Title
Gut microbes in healthy dogs have individualized responses to synbiotic supplementation that lead to increased abundances of probiotic strains: A randomized controlled trial

Authors
Jirayu Tanprasertsuk, PhD
Aashish R. Jha, PhD
Justin Shmalberg, DVM, DACVN, DACVSMR
LeeAnn M. Perry, MS
Heather Maughan, PhD
Ryan W. Honaker, PhD

Affiliation
1 NomNomNow Inc, Oakland, CA 9460, USA
2 Department of Biology, New York University Abu Dhabi, Abu Dhabi, UAE
3 Department of Comparative, Diagnostic, and Population Medicine, College of Veterinary Medicine, University of Florida, Gainesville, FL 32611, USA
4 Ronin Institute, Montclair, NJ 07043, USA

Corresponding author
Ryan W. Honaker, PhD
ryan.honaker@nomnomnow.com
Director of Microbiology
NomNomNow Inc, Oakland, CA 9460, USA
Abstract

Background: Probiotics ameliorate gastrointestinal symptoms in dogs. However, the effect of probiotics in a healthy population, as well as factors contributing individualized responses, remains largely unknown. This trial examined gut microbiota (GM) and health outcomes in household dogs after synbiotic (SN) supplementation containing probiotics and inulin. Healthy dogs were randomized to receive SN (50 mg/d inulin and 20 billion total CFU/d of *L. reuteri, P. acidilactici, E. faecium, L. acidophilus, B. animalis, L. fermentum, L. rhamnosus*) or placebo (PL) for 4 weeks. Owners completed a health survey and collected stool samples for GM profiling (metagenomic sequencing) at baseline and week 4 in both groups, and at week 6 in the SN group.

Results: A significant shift (p<0.001) in β-diversity was observed in the SN (n=24), but not PL group (n=19), relative to baseline at week 4. Forty-five bacterial species, 43 (96%) of which were Lactobacillales, showed an increase in the abundances (≥2 fold change, adjusted p<0.05) at week 4. *E. coli* also decreased at week 4 in the SN group (2.8 folds, adjusted p<0.01). The altered taxa largely returned to baseline at week 6. The degree of changes in the β-diversity was associated with GM at baseline. Specifically, dogs with higher Proteobacteria and lower Lactobacillales responded more robustly to supplementation. Dogs fed SN tended to have lower diarrhea incidence (0% vs 16%, p=0.08).

Conclusions: SN supplement had an impact on the change of gut microbiota in healthy household dogs as characterized with metagenomic sequencing. Findings warrant further investigation with longer duration or in populations at risk of gastrointestinal diseases. The magnitude of response to the supplement was associated with microbial profile at baseline. To our knowledge, this is the first study documenting such association that may provide a basis of personalized nutrition in companion dogs.

Key words: diarrhea, dogs, gastrointestinal health, gut microbiome, metagenomic sequencing, probiotics, synbiotics
List of abbreviations

| Abbreviation | Description                                      |
|--------------|--------------------------------------------------|
| BCS          | body condition score                             |
| FDR          | false discovery rate                             |
| GI           | gastrointestinal                                  |
| GM           | gut microbiome                                   |
| HR           | high-responder                                   |
| KEGG         | Kyoto Encyclopedia of Genes and Genomes          |
| KO           | KEGG Orthology                                   |
| LR           | low-responder                                    |
| MR           | mid-responder                                    |
| PCoA         | principal coordinate analysis                    |
| PERMANOVA    | permutational multivariate analysis of variance  |
| PL           | placebo                                          |
| SN           | synbiotic                                        |
1. Background

Probiotics are live bacterial cells that are ingested, frequently with the goal of maintaining or improving gastrointestinal health. Probiotics can reduce diarrhea, improve symptoms of irritable bowel disease, modulate the immune system, and inhibit pathogen colonization, among other benefits [1]. The underlying mechanisms of probiotic function rely on interactions between members of the gut microbiota (GM) and host cells. For example, incoming probiotic bacterial cells have been demonstrated to prevent colonization of pathogens by producing toxins, stimulating the immune system, or competitively excluding pathogens from the niche [1,2].

Probiotics and their health benefits have also been demonstrated in dogs [3]. Dogs and humans suffer from some of the same gastrointestinal disorders, and compositions of their GMs are similar [4–6]. Probiotics have improved symptoms in dogs suffering from diarrhea, gastroenteritis, allergy, or other ailments [7–10], and reduced abundances of some pathogens, such as C. difficile [10], despite some conflicting data showing no effects on diarrheal symptoms [11,12]. Additionally, an improvement toward ideal body weight with probiotic supplementation has also been observed in underweight dogs [13,14]. While clinical or biological outcomes were improved in these trials, changes in microbial composition were measured in only a limited number of studies [14–18]. Most of these included dogs with varied severity of diarrhea with a primary aim to improve diarrhea symptoms [14,15,18].

The efficacy and function of probiotics in a generally healthy canine population remain largely unknown. The gastrointestinal tract of healthy and diseased dogs likely provides different environments for bacterial growth and colonization [19]. Therefore, the investigation of probiotic supplementation in promoting and maintaining health throughout the life span is warranted. Of particular importance is whether probiotics can be used for prophylaxis in healthy dogs to prevent diarrhea and other gastrointestinal problems [20], and whether some healthy dogs may benefit more from supplementation than others based on baseline characteristics and microbiome composition. Moreover, the successful intestinal colonization of probiotic mixture has been reported in only certain breeds of healthy dogs [13,16,17]. Therefore, there is a need to expand the investigation to include a representative population of household dogs consisting of a heterogeneity of breeds and a range of ages.

Additionally, inulin is a common prebiotic found in dog food and supplements. Prebiotics are oligo- or poly-saccharides that stimulate the growth of beneficial bacteria in the gut. In order to investigate the combined effect of probiotics and prebiotics on the GM profile in healthy dogs, a synbiotic (SN) supplement (a combination of probiotics and prebiotics) was formulated based on reviewing the existing probiotic literature to include bacterial species with demonstrated effects on various health outcomes, including gastrointestinal (GI) health [8,10,21]. This high-dose formula contained some canine-derived probiotic strains as well as inulin as a prebiotic. Herein we describe the results from a randomized control trial examining the effects of this formula in healthy household dogs. GM was characterized by metagenomic sequencing, which provides superior taxonomic resolution as well as inferred function compared to the more commonly used
16S amplicon sequencing frequently used in microbiome research [22]. Differential responses to the supplement are also examined.

2. Methods

2.1 Animals and intervention
Fifty-one dogs of varied breeds were randomized to receive a daily dose of SN (n=25) or placebo (PL, n=26) for 4 weeks. Inclusion criteria included: aged 1-12 years, fed 2 meals per day (one or more of the four Nom Nom recipes with treats <10% of overall intake, ingredients and nutritional profiles available on www.nomnomnow.com), absent of gastrointestinal issues (including chronic diarrhea/vomiting or diarrhea/vomiting within 30 days prior to enrollment), absent of any infections or major chronic diseases (severe allergies, pancreatitis, diabetes, kidney disease or failure, liver disease, heart disease, cancer, severe gastrointestinal issues when young [<6 months old], surgery within the last 3 months prior to the enrollment), not pregnant or lactating at the time of enrollment, and limited to one dog per household. Dogs were not eligible to participate if they were fed any prebiotics, probiotics, cultured foods, or antibiotics within the last 3 months, or had a significant change in their diet within the last month prior to enrollment.

All owners were asked to collect stool samples using Nom Nom Plus Microbiome Testing Kits and complete a comprehensive health survey at baseline and at 4 weeks (the end of the intervention period). Additional stool samples were collected at week 6 in the SN group (2 weeks after stopping the supplement). The SN used in the study was Nom Nom Plus Full Spectrum Probiotics for Dogs, using a daily dose of 2 g containing 50 mg of inulin (derived from chicory root) and 20 billion live total CFU of a combination of the following species: Lactobacillus reuteri (canine-derived), Pediococcus acidilactici (canine-derived), Enterococcus faecium (canine-derived), Lactobacillus acidophilus, Bifidobacterium animalis, Lactobacillus fermentum, and Lactobacillus rhamnosus. These probiotic strains were chosen based on their individual benefits as previously reviewed [8,10,21]. Owners were instructed to mix one dose (2 g scoop) of the supplement into the dog’s meal once daily in the morning. An email reminder was sent out to all owners once a week to ensure adherence to the baseline diet, supplement and medication usage, and exercise habits, as well as providing the opportunity to report any adverse effects that were observed during the study period.

2.2 Health survey
Information on age, sex, body condition score (BCS), ideal body weight, physical activity level, neutered status, stool quality scale [23], and defecation and flatulence frequency was obtained through an online survey at baseline. Written descriptions and images were provided for the stool quality scale in the survey. At the end of week 4, dog owners completed a second set of questionnaires on overall health, physical activity, body weight, appetite, coat condition, stool quality scale, defecation and flatulence frequency, and incidence of diarrhea and/or vomiting.
2.3 GM DNA extraction
All stool samples were processed and sequenced in a single batch at Diversigen, Inc., USA. DNA extraction and sequencing library construction protocols were performed as previously described [24], with the exception that DNA was extracted using the Zymo DNA isolation kit (Zymo Research, USA) rather than Mo Bio Powersoil kit.

2.4 GM shotgun metagenomic sequencing and annotation
Shotgun metagenomic sequencing was performed with BoosterShot™ at Diversign, Inc., USA as previously described [24]. For quality control, single end shotgun reads were trimmed and processed using Shi7 [25]. The sequences were then aligned to the NCBI RefSeq representative prokaryotic genome collection at 97% identity with BURST using default settings [26]. Taxa present in < 5% of the samples were removed. The resulting taxonomy table was also aggregated at higher taxonomic levels.

2.5 GM functional annotation
Kyoto Encyclopedia of Genes and Genomes Orthology groups (KEGG KOs) were observed directly using alignment at 97% identity against a gene database derived from the strain database used above. The KO table contained the directly observed KO counts within each sample. KO terms present in < 5% of the samples were removed as part of the quality filtering process.

2.6 Statistical analysis
Continuous variables are expressed as mean±SD, except for the fold change data which are expressed as mean±SEM. Categorical variables are presented as count (%). All analyses were performed using R Studio version 1.2.5033. Statistical significance was set at $\alpha=0.05$. Subject characteristics at baseline and health survey data at week 4 were compared between the SN and PL groups using two-sample t-test and Fisher’s exact test for continuous and categorical variables, respectively.

Species richness and Shannon's diversity indices were computed by rarefying samples to various depths starting from 20,000–380,000 sequences per sample and increasing depth by 20,000 reads. One hundred iterations were performed at each depth and the mean values were used as the estimate of these measures in each sample (Supplemental Figure 1). To investigate the effect of SN on $\alpha$-diversity, the species evenness, richness, and Shannon’s as well as Simpson’s diversity indices were calculated at sequencing coverage of 380,000 reads, listed in Supplemental Table 1. Wilcoxon signed rank test was used to compare changes of alpha diversity metrics (evenness, richness, diversity indices) from week 0 to week 4 in each group, and Wilcoxon rank sum test was used to compare these metrics at each time point between SN and PL, as well as changes from week 0 to week 4 between SN and PL.

The non-rarefied count data were log-transformed and principal coordinate analysis (PCoA) was performed in R using the Bray-Curtis distance calculated with the vegan package at the species level [27]. Permutational multivariate analysis of variance (PERMANOVA) was performed using
Bray-Curtis distance with 10,000 randomizations by including groups and timepoints to assess differences in community composition using the vegan package [27]. Differential abundance of bacterial taxa and KO terms between groups or timepoints was assessed at the species level using a negative binomial generalized linear model (GLM) using the differential expression analysis for sequence count data version 2 (DESeq2) package [28]. Taxa with absolute log₂(fold change [FC])>1 and adjusted p-values<0.05 were considered significant. The adjustment was performed with a false discovery rate (FDR) for multiple comparisons.

3. Results

3.1 Subject characteristics
Twenty-four dogs in the SN group and 20 dogs in the PL group completed the trial by providing health assessment surveys and stool samples at weeks 0 and 4 (Figure 1). Twenty-one dogs in the SN group also provided stool samples at week 6. Seven dogs did not complete the study for the following reasons: vomiting and diarrhea one day prior to starting the supplement (n=1); lost-to-follow-up (n=5). The dropout rates between the SN and PL were not statistically different (p=0.10, Fisher exact test). One dog in the PL group was excluded from the analysis due to antibiotic use for bacterial dermatitis before completing the study. Dogs were 5.6±3.0 years old and 67% were male. Seventy-four percent had a BCS of 4-5 (ideal body condition) at the time of enrollment, while their ideal body weight was 11.5±10.2 kg (reflecting the diversity in breeds). Data on breeds and diet are available in Supplemental Table 2. The two groups did not significantly differ in age, sex, BCS, ideal body weight, physical activity level, and neutered status (Table 1). Fecal score was significantly lower (firmer stool) in the SN group at baseline (p=0.010). Subjects missed taking the supplement 0.5±0.9 and 0.9±1.1 days in SN and PL, respectively, but none missed >3 days during the 4-week study period.

3.2 Comparisons of GM between SN and PL

3.2.1 GM diversity
A total of 107 samples were collected from 19 subjects in the PL group and 24 subjects in the SN group (2 samples from each of the 19 subjects in the PL group, 3 samples from each of the 21 subjects and 2 samples from each of the three subjects in the SN group). Two samples from one subject were processed on a different sequencing batch and excluded from subsequent GM analyses to eliminate any batch effect (Figure 1).

A total of 8,486 taxa were identified among all 105 samples, with an average sequencing depth of 1,538,444±505,664 reads per sample, ranging from 389,644 to 2,903,240 reads (Supplemental Figure 2A). Nineteen taxa (0.22%) were removed from subsequent analyses because the taxon was present in <5% of the samples (Supplementary Figure 2B). The removal of these taxa did not significantly change the average number of reads per sample or the range of sequencing coverage.
At sequencing depth of 380,000 reads, alpha diversity (measured using species evenness, richness, and Shannon’s and Simpson’s diversity indices) was not significantly different among the different time points in both the SN and the PL groups (Supplemental Table 1). Changes at week 4 from baseline for these measures were also not significantly different between the two groups.

PCoA was used to investigate changes in β-diversity. The PCoA plot in Figure 2A shows the first two principal coordinate axes (PCoA1 and PCoA2), which respectively explain 13.9% and 9.8% of the variation at the species level. Figure 2B shows the next two axes, with PCoA3 (7.3%), and PCoA4 (4.74%). The eigenvalues for the first 20 PCoA axes are displayed in Supplemental Figure 3. There were no differences in spatial separation among the treatment groups or timepoints along the first two PCoA axes (p=0.093, PERMANOVA using the Bray-Curtis distance matrices). Scores from the first three principal coordinates (PCoA 1-3) were not significantly different among timepoints within each group, or at each timepoint between groups (Figure 3). PCoA 1-3 scores at baseline were also not associated with age, sex, neutered status, BCS, ideal weight, physical activity level, stool quality score, and defecation frequency across SN and PL groups (data not shown).

However, as shown in Figure 3, scores of the fourth principal coordinate axis (PCoA4) in the SN group at week 4 (0.072±0.087) were significantly different from PCoA4 scores at week 0 (0.003±0.056, FDR-adjusted p=0.002) and week 6 (-0.033±0.063, FDR-adjusted p <0.001). This difference was not observed in the PL group, and not between week 0 and week 6 in the SN group. In other words, a shift in PCoA4 score, which explains 4.74% of the overall variation at the species level, was observed after 4 weeks of SN administration, and it returned to baseline 2 weeks after stopping the intervention. Further, the majority of the subjects (n=20, 87%) in the SN group demonstrated a consistent shift in the same direction on the PCoA4 axis - increasing from week 0 to week 4 (also see Section 3.3.1 below). This proportion was significantly different than that in the PL group whose PCoA4 score increased in 11 dogs (58%) and decreased in 8 dogs (42%) between weeks 0 and 4 (p=0.042, Fisher exact test), showing inconsistent shifts between the two treatment groups. Based on these results, it can be concluded that SN had a small but significant effect on the overall GM composition.

### 3.2.2 GM abundance

The phyla Firmicutes (SN: 60.9±30.0%, PL: 60.5±30.0%), Proteobacteria (SN: 22.7±24.5%, PL: 25.3±29.2%), Bacteroidetes (SN: 9.4±15.0%, PL:9.6±10.3%), and Actinobacteria (SN: 6.7±9.1%, PL: 4.3±5.8%) constituted the majority of the gut bacteria in samples collected at baseline (Supplemental Figure 4). No significant changes in the relative abundance of any phylum was observed across timepoints in either group. However, differences were detected at the order level. Subjects receiving the SN showed an increase in the abundance of the Bifidobacteriales order at week 4 as compared to baseline (FDR-adjusted p=0.001) and week 6 (FDR-adjusted p=0.007, Kruskal Wallis test). This change was not observed in the PL group. *Bifidobacterium animalis*, a species present in the SN supplement, belongs to the Bifidobacteriales order.
GM abundances from samples collected at weeks 4 and 6 were compared with those collected at week 0 at the species level (Figure 4). As listed in Table 2, the abundances of 45 species were shown to be significantly higher at week 4 as compared to week 0 in the SN group. All of the 7 bacterial species present in the SN supplement were among these identified species: *Lactobacillus reuteri* (log$_2$FC=6.43±1.00, adjusted p<0.001), *Pediococcus acidilactici* (log$_2$FC=6.53±1.10, adjusted p<0.001), *Enterococcus faecium* (log$_2$FC=3.00±0.82, adjusted p<0.001), *Lactobacillus acidophilus* (log$_2$FC=6.76±0.88, adjusted p<0.001), *Bifidobacterium animalis* (log$_2$FC=6.80±0.93, adjusted p<0.001), *Lactobacillus fermentum* (log$_2$FC=3.54±0.88, adjusted p<0.001), and *Lactobacillus rhamnosus* (log$_2$FC=7.18±1.35, adjusted p<0.001). The abundances of these species returned to baseline at week 6, 2 weeks after stopping the SN, except for *Lactobacillus acidophilus* whose abundance remained significantly higher than at baseline (log$_2$FC=3.48±1.07, adjusted p=0.037). No significant changes in the abundances of these species presented in SN were observed in the PL group between weeks 0 and 4.

Increases in the abundances of additional species were also observed at week 4 in the SN group (Table 2). They included 13 additional species of *Lactobacillus* (including *L. frumenti*, *L. vaginalis*, *L. plantarum*, *L. intestinalis*, *L. murinus*, *L. inlguviei*, *L. salivarius*, *L. taiwanensis*, *L. hominis*), and 22 species of *Enterococcus* (including *E. durans*, *E. villorum*, *E. pseudoavium*, *E. malodoratus*). The abundances of these bacteria did not increase at week 4 in the PL group and all returned to baseline at week 6 in the SN group. *Lactobacillus* and *Enterococcus* belong to the Lactobacillales order, which accounted for 96% of the species that showed a significant increase in the abundances at week 4. Moreover, the abundances of 15 known and 2 unknown species significantly decreased after 4 weeks of SN but not PL supplementation. They belonged to the genera *Clostridium*, *Arthrobacter*, *Kurthia*, *Lactobacillus*, *Timonella*, *Bacteroides*, *Lactococcus*, and *Streptococcus*. The abundances of these species returned to baseline at week 6, except for *Kurthia sp. Dielmo* which remained statistically lower at week 6 (log$_2$FC=-7.76 ± 2.07, adjusted p=0.012). The abundance of an unknown species of *Arthrobacter* also decreased at week 4 (log$_2$FC=-7.83±1.45, adjusted p<0.001) and remained lower than baseline at week 6 (log$_2$FC=-5.52±1.42, adjusted p<0.001).

Changes in the abundances of 21 species of GM were also observed at week 4 in dogs receiving PL (Table 2). These 21 identified species did not overlap with the identified species in the SN group with the single exception of *L. pisicum* which decreased at week 4 in the both SN and PL groups.

### 3.2.3 GM functional data

A total of 4,651 KO terms in 105 samples were identified, and after filtering, 3,615 remained for subsequent analyses. In the PL groups, one KO term showed a significant increase while one KO term showed a significant decrease in the abundance at week 4 compared to baseline. On the other hand, a significant increase in the abundance of 15 KO terms was observed in the SN group after 4 weeks as listed in Table 3. These KOs are associated with multiple metabolic pathways including: aromatic compound degradation (K18364), biosynthesis of macrolides.
(K16001), starch and glucose metabolism (K16147, K00689), pentose phosphate pathway (K01621), and lipoic and propionic acid metabolism (K16869, K01699). Additionally, a significant decrease in 2 KO terms (K11521, K11384) was observed. Significant changes were no longer observed at week 6.

3.3 Heterogeneous response to SN

3.3.1 High-, mid-, and low-responders

As demonstrated in Figure 5A, while the average effect of SN on GM composition was observed at week 4, the magnitude of the response varied among individual subjects within the SN group. In order to investigate this heterogeneity of response to SN, the subjects were divided into tertiles based on the degree of PCoA4 change from week 0 to week 4, with subjects in the first tertile labeled high-responders (HR, maximal PCoA4 score increase, n=8), those in the second tertile labeled mid-responders (MR, n=7), and those in the final tertile labeled low-responders (LR, PCoA4 score decrease or minimal PCoA4 score increase, n=8).

As seen in Figure 5B, PCoA4 scores were not significantly different among HR, MR, and LR at baseline (p=0.397, Kruskal-Wallis test) or week 6 (p=0.367). However, they were significantly higher at week 4 in HR (0.132±0.037, FDR-adjusted p=0.003, Kruskal-Wallis test) and MR (0.091±0.076, FDR-adjusted p=0.043) compared to LR (-0.004±0.080), but not between MR and HR (FDR-adjusted p=0.536). While PCoA1, 2, and 4 scores did not significantly differ among HR, MR, and LR at baseline, PCoA3 scores were significantly higher in HR (0.084±0.117) than LR (-0.043±0.049) but not MR (-0.038±0.096) (FDR-adjusted p=0.031, Kruskal-Wallis test, Figure 5C). Additional differences in GM abundances are reported in section 3.3.2.

Subject characteristics at baseline were further compared between HR, MR, and LR, and were not statistically different (Supplemental Table 3). GM α-diversity metrics including evenness, Shannon’s and Simpson’s diversity indices were significantly lower in HR and LR compared to MR at baseline (p=0.022 for all metrics, Kruskal-Wallis test), but not at weeks 4 and 6 (Supplemental Table 4 and Supplemental Figure 5). Changes in these metrics from week 0 to week 4 or 6 were not different across the among groups.

3.3.2 HR and LR exhibited different GM at baseline and 4 weeks after SN supplementation

Significant differences in the abundances of 62 species of gut bacteria between HR and LR were observed at baseline, of which 51 species and 11 species were lower and higher in HR, respectively (Table 4, Figure 6A). Among these species that were underrepresented at the family level in HR, 21 (41%) were Enterococcaceae, 13 (25%) Streptococcaceae, 7 (14%) Leuconostocaceae, and 6 (12%) Lactobacillaceae family (Figure 6C). At the order level, all but three species (94%) were Lactobacillales (Figure 6D). For those 11 species whose abundances were significantly higher in HR, 9 (82%) species, including Escherichia coli and Escherichia albertii, were in the Enterobacteriaceae family, which belongs to the Enterobacterales order. The other two species were Achromobacter and one unknown species that belonged to the Gammaproteobacteria class. This observation likely reflected the difference in PCoA3 scores
between HR and LR at baseline (see section 3.3.1). None of the abundances of species present in the SN supplement differed between the guts of HR and LR at baseline.

After 4 weeks of SN supplementation, the abundance of 53 species significantly increased in HR, 51(96%) of which belong to the order Lactobacillales (Supplemental Table 5), while no significant increase of any species was observed in LR. Of these 53 species, twenty one species were identified as having significantly higher abundances in HR as compared to LR at week 4 (Table 5, Figure 6B). None of these species were overrepresented in HR at baseline. Among these 21 species, 14 (67%) belonged to the Enterococcaceae family and 5 (24%) belonged to the Lactobacillaceae family (Figure 6C). At the order level, all but one species (95%) were Lactobacillales, similar to those species in the Lactobacillales order that were present in low levels in HR at baseline (Figure 6D). Lactobacillus reuteri (log$_2$FC=4.84±1.39, adjusted p=0.015) and Enterococcus faecium (log$_2$FC=4.19±1.25, adjusted p=0.018), both of which were in the SN supplement, were among these 21 identified species. The species in the Enterobacteriaceae family which had been overrepresented in HR at baseline were no longer overrepresented in HR at week 4. This was further supported by the observation that PCoA3 scores were no longer different among HR, MR, and LR at week 4 (Figure 5C). Conversely, 14 species were observed to have lower abundances among HR at week 4, 7 (50%) and 4 (29%) of which belonged to the Streptococcaceae family and the Bacteroidaceae family, respectively. At the order level, 7 (50%) were Lactobacillales and 5 (36%) were Bacteroidales. At week 6 (2 weeks after stopping the SN supplementation), abundances of all species did not significantly differ between HR and LR.

The average relative abundance of GM in HR and LR at the family and order levels are also shown in Supplemental Figure 6. The figure reflects the changes at the species level previously described, but they were not significant at the family and order levels in either HR or LR after the adjustment for multiple comparisons.

### 3.4 Health outcomes

No health outcomes and adverse effects were statistically different between treatment and placebo groups at the end of the intervention at week 4 (Table 6). While 16% of dogs in the PL group had an incidence of diarrhea during the 4-week period, none of the dogs in the SN group experienced diarrhea. After starting the supplement, vomiting was reported in 4 dogs (21%) in the PL group and 1 dog (4%) in the SN group during the 4-week study period. Although both the diarrhea and vomiting incidences were reduced in the SN group, neither reached statistical significance. Other reported adverse effects included constipation (n=1) in the SN group and itching (n=1) in the PL group.

### 4. Discussion

The primary goal of this randomized control trial was to identify the effects of a multi-species SN on the gut microbial community composition and function in healthy dogs. A limited number of studies have investigated the effect of probiotic administration on the fecal microbiota in healthy dogs, but include only Alaskan Husky sled dogs, Beagles, and Boxers [13,16,17]. The present
study was a defined cohort of household dogs representative of pet dogs in the United States. The cohort comprised a diversity of ages and breeds, included both males and females, and had a relatively large sample size. Therefore, these findings should be more applicable to general dog populations than studies performed in laboratory or working dogs. Moreover, while a probiotic consisting of a single strain of bacteria was chosen as the treatment in many studies [13,29–36], the present study adopted a SN supplement consisting of inulin and seven bacterial species, three of which have canine origin. Finally, the use of metagenomic sequencing, which has not been widely used in the characterization of the canine microbiota [7,37–39], increases taxonomic resolution and therefore detection of bacterial species and diversity, and is superior in functional prediction compared to the more commonly used 16S amplicon sequencing [22].

While a variety of changes in bacterial species abundances were observed during the four week trial period in both the SN and PL groups, the greatest number and significance of differences were observed in the SN group. These differences were also reflected in changes of β-diversity along the PCoA4 axis solely in the SN group, indicating that these changes were most likely caused by the supplement. Additionally, SN led to increased abundances of the bacteria present in the supplement, which included species of *Lactobacillus*, *Enterococcus*, *Bifidobacterium*, and *Pediococcus*. The high abundances of DNA from these probiotic bacteria in feces indicate that these bacteria likely survived gastric transit. These probiotic strains have demonstrated health benefits such as ameliorating diarrheal symptoms, improving stool quality, and improving the intestinal barrier integrity in dogs [7,40,41].

Dogs given the supplement also had decreased abundances of three species of *Clostridium* that are rare potential pathogens (*C. celatum, C. baratii, and C. disporicum*) [42–44] and several species with evidence of opportunistic pathogenicity (e.g., *Citrobacter* spp. and *Bacteroides* spp.) [45–48]. However, the pathogenicity of these *Clostridium, Citrobacter, and Bacteroides* species has been documented only in humans. These results are consistent with probiotic bacteria reducing pathogen load of the intestines by preventing pathogen colonization, particularly in the case of *Clostridium* species [49,50].

As demonstrated by the small changes in the abundance of the KO terms, overall GM function did not differ drastically before and after SN treatment. This observation likely reflects the relatively small, albeit significant, effects of SN use in healthy dogs as well as no reported overall change in health outcomes. Further investigation incorporating other -omics approaches such as transcriptomics and metabolomics may provide better insights into direct or indirect synbiotic functionality.

In dogs given the SN, changes in the β-diversity (PCoA4) returned to baseline after the supplement use was discontinued for 2 weeks. The abundances of six out of the seven probiotic species also returned to baseline. The one exception was *L. acidophilus*, whose abundance in feces remained high for at least two weeks after probiotic use was discontinued, indicating that *L. acidophilus* may have more permanently engrafted in the gut. Otherwise this observation indicates that the probiotic bacteria did not permanently colonize the GI tract at detectable
levels. In contrast, the abundances of two species, Kurthia sp. Dielmo and an unknown Arthrobacter sp., did not return to their baseline levels after being reduced by SN use. Little is known about Kurthia sp. Dielmo, and Arthrobacter species are generally found in soil, but have been isolated from oral cavities of healthy dogs [51–53]. With the exception of these examples, our findings corroborated previous findings that GM changes occur in a relatively short period of time (within 6-15 days) after stopping supplementation [14,17,30,35,36]. They also support that engraftment of probiotics is generally uncommon and not required for their beneficial effects [54], and that continued administration of probiotics or synbiotics may be necessary to produce prolonged effects.

Changes in community composition after the SN treatment were most striking in a subset of the dogs, termed “high-responders”. The abundances of 53 species increased only in the high-responders, 96% of which belonged to the order Lactobacillales. These changes were no longer evident two weeks after SN use was discontinued. Interestingly, changes in the abundances of these 53 species were not observed among the “low-responders” despite their receiving the same treatment throughout the four weeks.

This raises the question of what factors contribute to such differential responses in this cohort of healthy dogs. We identified that baseline GM composition of high- versus low-responders significantly differed in β-diversity (PCoA3) and the abundances of some bacterial species. These differences may be the reason certain dogs were more responsive to the effects of SN treatment than others. Specifically, species belonging to the Lactobacillales order were underrepresented in high-responders prior to supplementation. One possibility is that the Lactobacillus niche in low-responders is already being utilized by other lactic-acid bacteria, therefore they were more resistant to any change induced by the supplement. Baseline compositional differences that result in varying magnitudes of response to a treatment are increasingly common in the literature. With regard to probiotic supplementation, this observation is consistent with studies in human subjects that have identified certain individuals whose gut microbial communities respond to probiotics whereas the communities in other individuals exhibit lower or no responses [55,56]. To our knowledge, this is the first study documenting such an effect in companion dogs. Rather than a one-size-fits-all regimen, the results from our study support the notion that the prediction of response to SN supplementation and tailored probiotic recommendation may be possible based on their identified baseline microbiota [55].

In the high-responders, many of the bacteria whose abundances were significantly reduced by the SN are known pathogens [57], including species of Shigella and Escherichia [58,59]. These dogs did not have reported clinical symptoms of GI diseases, so the clinical impact of this change by SN is unknown. It is intriguing to speculate that the presence of these pathogens may indicate increased susceptibility or predisposition to developing GI disorders or diseases, and that SN treatment may have a prophylactic effect by reducing their abundances as previously reported [60–62]. Consistent with this was our finding that the PL group had a non-significant trend toward higher incidence of diarrhea than SN.
5. Conclusion
SN administration for four weeks caused a small but significant shift in the GM profile and predicted function in healthy dogs. The shift included an increase in the abundance of bacteria contained in the SN and a decrease in potentially pathogenic bacteria, and GM composition largely returned to baseline two weeks after the termination of SN treatment. Heterogeneity of treatment response was observed. Dogs whose GM at baseline included low levels of Lactobacillales and high levels of several pathogens, despite having no clinical symptoms, responded to the SN supplementation to a greater extent by reducing pathogen load and increasing abundances of beneficial lactic-acid bacteria, among the others. Future trials with longer duration in healthy dogs or in a population at higher risk of diarrhea are respectively warranted to investigate the possible effect of SN supplementation on health maintenance and disease prophylaxis.

6. Declarations

6.1 Ethics approval and consent to participate
Consent was received from dog owners prior to enrolling their dogs in the study. Since the study gathered information focused solely on dogs, it does not meet the definition of human subject research and therefore does require review by an Institutional Review Board. Because fecal samples were collected non-invasively by the dog owners, no institutional animal ethical review was required.

6.2 Consent for publication
Not applicable - the manuscript does not contain data from any individual subject.

6.3 Availability of data and material
Survey data included in this published article and its supplementary information files. Fastq files and OTU tables are available from the corresponding author RWH upon request.

6.4 Competing interests
JT, JS, LAMP, RWH are employees of and/or hold stocks or stock options in NomNomNow Inc. ARJ and HM are contracted by NomNomNow Inc as consultants.

6.5 Funding
NomNomNow, Inc. provided support in the form of salaries for all authors, but did not have any additional role in the preparation of the manuscript.

6.6 Authors’ contributions
JT designed and executed the study, analyzed the data, interpreted the results, and wrote the manuscript. ARH analyzed the data and interpreted the results. JS designed the study and interpreted the results. LAMP interpreted the results and wrote the manuscript. HM interpreted the results and wrote the manuscript. RWH designed the study, analyzed the data, interpreted the results, and wrote the manuscript. All authors read and approved the final manuscript.
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Figures

Figure 1. Trial flowchart

Figure 2. Principal coordinate analysis (PCoA) plot. (A) PCoA1 (Axis 1) and PCoA2 (Axis 2) respectively explained 13.9% and 9.8% of the variance of the abundance of gut microbiota at the species level (105 samples from 42 dogs). PERMANOVA using Bray-Curtis distance showed no spatial separation among treatment groups (placebo and probiotics) or timepoints (weeks 0, 4, 6) based on PCoA1 and PCoA2 scores. (B) PCoA1 and PCoA4 (explaining 4.7%).

Figure 3. Scores of the first 4 PCoA axes in subjects receiving synbiotic (SN, n = 23) or placebo (PL, n = 19) at weeks 0, 4, and 6. PCoA4 score in the synbiotic group at week 4 was significantly different from that at week 0 (adjusted p = 0.002) and week 6 (adjusted p < 0.001). P value adjustment for pairwise comparisons was performed using false discovery rate.

Figure 4. Volcano plots demonstrating the fold-change (FC) in the abundance of gut bacteria at the species level (A) at week 4 compared to week 0 in the synbiotic group (n=23); (B) at week 6 compared to week 0 in the synbiotic group (n=21); and (C) at week 4 compared to week 0 in the placebo group (n =19). Vertical dashed lines show log₂FC at 1 and -1 (i.e. FC at 2 and -2). Horizontal dashed line shows -log₁₀(adjusted p) = 2 (i.e. adjusted p = 0.01). Points are colored by phylum.

Figure 5. Varying degrees of PCoA4 changes from week 0 to week 4 were observed among subjects receiving the synbiotic supplement. (A) PCoA4 score at week 4 increased in 20 dogs (87%) as compared to baseline in the synbiotic group, while the direction of change was less consistent in the placebo (PL) group - increased in 11 dogs (58%) and decreased in 8 dogs (42%). Subjects in the synbiotic group were divided into tertiles based on the degree of PCoA4 changes between week 0 and week 4, with subjects in the first tertile labeled high-responders (HR, maximal PCoA4 score increase, n = 8), those in the second tertile mid-responders (MR, n = 7), and those in the third tertile low-responders (LR, PCoA4 score decrease or minimal PCoA4 score increase, n = 8). (B) PCoA4 scores at week 4 were significantly higher in HR (0.132 ± 0.037, FDR-adjusted p = 0.003, pairwise Wilcoxon rank sum test) and MR (0.091 ± 0.076, FDR-adjusted p = 0.043) as compared to LR (-0.004 ± 0.080). (C) PCoA3 scores were significantly higher in HR (0.084±0.117) than LR (-0.043±0.049) but not MR (-0.038±0.096) at baseline (FDR-adjusted p=0.031, Kruskal-Wallis test). PCoA3 scores were not significantly
different among groups at week 4 or week 6. Values sharing the same superscript are not statistically significant from each other.

**Figure 6.** Volcano plots demonstrating fold-change (FC) abundance of gut bacteria between HR (high-responders, n=8) and LR (low-responders, n=8) among dogs receiving the probiotics at (A) week 0 and (B) week 4. Numbers of species with significantly different abundances are shown at (C) the family level and (D) the order level.

**Supplemental Figure 1.** Rarefaction curves demonstrate sufficient sequencing coverage to calculate species (A) richness and (B) Shannon’s diversity index in subjects receiving synbiotic (n = 23) or placebo (n = 19). Species richness was calculated from 10000 to 380000 reads. Each point represents a mean and each error bar represents a standard deviation at each rarefaction depth.

**Supplemental Figure 2.** Metagenomic sequencing data quality control. Plots show the abundance (x-axis, as count) and the prevalence (y-axis, as percentage of all samples) of each read for all phyla (A) pre- and (B) post-filtering. Each data point represents a read. As a part of filtering process, 19 taxa from phyla Candidatus Kryptonia, Candidatus Saccharibacteria, Chloroflexi, Deinococcus-Thermus, Gemmatimonadetes, Nitrospirae, Planctomycetes, Synergistetes were removed because each represented <5% of samples or belonged to an unknown phylum.

**Supplemental Figure 3.** Scree plot showing eigenvalues of the first 20 principal coordinate axes.

**Supplemental Figure 4.** Relative abundance at the phylum level in the samples collected at different time points in dogs receiving placebo or synbiotic.

**Supplemental Figure 5.** (A) Evenness, (B) richness, (C) Shannon’s and (D) Simpson’s diversity indices of the gut bacteria among HR (high-responders, n=8), MR (mid-responders, n=7), and LR (low-responders, n=8), at different time points.

**Supplemental Figure 6.** Average relative abundance in high-responders (HR, n=8) and low-responders (LR, n=8) at baseline and week 4 at the (A) family and (B) order levels. The legend shows only those whose relative abundance >1% at any time point.
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