). Collectively, the current findings suggested that multi-strain probiotic formulations hold promise for promoting gut and oral health through modulation of key microbial taxa, even in the absence of notable shifts in overall microbial diversity.

CONCLUSIONS

This study demonstrated that supplementation with new *L.* strains, particularly *L. fermentum* CM14-8 (TISTR 2720), promoted beneficial shifts in the gut microbiota of healthy dogs which significantly increased the level of *Faecalibacterium* spp. In the oral microbiome, there were reductions in the relative abundance of the Desulfobacterota following treatment with several *L.* strains, especially *L. acidophilus* Im10, *L. rhamnosus* L12-2, *L. fermentum* CM14-8, and the mixed-strain formulation. *L. fermentum* CM14-8 may confer selective health benefits by enhancing protective taxa in the gut microbiota and suppressing potential pathogens in the oral microbiota. These findings emphasize the clinical potential of specific probiotic strains, particularly *L. fermentum* CM14-8, as candidates for targeted modulation of the gut and oral microbiota in dogs. Future studies should include SCFA quantification, inflammatory cytokines, immune biomarkers, and longitudinal trials in dogs with gastrointestinal or periodontal disorders to use for preventive and therapeutic clinical applications.

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AUTHOR'S CONTRIBUTIONS

Attawit Kovitvadhi: Conceptualization (lead); review and editing (equal). **Kamonporn Panja:** Conceptualization (equal); writing – original draft (lead); formal analysis (lead); writing – review and editing (lead).
Sathita Areerat, Pipatpong Chungdang, Pornsucha Palaseweenun, Nattaphong Akrimajirachoote, Suthkamol Suttikul, Nantana Bumrungchue, Pennapa Chonpathompikunlert, Kanidta Niwasabutra: Methodology (equal); review and editing (equal).
Supparin Mahasawasde: writing – review and editing (equal).

CONFLICT OF INTEREST

The authors declare no conflict or competing of interest

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