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Article

The Canine Gut Health: Impact of a New Feed Supplement on Microbiota Composition

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Simple Summary: The community of microorganisms and their genes in the gastrointestinal (GI) tract, collectively known as the gut microbiome, play multiple roles in canine health. Disruption, due to the lack of beneficial organisms or the overgrowth of pathogenic organisms, of the delicate balance of the microbes in the canine GI microbiome can lead to dire consequences including inflammatory bowel disease and diarrhea. Here, we provide evidence that a novel dietary supplement containing antioxidants can promote the growth of beneficial bacteria, including *Bifidobacterium* and *Lactobacillus*, that are known to be anti-inflammatory, while not disrupting the overall gut microbiome composition of healthy adult dogs when compared to a controlled group that experienced shifts in their gut microbiome after same period of time.

Abstract: This study aimed to determine the impact of a novel formulation of a supplement composed of the natural ingredients: bromelain, quercetin, and *Lentinula edodes*, on the gut microbiota of healthy adult dogs. Adult healthy female dogs were administered either a placebo (CTR, n = 15) or the supplement (TRT, n=15) over 28 days. Stool samples were collected for 16S rRNA sequencing before supplement administration (T0), at completion of supplement administration (T28), and one week after the end of supplement administration (T35) to characterize changes in the gut microbial communities. QIIME was used to determine both alpha- and beta-diversity and ANCOM-BC was used to identify differences in taxonomic abundances before and after supplementation. We found a significant decrease in overall diversity in CTR group, but no significant differences in overall diversity in the TRT group over time. Furthermore, we found differences in the abundance of several taxa in both CTR and TRT groups but differences in abundance of beneficial bacteria were more pronounced in TRT group. Specifically, we found increases in the abundance of sequences belonging to the genera *Bifidobacterium*, *Lactobacillus*, and *Pediococcus* at T28 in the TRT group with significant increases in *Bifidobacterium* and *Lactobacillus* persisting at T35 when compared to T0. Importantly, members of these genera are known to be pro anti-inflammatory and associated with a healthy gut microbiota in dogs. The results of our study show the potential of our supplement to selectively enhance specific beneficial bacterial taxa, offering a targeted approach to modulating the gut microbiome without causing disruptions to the overall equilibrium.

Keywords: gut microbiota; dogs; bromelain; quercetin; *Lentinula edodes*; healthy

1. Introduction

Research on the gut microbiota has attained attention in recent years largely because of its relationship to gastrointestinal (GI) and extra gastrointestinal (extra-GI) diseases. The gut microbiota refers to the group of microorganisms such as bacteria, viruses, archaea, protozoa, and fungi that coexist symbiotically in the host GI tract (Hou et al. 2022; de Vos et al. 2022; Pilla and Suchodolski 2019). They perform various functions in several important processes including energy metabolism, immunomodulation, digestion, fermentation, and protection against pathogens. Non-digestible compounds in food, such as plant-derived polysaccharides, and nutrients that escape from small intestines digestion can also be degraded by these microorganisms. Moreover, the gut microbiota also plays an important role in the homeostasis of the host by modulating the function of several vital systems, such as the cardiovascular, renal, and immune systems[1–3].

From an immunological perspective, specific microorganisms can be recognized as pathogenic by the host immune system. However, the majority of gut microorganisms are non-pathogenic and, instead, co-exist with enterocytes in an interdependent relationship[4]. A balanced and well-functioning microbiota can be both pro-inflammatory and anti-inflammatory to maintain a balance that avoids over-inflammation while responding quickly to infections[5]. The types of microorganisms that make up the microbiota vary from site to site and species to species[6,7].

In animals, the health status of the gut microbiome is connected to a variety of diseases. For example, dysbiosis, or an imbalance in the microbiome, has been associated with a number of inflammatory diseases such as inflammatory bowel disease (IBD) and leaky gut syndrome in dogs. Previous research reported that dogs living in breeding conditions are more vulnerable than companions to chronic stress, which is linked to more gut disorder occurrences and increased dysbiosis. Intestinal dysbiosis is frequently associated with a decrease in microbial diversity in dogs with IBD[8]. Diarrhea, colitis, and constipation are other diseases that affect millions of humans and dogs around the world and changes in the gut microbiota play a significant role in the onset and progression of these disorders[1,2]. Similarly, an imbalanced gut microbiota of dogs has also been linked to metabolic complications in the gut and many GI disorders, in which a classical treatment would involve the use of antimicrobial drugs and antibiotics[2,3]. However, it should be noted that antibiotic treatment increases the risk of developing antimicrobial resistance (AMR) and poses threats on gut microbiota such as reduction of species diversity, alteration of metabolic activity, and consequent antibiotic-resistant organisms, which in turn can lead to antibiotic-associated diarrhea and recurrent infections[3,9]. Thus, there is a growing need for treatments that do not rely on antimicrobials, such as the use of natural agents, which do not pose a risk of AMR[10,11].

Nutraceuticals are a wide range of natural substances with health benefits and fewer side effects. They provide health benefits to the body and aid in the prevention of diseases[12]. A large variety of nutraceutical groups have antioxidant activity, making them useful agents in a variety of disorders[13] which include oxidative stress, a major component in several gastrointestinal disorders[14]. Among these nutraceuticals are bromelain (B), *Lentinula edodes* (LE), and quercetin (Q).

Bromelain (B), is a complex mixture of enzymes and bioactive compounds naturally occurring in various parts of pineapple, including its stems, fruit, and roots [15]. Among its constituents, proteinases and proteases are the key enzymes responsible for protein breakdown within the body. Notably, bromelain can easily be absorbed in the body without interfering with protein digestion in the gastrointestinal tract. In addition to its antibacterial and anti-inflammatory properties, bromelain has demonstrated efficacy in managing various gastrointestinal ailments such as dyspepsia [16].

Quercetin (Q), is a significant polyphenolic flavonoid, present in a wide range of fruits and vegetables, including apples, onions, grapes, berries, and capers [17]. Human studies has indicated that quercetin, in addition to being a potent antioxidant, has valuable anti-inflammatory qualities [13]. Additionally, it has been shown to improve intestinal dysbiosis (HFD) in mice fed a high-fat diet by attenuating lipoprotein-dependent activation of the lipoprotein-dependent pathway (TLR-4) [18].

Lastly, mushrooms contain a wide variety of bioactive substances including polysaccharides, dietary fibers, minerals, and proteins[19,20]. *Lentinula edodes* (LE), in particular, is a mushroom used in supplement preparations and has been shown to possess anti-inflammatory, antioxidant, and anti-

diabetic properties in both humans and animal models[21–23]. In addition to their biological effects, there has also been work focusing on regulating gut microbiota through the use of mushrooms' polysaccharides[24,25].

Here, we sought to evaluate the activity of a formulated antioxidant-rich supplement composed of the natural ingredients B, Q, and LE on the gut microbiota of healthy adult dogs and determine if this new formulation impacts microbiota stability in the gut when compared to a placebo.

2. Materials and Methods

2.1. Animals and study design

In a double blinded randomized controlled trial, a total of 30 adult female American Staffordshire Terrier dogs, weighing 17 ± 1.5 kg and 5 ± 1 years old were selected from Ente Nazionale Cinofilia Italiana (ENCI/www.enci.it), a registered dog breeder in Italy. Animals participating in this study were the same as those reported[14]. The study was performed in strict compliance with the guidelines of the Ministry of Health for the use of animals in research. Before signing a formal informed consent, the overall objectives of the study were disclosed to the breeder. Both study protocols and the use of supplements were approved by the Ethical Animal Care and Use Committee of the University of Turin on 19 July 2022 under protocol number 2741.

The health status of all animals was evaluated by a veterinarian through a general physical examination followed by a copromicroscopic analysis of their feces. All animals were found to be healthy with no underlying conditions that required treatment. The 30 dogs were randomly allocated to two groups: a control (CTR, $n = 15$) and treatment (TRT, $n = 15$) group. Dogs were housed in cages (3 dogs per cage), with an area of 6 ± 2 square meters. To ensure animal welfare following the principles of animal welfare legislation (Regolamento regionale 11 novembre 1993, Italy), dogs were provided with an open space of the same size as their box thus avoiding public stress due to collective manipulation. Animals in both groups received a commercially available diet ("Royal Canin, Medium Adult Dry Dog Food") (Supplemental Table 1) from 7 days prior to the commencement of the study to allow the GI system to adapt to the new diet. The daily calories intake of each group was calculated per FEDIAF Nutritional Guidelines (FEDIAF, 2021) using the following equation such that "ME" is metabolizable energy and "BW" is body weight:

$$\text{ME (kcal/day)} = 110 \text{ kg BW}^{0.75} \quad (1)$$

In both the CTR and TRT groups, each participant was given either a placebo of maltodextrin powder, or a formulated feed containing a mixture of nutraceutical ingredients in their food once a day for 28 days. Dosage: 1 g / 10 Kg of BW (Atuahene et al. 2023).

2.2. Formulated Feed Composition

The formulated feed consisted of a mixture of nutraceuticals including 1.35% each of bromelain (B) and quercetin (Q), and 1.00% Lentinula edodes mushrooms (LE). Exact compositions of the formulated feed were previously reported[14] and can be found in Supplementary Table 2.

2.3. Safety and Nutritional Parameters

An experienced veterinarian measured the body weights (BW) at T0 (baseline) and then at T28 (28 days after the beginning of the feed supplement administration) for all animals. A Body Condition Score (BCS), using a scale of 1 to 9, was determined through a physical (visual) examination and palpation at T0 and T28 by the same experienced veterinarian. Additionally, the fecal pH was determined using a calibrated pH instrument at T0 and T28 (HI 9125 pH/ORP meter; Hanna Instruments, Bedfordshire, UK). The data for these parameters have been reported previously[14].

2.4. Fecal Sample Collection

Fresh fecal samples were collected in a sterile plastic bag using a sterile spatula and temporarily held at 4 °C freezer until moving into a -80°C freezer until sequencing, as previously described[14]. To minimize bias, a non-biased blinded procedure was employed for the identification of all samples. Microbiome analysis was subsequently conducted on fecal samples collected at T0, T28, and T35.

2.5. Fecal DNA extraction, PCR Amplification and Amplicon Target Sequencing

Microbial DNA was extracted from a total of 150 mg of fecal samples using a DNA Miniprep kit (Zymo Research; Irvine, CA, USA) following the manufacturer's protocols, including a bead beating step. Total DNA was quantified using a Qubit™ 3 Fluorometer (Thermo Scientific; Waltham, MA, USA), and subjected to a PCR step where the V3-V4 regions of the 16S rRNA gene were amplified using indexed primers[26] and DNA was purified using a Nextera DNA Library Prep Kit (Illumina; San Diego, CA, USA), according to the manufacturer's instruction. The generated amplicons were sequenced on an Illumina MiSeq (San Diego, CA, USA) using a 2 x 250 bp paired-end v2 sequencing kit.

2.6. Computation and Sequence Analysis

The resulting raw sequences (FASTQ) were processed using the same standard operation procedure as previously described[27]. Briefly, sequences were filtered and processed using DADA2 in the Quantitative Insights Into Microbial Ecology 2 (QIIME 2) program[28]. Chimeric sequences were identified and subsequently removed from the dataset and the remaining sequences were then grouped into Amplicon Sequence Variants (ASVs) and assigned taxonomy using the Greengenes database (version 13.8). The ASV abundance tables, the taxonomy tables and associated phylogenetic trees were imported into R v4.3.2 [29] and used to build a phyloseq object[30].

2.7. Taxonomic Statistical Analysis

The phyloseq object, containing the ASV abundances, was used to calculate alpha- and beta-diversity along with differential abundance analyses of taxa. For alpha-diversity, samples were rarefied down to the read depth of the sample with the lowest read depth and Shannon's diversity, the observed species and the inverse Simpson metrics calculated. Beta-diversity was conducted using Principal coordinate analysis (PCoA) using the Bray-Curtis dissimilarity matrix in the vegan package[31]. The Bray-Curtis dissimilarity matrix was further used in a PERMANOVA analysis with the adonis function of the vegan package[32] with 1,000 permutations. For diversity metrics, the threshold for significance was set at $p < 0.05$.

To examine if individual ASVs changed over time, differential abundance analysis was conducted using the ancombc2 function in the ANCOM-BC package[33] in R. P-values were adjusted using the false discovery rate procedure and ASVs with a corrected p-values < 0.1 were considered significant.

3. Results

A total of 5,346,677 reads were generated across the 90 samples. After filtering and sequence clean-up an average read count per sample of $60,075 \pm 4122$ were retained, which clustered into 3,038 distinct amplicon sequence variants (ASVs) that classified into 15 phyla, 103 families and 153 genera. To ensure comprehensive alpha and beta diversity analyses, samples were rarefied based on the sample with the lowest read count, which was 51,499 reads. The rarefaction curves, depicted in Supplementary Figure 1, indicated that this read depth was sufficient to capture most of the diversity within the samples.

At the phylum level, the taxonomic distribution displayed remarkable consistency across all samples. The Firmicutes emerged as the predominant phylum, followed by the Actinobacteriota and Fusobacteriota, as illustrated in Figure 1. This uniformity underscores the substantive presence and importance of these phyla within the studied microbiome.

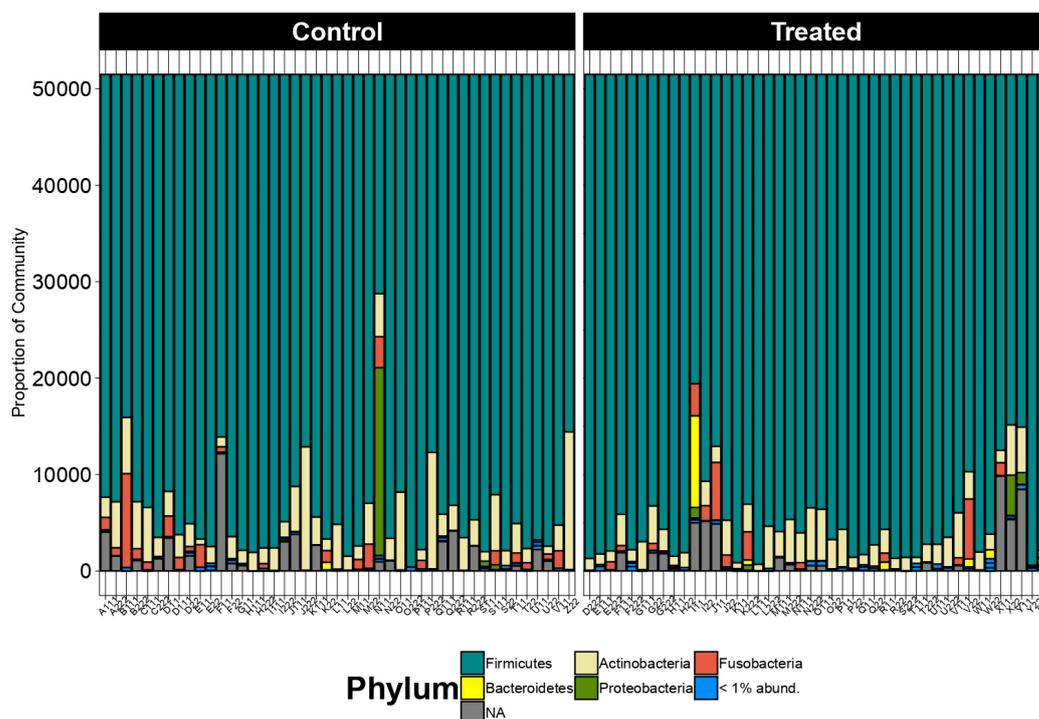


Figure 1. Taxonomic distribution for all dogs across samples from control and treatment groups by phyla.

The impact of the supplement on microbiome diversity was rigorously assessed. Comparative analysis across treatments at T0, T28, and T35 revealed no discernible differences in alpha diversity metrics between the treatment groups (Supplementary Figure 2). Moreover, as shown in Figure 2, within the TRT group, assessment of diversity alterations over time exhibited no significant changes in alpha diversity.

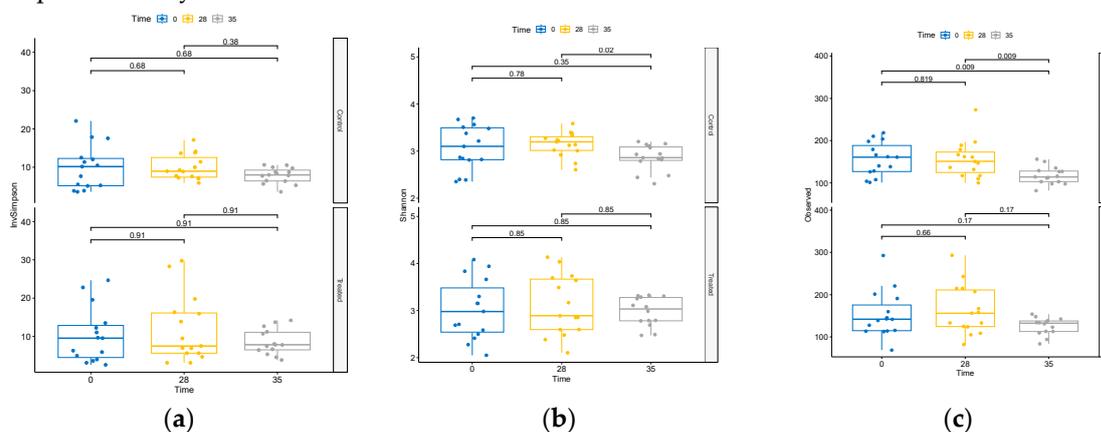


Figure 2. Changes in alpha diversity as measured by a) inverse Simpson index, b) Shannon diversity index and c) observed ASVs at T0, T28, and T35 in treated and control groups.

Contrarily, within the CTR group, we observed a decline in alpha diversity at day 35 (Figure 2). This decline was evident in both the total observed ASVs and the Shannon's index, a widely used metric that accounts for species richness and evenness in data evaluation, highlighting a substantial reduction in diversity within this group at that specific time point.

Our analysis of beta diversity showed a shift in the overall gut bacterial communities over time point (Figure 3). This was confirmed using an adonis test, which showed that the observed separation is statistically associated with time point differences. The identified changes were evident in both the TRT and CTR groups, signifying shifts in the bacterial communities within these cohorts. Moreover,

a modest statistically significant dissimilarity was noted when comparing the bacterial compositions of dogs between the TRT and CTR groups (p-value = 0.02), as shown in Supplementary Figure 3.

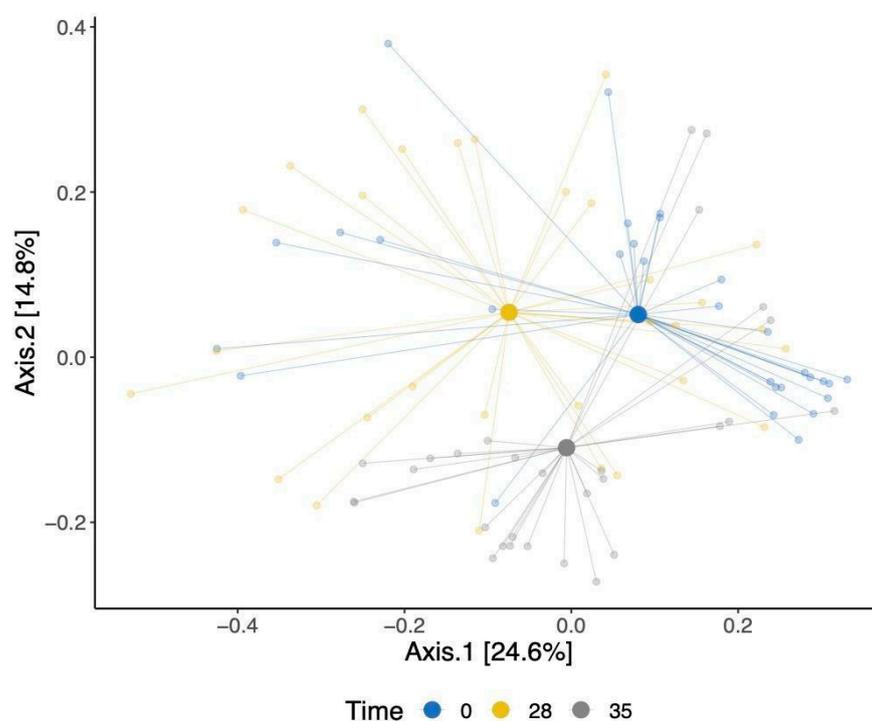


Figure 3. A principal coordinate analysis of Bray-Curtis's dissimilarity matrix for β diversity for all dogs colored by timepoints. .

We then identified ASVs that were determined to be differentially abundant in the TRT group between T0 and T28. Several ASVs were found to be differentially abundant in this group over time (Table 1) including increased abundance of ASVs belonging to the Bifidobacterium ($\beta = 0.065$, SD = 0.014, corrected p-value < 0.001), Lactobacillaceae (unclassified at genus level) ($\beta = 0.104$, SD = 0.022, corrected p-value < 0.001) and Peptostreptococcaceae (unclassified at genus level) ($\beta = 0.05$, SD = 0.015, corrected p-value = 0.019). There was also a decrease in ASVs belonging to the Fusobacteria (unclassified at species level) ($\beta = -0.070$, SD = 0.023, corrected p-value = 0.049), Peptococcus ($\beta = -0.059$, SD = 0.010, corrected p-value = 0.007) and Dorea ($\beta = -0.053$, SD = 0.015, corrected p-value = 0.019).

Table 1. Differentially abundant ASVs by time within treated dogs as determined by ANCOM-BC (T0 vs T28).

Taxonomic classification	Higher Abundance Timepoint	beta coefficient	Standard deviation	W statistic	p-value	Corrected p-value	ASV code
Fusobacteria_unclassified	T0	-0.047	0.016	-2.972	0.003	0.050	ASV334
Fusobacteria_unclassified	T0	-0.070	0.023	-3.025	0.002	0.049	ASV029
<i>Bifidobacterium</i> sp	T28	0.065	0.014	4.698	0.000	0.000	ASV073
<i>Bifidobacterium animalis</i>	T28	0.089	0.023	3.925	0.000	0.005	ASV117
<i>Bifidobacterium breve</i>	T28	0.070	0.023	2.993	0.003	0.050	ASV007
Lactobacillaceae_unclassified	T28	0.104	0.022	4.697	0.000	0.000	ASV256
Lactobacillaceae_unclassified	T28	0.063	0.017	3.827	0.000	0.006	ASV585
Lactobacillaceae_unclassified	T28	0.111	0.025	4.489	0.000	0.001	ASV098
<i>Lactobacillaceae hamsteri</i>	T28	0.098	0.027	3.568	0.000	0.012	ASV440
<i>Anaerorhabdus furcosa</i>	T28	0.055	0.016	3.318	0.001	0.022	ASV269
Bacillaceae_unclassified	T28	0.047	0.016	2.847	0.004	0.070	ASV706
<i>Lactobacillus hamsteri</i>	T28	0.064	0.023	2.715	0.007	0.099	ASV408
<i>Peptococcus</i> sp	T0	-0.059	0.016	-3.590	0.000	0.012	ASV519
Peptostreptococcaceae_unclassified	T28	0.050	0.015	3.387	0.001	0.019	ASV003
Peptostreptococcaceae_unclassified	T28	0.046	0.015	3.140	0.002	0.037	ASV006
<i>Dorea longicatena</i>	T0	-0.053	0.015	-3.418	0.001	0.019	ASV262

We also observed ASVs that were differentially abundant in the placebo group between T0 and T28 (Supplemental Table S2). These included increased abundances of ASVs belonging to the *Bifidobacterium* ($\beta = 0.019$, $SD = 0.007$, corrected p-value = 0.081) and *Lactobacillaceae* (unclassified at genus level) ($\beta = 0.084$, $SD = 0.027$, corrected p-value = 0.058). There were also decreases in the abundances of ASVs belonging to the *Fusobacteria* (unclassified at species level) ($\beta = -0.058$, $SD = 0.021$, corrected p-value = 0.081). Although there were changes in both the TRT and CTR groups, differences in the CTR group were less pronounced than changes in the TRT group as observed by beta coefficients.

We then conducted a pairwise analysis of differences in relative abundance of the three enriched genera within our TRT group between time points using pairwise t-test (Figure 4). We found a significant increase in *Lactobacillus* (p-value = 0.00064) and *Bifidobacterium* (p-value = 0.043) from T0 to T28. We also observed significant increases in the abundances of the *Lactobacillus* (p-value = 0.00096) and *Bifidobacterium* (p-value = 0.002) from T0 to T35.

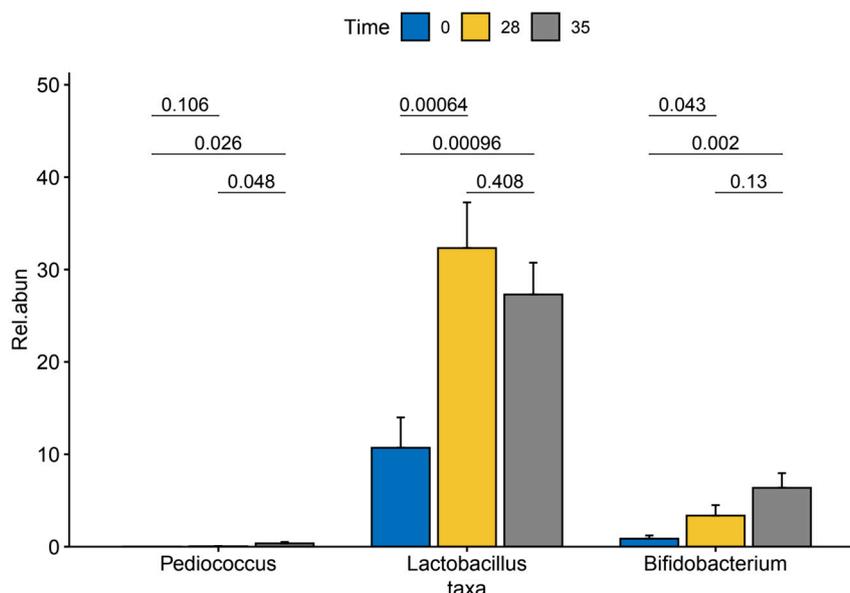


Figure 4. Relative abundance of *Pediococcus*, *Lactobacillus* and *Bifidobacterium* at time points T0, T28 and T35 in treated group.

4. Discussion

This study provides evidence that the use of a novel supplement that includes a blend of nutraceuticals in the form of B, Q and LE results in a shift in the gut microbiome of healthy dogs after four weeks of treatment. Although we did not observe changes in alpha diversity after the supplementation in the TRT group as measured by inverse Simpson's Index, Shannon's Diversity Index or observed species, we found shifts in individual taxa over time. It has previously been shown that supplementation of this blend of nutraceuticals improves stress and inflammatory outcomes and also increases the presence of short-chain fatty acids (SCFAs) in this cohort of dogs[14]. We postulate that the shift in the gut microbiome has a hand in modulating improvement in these biomarkers. While there were decreases in the alpha diversity over time, specifically in observed ASVs and Shannon Diversity in the control group these differences were likely due to the natural fluctuations of the gut microbiome.

ASVs belonging to the family *Bifidobacteriaceae* and *Lactobacillaceae* were increased after supplementation administration in the TRT group. Members of the *Bifidobacteria* and *Lactobacillaceae* include commensal bacteria that produces several SCFAs including propionate, acetate and formate as a product of carbohydrate fermentation[34]. While *Bifidobacteria* tends to have a lower abundance in dogs, when compared to humans, it provides metabolites that promote anti-inflammation in dogs, such propionate, which decreases the production of cytokines that promote inflammation including IL-6 and IL-8[35]. We also observed decreases in ASVs belonging to *Peptococcus* and *Dorea longicatena*, both of which are SCFA-producers and known to be residents in the guts of healthy canines under the state of eubiosis[36] (Suchodolski et al. 2013). We postulate that this reduction in these groups of bacteria may be a result of the increase of the other SCFA-producers including *Lactobacillaceae*. Panasevich et al. showed, in a cohort of healthy adult Beagles, that after administration of nonviable *Lactobacillus acidophilus* plus prebiotics the dogs had a significant increase in total SCFA, significant increase in abundance of bacteria belonging to *Lactobacillaceae* and a decrease in *Dorea* when compared to dogs on a control diet [37].

Additionally, the ASVs belonging to *Dorea longicatena* may have a role in levels of cortisol, the hormone that responds to stress, in the dogs. Associations between cortisol and gut microbial abundance in dogs have been already explored[2,38]. Briefly, in a randomized study of 20 5-month-old beagles examining the safeness of softening dog food with water, dogs that consumed softened dog food had higher levels of cortisol and higher levels of pathogenic bacteria including

Enterococcus[38]. In our prior study, we found that cortisol decreased in the dogs 28 days after supplementation administration[14]. Moreover, work in our group has previously shown that a 35-day *Saccharomyces boulardii* supplementation resulted in a decrease in cortisol levels and a decrease in ASVs belonging to the genus *Dorea* [2] as in the current study.

We also observed a decrease in ASVs belonging to the *Bacteroides* in the TRT group. *Bacteroides* are among the gut microbes that can metabolize tryptophan into various metabolites including indole and the indole-related skatole[39,40]. Indole is involved in intra-bacterial communication and has been linked to the reduction in motility[41], cytotoxicity[42] and invasiveness [43] of pathogenic bacteria. Despite these benefits, excess indole can be detrimental to the host. In studies involving rodent models, germ-free rodents that were colonized with indole-producing *E. coli* had significantly higher emotional responses to mildly stressful events in study involving mice[44] and increased anxiety-like behavior in rats[45] when compared to rodents that were colonized with non-indole-producing *E. coli*. This provides a possible link between the decreased *Bacteroides* and our previous observation of decreased cortisol and the decreased indole in this cohort of dogs[14].

Our work here shows that the dogs in the TRT group had an increase in abundance of ASVs belonging to the *Lactobacillaceae* family. Members of *Lactobacillaceae* family have been inversely associated with calprotectin, a protein that is released by neutrophils which are white blood cells. The presence of calprotectin in feces is indicative of gastrointestinal inflammation[46]. In our prior study, calprotectin was significantly reduced over the course of supplementation administration in dogs[14]. Similarly, a double-blind study of 22 dogs, given either a Lactic-acid bacteria (LAB) supplement containing *Limosilactobacillus fermentum*, *Lactiacaseibacillus rhamnosus*, and *Lactiplantibacillus plantarum* or placebo over 7 days, showed that all dogs given the LAB supplement had negligible levels of calprotectin while five dogs given placebo had increased calprotectin after supplementation[47].

Our study used a double blinded randomized controlled design, an optimal study design which guaranteed high quality of the evidence. However, this study is limited by the use of 16S rRNA sequencing which is able to examine the structural profiles of the gut microbial communities and not the functional aspects of the gut microbiome which would be attainable if we conducted whole-genome metagenomics, metatranscriptomics or meta-proteomics. However, we note that we have previously reported on fecal measures in our prior study[14], including the sum of all SCFA that are bacterial-derived. This information provides valuable insights into the functional roles of certain members of the gut microbiome. An additional limitation of this study is that we observed changes in features of the microbiome over time in the placebo group, namely decreases in observed species after administration of the placebo supplement in addition to changes in individual ASV abundances over the course of placebo supplementation. Despite this, the differences observed in ASVs abundances were more pronounced in the TRT group providing some evidence that the supplement might be contributing to the shift in the microbiome. We also found that there is an overall difference in the microbial communities between the two groups (Supplemental Figure 3) suggesting a nuanced but noteworthy divergence between the microbiota of these distinct experimental cohorts.

5. Conclusions

Our study demonstrates that a supplement containing Q, B, and LE alters the gut microbiota of adult dogs after a 4-week treatment. Although promoting the growth of various microbes, the supplement does not disturb the overall gut microbiome diversity. This suggests its potential as a microbial modulator without compromising the overall balance, making it a candidate for investigating its role alongside or in support of antibiotics.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org. Figure S1: Rarefaction curves for all samples for treated and control groups; Figure S2: Alpha diversity analysis of treated vs control groups at T0, T28 and T35; Figure S3: A principal coordinate analysis using Bray-Curtis dissimilarity matrix for β diversity colored by treated and control groups; Table S1: Composition of nutraceutical and placebo; Table S2: Differentially abundant ASVs by time within control group as determined by ANCOM-BC

Author Contributions: Conceptualization, G.M. and D.A.; methodology, D.A., G.M., G.S. and B.S.; software, B.S., I.Z.C. and D.A.; validation, G.M., E.M., B.S. and G.S.; formal analysis, I.Z.C., B.S. and D.A.; investigation, D.A., G.M. and B.S.; resources, G.M. and B.S.; data curation, D.A.; writing—original draft preparation, D.A.; writing—review and editing, E.M., G.S., I.Z.C., D.A., B.S., F.B., and G.M.; visualization, D.A., I.Z.C. and G.S.; supervision, G.M. and G.S.; project administration, E.M. and G.M. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: Regulations for the care and use of animals set forth by the Italian Ministry of Health were followed during this study's execution (D.L. n. 26, 2014), and Regulation (EC) N. 767/2009 was used to regulate the use of supplements. This study was approved by the Ethical Animal Care and Use Committee of the University of Turin (19 July 2022; protocol n. 2741).

Informed Consent Statement: A written informed consent form was signed by the dog breeder after being apprised of this study's procedures.

Data Availability Statement: Data available upon request to the first author.

Conflicts of Interest: The authors declare no conflict of interest.

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