

A specific blend of prebiotics and postbiotics improved the gut microbiome of dogs with soft stools in the in vitro Simulator of the Canine Intestinal Microbial Ecosystem

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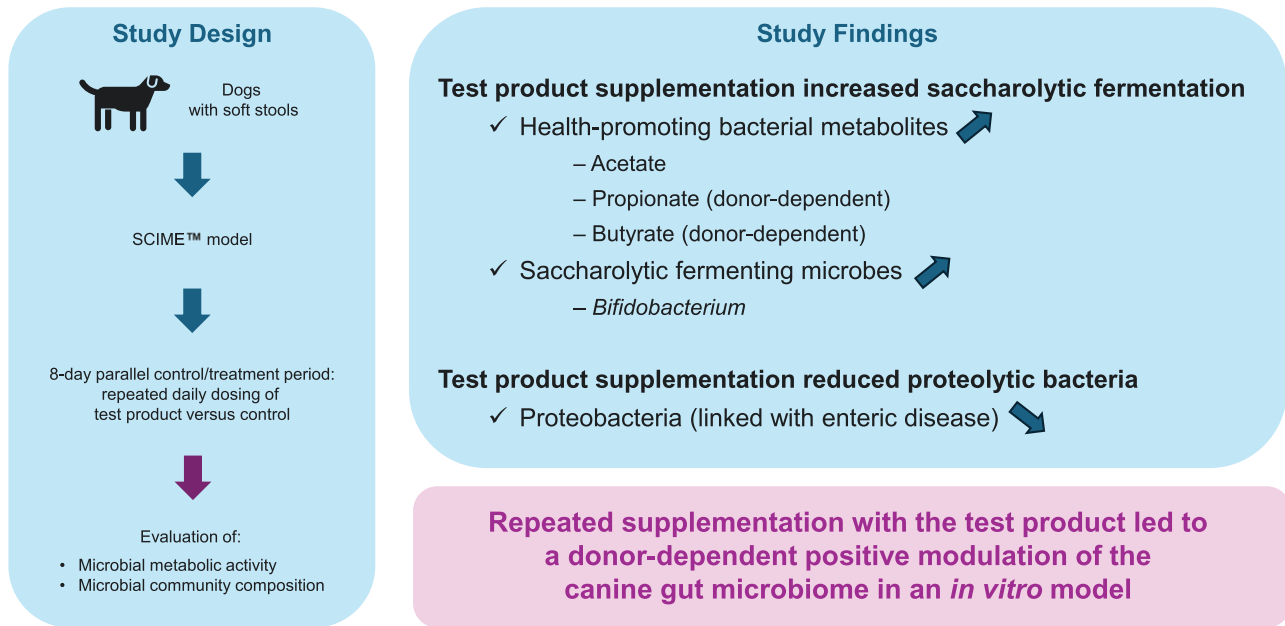
Abstract

The Simulator of the Canine Intestinal Microbial Ecosystem (SCIME) allows for the study of the long-term effects of food, supplements, or ingredients on the canine gut microbiome in a simulated proximal and distal colon. This model has been used to evaluate the impact of repeated administration of a test product blend composed of a mixture of baobab fruit pulp, acacia gum, heat-killed *Lactobacillus helveticus* HA-122, and specific fractions of selected inactivated yeast strains (including *Saccharomyces cerevisiae* AQP 12260 and AQP 12988 and *Cyberlindnera jadinii* AQP 12549), on the activity and composition of the gut microbiome of canine donors with soft stools. The SCIME colonic reactors were inoculated with fecal material from 3 different canine donors. After 2 d of stabilization, the 8-d parallel control/treatment period was initiated; reactors were fed with SCIME nutritional medium with or without test product. Changes in microbial metabolic activity were assessed by measuring levels of acetate, propionate, butyrate, lactate, branched short-chain fatty acids, and ammonium. Changes in microbial community composition were assessed using 16S-targeted Illumina sequencing. Overall, test product supplementation resulted in increased saccharolytic fermentation, as evidenced by increases in the health-promoting bacterial metabolites such as propionate (donor-dependent), acetate, and butyrate (donor-dependent) as well as increased abundances of several saccharolytic fermenting microbes, including *Bifidobacterium*. Conversely, proteolytic bacteria like Proteobacteria were reduced with the test product compared to control. Repeated supplementation with the test product was therefore able to induce—in vitro—a positive modulation of the microbiome originated from dogs with soft stools.

Lay Summary

Dogs with digestion problems can have an unbalanced digestive flora. This means the proportion of healthy and unhealthy bacteria that live in their gut is not well balanced. We used an experimental model to mimic the gut bacteria of dogs with soft stools. In the model, we looked at how a test product affected the gut bacteria. The test product was a mixture of baobab fruit pulp, acacia gum, a heat-killed bacteria called *Lactobacillus helveticus* HA-122, and parts of specific yeast strains that are no longer alive. When the test product was added, the bacteria made more healthy products like propionate compared to when it was not added. Other healthy products were increased depending on the dog's original microbiota. The types of bacteria in the model were also different depending on whether the test product was added. There was an increase in beneficial bacteria when the test product can improve the gut flora of dogs with soft stools and this effect may vary depending on the original flora of the animal.

Graphical Abstract



Test product: baobab fruit pulp, acacia gum, heat-killed *Lactobacillus helveticus* HA-122, specific fractions of inactivated yeast strains

Key words: dog, in vitro simulation, intestinal health, Simulator of the Canine Intestinal Microbial Ecosystem

Abbreviations: bSCFA, branched short-chain fatty acid; DAPC, discriminant analysis of principal components; DC, distal colon; M-SCIME, Mucosal Simulator of the Canine Intestinal Microbial Ecosystem; OTU, operational taxonomic Units; PC, proximal colon; SCFA, short-chain fatty acid; SCIME, Simulator of the Canine Intestinal Microbial Ecosystem

Introduction

The gut microbiome is a complex collection of microorganisms that is mostly composed of bacteria (Suchodolski, 2011). These bacteria perform several important digestive functions, including the fermentation of non-digestible dietary fibers (Makki et al., 2018). Other important roles of the gut microbiome include providing protection against pathogens, producing important health-related metabolites, and aiding in the development of a healthy immune system (Suchodolski, 2011). Through these activities, a healthy gut microbiome supports the health of its host. Bacteria in the canine gastrointestinal tract generally belong to 1 of the 5 phyla: Firmicutes, Fusobacterium, Bacteroidetes, Proteobacteria, and Actinobacteria (Suchodolski et al., 2008; Honneffer et al., 2017). Firmicutes, Fusobacterium, and Bacteroidetes are the predominant phyla in the fecal microbiome of healthy dogs (Middelbos et al., 2010; Hand et al., 2013). It has been reported that the fecal microbiome of dogs with acute and chronic gastrointestinal diseases is substantially altered relative to that of healthy dogs (Suchodolski et al., 2012), resulting in dysbiosis which encompasses alterations in microbiome diversity and/or structure and functional changes, such as adjustments in microbial metabolite production. A physiological outcome is the production of soft or diarrheic stools.

As mentioned, fermentation of dietary fibers or carbohydrates, termed saccharolytic fermentation, is an important function of the gut microbiome. Short-chain fatty acids (SCFAs) are key byproducts of microbial fermentation that have health-promoting effects. The most abundantly produced SCFAs are acetate, propionate, and butyrate (Sunvold et al., 1995). These metabolites are utilized as an energy source for intestinal epithelial cells, have anti-inflammatory properties, and are involved in the regulation of intestinal

motility (Arpaia et al., 2013; Morrison and Preston, 2016; Priyadarshini et al., 2018). SCFAs are produced by some bacteria that are commonly present in the gut microbiome. Many members of the Firmicutes phylum are SCFA producers, suggesting their importance in gut health (Deleu et al., 2021). Lactate is another important byproduct of bacterial fermentation. The gut microbiome includes both lactate-producing and lactate-utilizing bacteria. Lactate produced by lactic acid bacteria can lower the pH of the gut, which has an antimicrobial effect (Wang et al., 2020). Additionally, lactate can be converted to butyrate and/or propionate by lactate-utilizing bacterial species, including members of the Firmicutes phylum, in a process termed cross-feeding (Wang et al., 2020). Following carbohydrate depletion, proteins are fermented by the gut microbiota, resulting in the production of branched SCFAs (bSCFAs) and ammonium along with other compounds such as p-cresol and phenols, with the latter being associated with direct and indirect adverse health effects (Gozdzik et al., 2023).

Prebiotics and probiotics have been shown to improve clinical signs of gastrointestinal disease, including diarrhea. Prebiotics and probiotics, alone or in combination, can help reestablish beneficial bacteria in the canine gut and restore SCFA production (Pilla and Suchodolski, 2021). This study evaluated a specific blend containing 2 prebiotics (baobab fruit pulp and acacia gum) and 2 types of postbiotics (heat-inactivated *Lactobacillus helveticus* HA-122 and fractions of selected inactivated yeast strains including *Saccharomyces cerevisiae* AQP 12260 and AQP 12988 and *Cyberlindnera jadinii* AQP 12549). Both baobab fruit pulp and acacia gum have been shown to have prebiotic properties (Calame et al., 2008; Foltz et al., 2021). Heat-killed whole-cell bacteria or yeast, as well as their derivatives (cell walls and cell extracts),

are nowadays considered postbiotics, defined as “a preparation of inanimate microorganisms and/or their components that confers a health benefit on the host” (Salminen et al., 2021), exerting similar effects as probiotics, conferring protection against enteropathogens, demonstrating immunomodulatory effects, and supporting the integrity of the intestinal barrier (Pique et al., 2019). Heat-killed *L. helveticus* HA-122 in particular (formerly *Lactobacillus acidophilus* HA 122) has demonstrated immunomodulatory effects (in vitro and in vivo) and beneficial effects on the gut barrier and digestive signs in different species, including humans (Taverniti and Guglielmetti, 2012; Martinelli et al., 2017; Rawling et al., 2023). Yeast derivatives are common dietary supplements for animals and are also considered postbiotics (Thorakkattu et al., 2022). Supplementation has been associated with several health effects, including immunomodulatory effects, improved intestinal health, and enhanced production of SCFAs (Xue et al., 2017; Lee et al., 2021). The yeast strain derivatives used in the test compound are isolated from selected strains (*S. cerevisiae* AQP 12260, *S. cerevisiae* AQP 12988, and *C. jadinii* AQP 12549). They have been selected by atomic force microscopy and single-molecule force spectroscopy for their capacity to bind microbial pathogens (Schiafone et al., 2014), their immunomodulatory properties, and their beneficial effects on the digestive system and animal growth (Guan et al., 2017; Rawling et al., 2019, 2021).

The Simulator of the Canine Intestinal Microbial Ecosystem (SCIME) is a semi-continuous gastrointestinal tract model that has been validated using a simultaneous in vivo–in vitro comparison (Duysburgh et al., 2020). The SCIME can be utilized to study the long-term effects of food products, supplements, or drugs on the colon-associated microbial community of dogs.

The aim of the study was to assess the impact of repeated administration of a test product composed of a specific blend of baobab fruit pulp, acacia gum, heat-killed *L. helveticus*, and selected yeast strain derivatives on the activity and composition of the gut microbiome of canine donors with soft stools. This study utilized the SCIME model and included 3 canine donors to allow for the assessment of interindividual variability.

Materials and Methods

Canine donors and fecal samples

Fecal samples were collected from 3 canine donors with moderate digestive problems and soft stools without the presence of parasites. Samples were collected in sampling boxes and, immediately after collection, an Anaerogen bag was added to the box, which was then sealed to remove all oxygen. Anaerobic phosphate buffered saline was added to the sample and a fecal slurry was prepared following homogenization using a stomacher. Large particles were removed by brief centrifugation (2 min at 500 g) and an equal volume of cryoprotectant solution (modified from Hoefman et al. [Hoefman et al., 2013]) was added under anaerobic conditions. Samples were snap-frozen in liquid nitrogen, stored at -80°C and used within a week. Reactors were inoculated with 5% v/v of a 20% w/v fecal slurry.

Test product

The test product was a proprietary mixture composed of baobab fruit pulp and acacia gum (200 mg/day), heat-killed

whole cell *L. helveticus* HA-122 (with maltodextrin as carrier; 30 mg/d), and specific fractions of inactivated yeast strains *S. cerevisiae* AQP 12260, *S. cerevisiae* AQP 12988, and *C. jadinii* AQP 12549 (170 mg/d). The test product was provided by Virbac SA (Carros, France).

SCIME model

Given the high similarity between the human and canine intestinal conditions, the reactor setup for the SCIME model was based on the Simulator of the Human Intestinal Microbial Ecosystem (Molly et al., 1993). Details regarding the setup and validation of the SCIME model have been previously published (Duysburgh et al., 2020). The SCIME model used in the present study followed the setup of Duysburgh et al. with some modifications to account for the limited amount of time that a possibly dysbiotic microbial community linked to a gastrointestinal condition can be maintained in vitro. Briefly, the SCIME included 3 reactors representing the canine gastrointestinal tract (stomach and small intestine simulated in a single compartment and implemented by modifying conditions over time), the proximal colon (PC), and the distal colon (DC). The compartments were temperature controlled at 39°C under anaerobic conditions, which was achieved by daily flushing with N_2 -gas. For each donor ($n = 3$), a control and treatment arm were executed in parallel rather than the sequential control and treatment periods used in the standard SCIME setup, in order to compare the evolution of the microbial communities in the presence and absence of a treatment (Figure 1). Colon reactors for the control and treatment arms were inoculated with appropriate fecal samples from each canine donor and the inoculum was allowed to grow and colonize the reactors for 2 d, while the reactors were fed with a basic SCIME nutritional medium (PDNM009, ProDigest, Belgium). After 2 d, the 8-d parallel control/treatment period was initiated. During this period, the reactors were fed with standard SCIME nutritional medium with or without test product supplementation to represent the treatment or control arms, respectively. Samples were collected from each colon vessel for analysis of microbial metabolic activity (lumen) and microbial community composition (lumen) on the first day (D1; after the first treatment was provided), third day (D3), fifth day (D5), and eighth day (D8) of the control/treatment period (Figure 1).

Microbial metabolic activity

Quantitative analysis of the SCFAs, namely acetate, propionate, and butyrate, and of the bSCFAs (isobutyric acid, isovaleric acid, and isocaproic acid), was performed using capillary gas chromatography coupled with a flame ionization detector. SCFAs were isolated using liquid-liquid extraction with diethyl ether, after the addition of 2-methyl hexanoic acid as an internal standard (De Boever et al., 2000; De Weirde et al., 2010). Lactate concentrations were measured using the Enzytec™ kit (R-Biopharm, Darmstadt, Germany) on an iCubio iMagic-M9 (Shenzhen iCubio Biomedical Technology Co. Ltd., Shenzhen, China), according to the manufacturer's instructions. Ammonium analysis was run on an AQ300 Discrete Analyzer (Seal-Analytical, Rijen, The Netherlands) using the indophenol blue spectrophotometric method according to the manufacturer's instructions. Each measurement was performed in a single repetition.

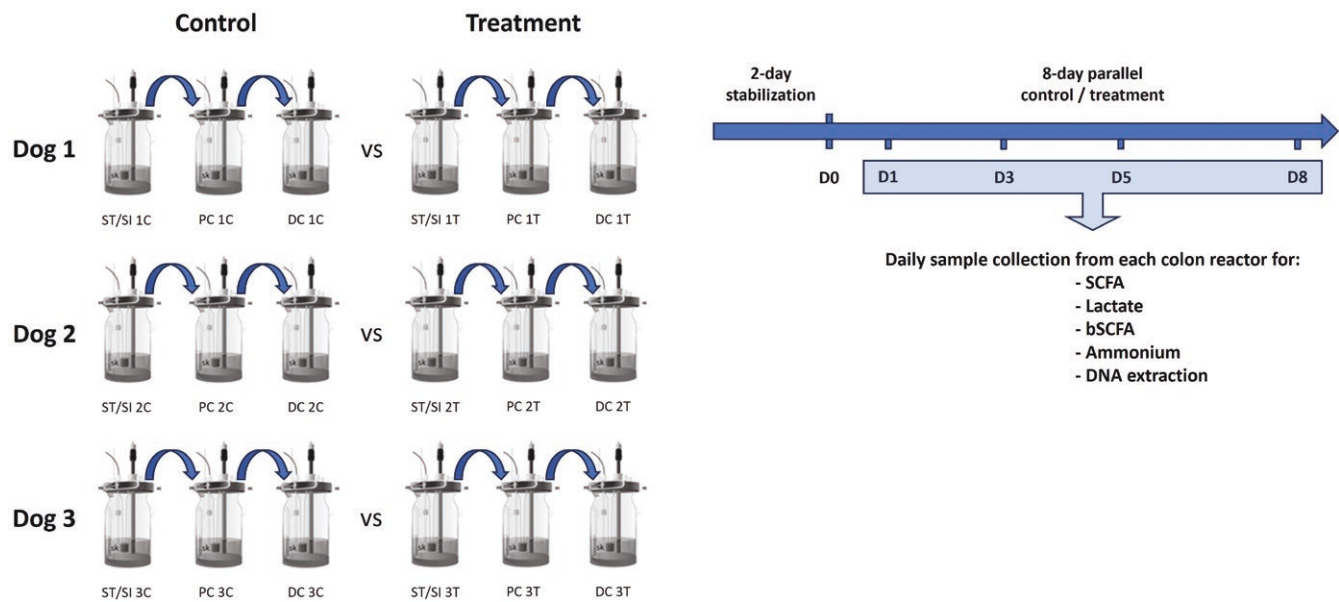


Figure 1. Schematic representation of the experimental design making use of a SCIME inoculated with the microbiota of 3 dogs with soft stools. The microbiota of each dog has been used to inoculate 2 systems, one acting as a control and the other treated with the test product. After 2 d of stabilization, the test product has been dosed in the 'treatment' arm of the study (D0). Samples for all analytical endpoints have been collected on D1, D3, D5, and D8 both in the proximal (PC) and distal colon (DC). bSCFA, branched short-chain fatty acid; D, day; DC, distal colon; PC, proximal colon; SCIME, Simulator of the Canine Intestinal Microbial Ecosystem; SCFA, short-chain fatty acid; ST/SI, stomach/small intestine reactor.

Microbial community composition

Total DNA was isolated from the collected luminal samples as described by Duysburgh et al. (Duysburgh et al., 2019). Briefly: the DNA was extracted from a pellet of bacterial cells originating from a 1 mL sample after centrifugation for 5 min at 7,700 g. A Fastprep-24 device (MP BioMedicals, Illkirch, France) was used for homogenization, which was performed twice for 40 s at 4 m/s; the sample was allowed to rest for 5 min between shakings. 16S-targeted Illumina sequencing utilized primers that span 2 hypervariable regions (V3–V4) of the 16S rRNA gene profiling (341F, 5'-CCTACGGG-NGGCWGCAG-3'; 785R, 5'-GACTACHVGGGTATCTA-AKCC-3'; Klindworth et al., 2013). A pair-end sequencing approach was used to sequence 2 × 250 bp and 424 bp amplicons were generated (LGC Genomics GmbH, Berlin, Germany). Fragments of this size are considered taxonomically more informative than smaller fragments. The MiSeq SOP, as described by the Schloss lab, was followed for read assembly and cleanup (Schloss and Westcott, 2011; Kozich et al., 2013). Briefly, mothur (v.1.44.3) was used to assemble reads into contigs, perform alignment-based quality filtering (alignment to the mothur-reconstructed SILVA SEED alignment, v138), remove chimeras (vsearch v2.13.3), assign taxonomy using a naïve Bayesian classifier (Wang et al., 2007) and SILVA NR v138_1, and cluster contigs into operational taxonomic units at 97% sequence similarity. Sequences that could not be classified and those classified as Archaea, Eukaryota, mitochondria, or chloroplasts were removed. The representative sequence was the most abundant sequence within an OTU. Reads with maximum abundances ≤5 across samples were considered artifacts or bacteria with no biological impact and were thus removed.

Samples that were analyzed by 16S rRNA targeted gene sequencing were also analyzed using flow cytometry to determine the number of total bacterial cells. This allowed for the

conversion of relative abundances into absolute abundances, which was done by multiplying the relative abundances of any population in a sample with the total cell count obtained for that sample using flow cytometry (Vandeputte et al., 2017). A BD Accuri C6 Plus Flow Cytometer (BD Biosciences, Franklin Lakes, New Jersey, USA) on the high flow rate was used to obtain cell counts. A threshold level of 700 on the SYTO channel was applied to separate bacterial cells from signal noise and medium debris. Parent and daughter gates were set as appropriate to determine all populations.

Statistical analysis

For statistical comparisons of microbial community activity, 2o-way ANOVA for repeated measures were used to compare the effect of repeated administration between both groups (interaction). One-way ANOVA for repeated measures were used to assess the effect of repeated administration in each group followed by the Tukey HSD test in case of significance to check where the difference lay. Two-tailed paired *t*-tests were used for the comparison of the test product supplementation arm with the control arm within each donor. Differences were considered statistically significant with a *P*-value < 0.05.

Beta diversity analysis was conducted by hierarchically clustering Euclidean distances between samples using Ward's minimum variance method. If absolute abundances were used (based on provided cell count data), a Variance Stabilizing Transformation was applied using DESeq2 v1.41.12 (Love et al., 2014) prior to the calculation of distances. Furthermore, a Discriminant Analysis of Principal Components (DAPC) plot was constructed with 2 discriminants and 80% of retained variance in the principal components using ADEGENET v2.1.10 (Jombart, 2008; Jombart et al., 2010).

A hierarchical Differential Abundance Analysis was conducted to identify the taxa most likely to explain differences between study arms. This was run on the Trimmed Mean of

M-values library sizes for each OTU using treeclimbR v0.1.5 and edgeR v3.42.4 (Huang et al., 2021). Benjamini–Hochberg multiple testing correction was used, and the alpha level was set at 0.05. Data from the treeclimbR analysis are shown in volcano plots, which are scatter plots that show statistical significance vs magnitude of change. The volcano plots classify the taxa into 4 distinct categories based on the abundance in compared study arms: not statistically significant and not biologically significant; biologically significant, but not statistically significant; statistically significant, but not biologically significant; or biologically and statistically significant. P -values ≤ 0.05 by the Kruskal–Wallis and Wilcoxon tests were considered statistically significant and a fold change (\log_2) > 2 was generally considered biologically relevant.

Results

Microbial metabolic activity

Measures of saccharolytic fermentation

In the PC, the acetate increased over time in both the test and control arms, with no difference between groups. The mean (SD) went from 21.3 (5.0) to 26.7 (4.1) mM in the control arm (+25%) and from 22.9 (8.0) to 29.1 (12.3) mM in the test arm (+27%), on days 1 and 8, respectively. Although the increase is lower with the control than with the supplement, it was significant only in the control group ($P = 0.026$, Figure 2A). There was however a significant difference between donors. In the DC, a similar increase over time was observed in both groups and tended to be significant with the test supplement ($P = 0.073$) but there was no difference between groups (Figure 2A). The mean (SD) went from 44.1 (4.6) to 47.6 (6.6) mM in the control arm (+8%) and from 44.8 (7.4) to 49.9 (4.3) mM in the test arm (+11%), on days 1 and 8, respectively. The difference between donors was also significant in the DC. SCFA and lactate levels for individual

donors can be found in Figure S1. Acetate production was significantly increased with the test product compared with the control for donor B in the PC ($P = 0.021$), and DC ($P = 0.020$; Figure S1A). The production also tended to increase in donor C in the PC ($P = 0.08$).

Propionate production in the PC significantly differed in the 2 groups over time ($P = 0.027$). There was no significant change with the control (from 42.4 ± 3.3 to 45.8 ± 3.1 mM on D1 and D8, respectively) while the production tended to increase over time with the supplement (from 40.8 ± 0.5 to 43.0 ± 0.6 mM, respectively, $P = 0.085$), and especially on day 3 (46.1 ± 3.9 mM, +13% vs day 1, $P = 0.068$) before decreasing again (Figure 2B). There was a significant difference between donors with the control but not with the test supplementation. In the DC, the production of propionate seemed to slightly increase in both arms on days 3 and 5, but there was no significant difference between time points and between groups. The mean (SD) went from 44.1 (3.4) to 46.6 (4.4) mM in the control arm (+6%) and from 42.8 (1.3) to 45.6 (4.1) mM in the test arm (+27%), on days 1 and 3, respectively. There was, however, a significant difference between donors in the control group. When considering the single donors, while propionate levels appeared numerically higher with the test product vs control for donor B (from D3), there was no significant difference in propionate levels between test product administration and the control for any donor in the PC (Figure S1B). In the DC, there was a significant decrease in propionate production with test product supplementation vs control for donor C ($P = 0.044$).

While butyrate levels seemed to increase over time in both groups in the PC and DC, there was no significant difference between time points and groups (Figure 2C). In the PC, the means (SD) went from 2.7 (4.2) to 6.2 (10.0) mM in the control arm (+131%) and from 2.4 (3.9) to 8.6 (9.2) mM in the test arm (+260%), on days 1 and 8, respectively. In the

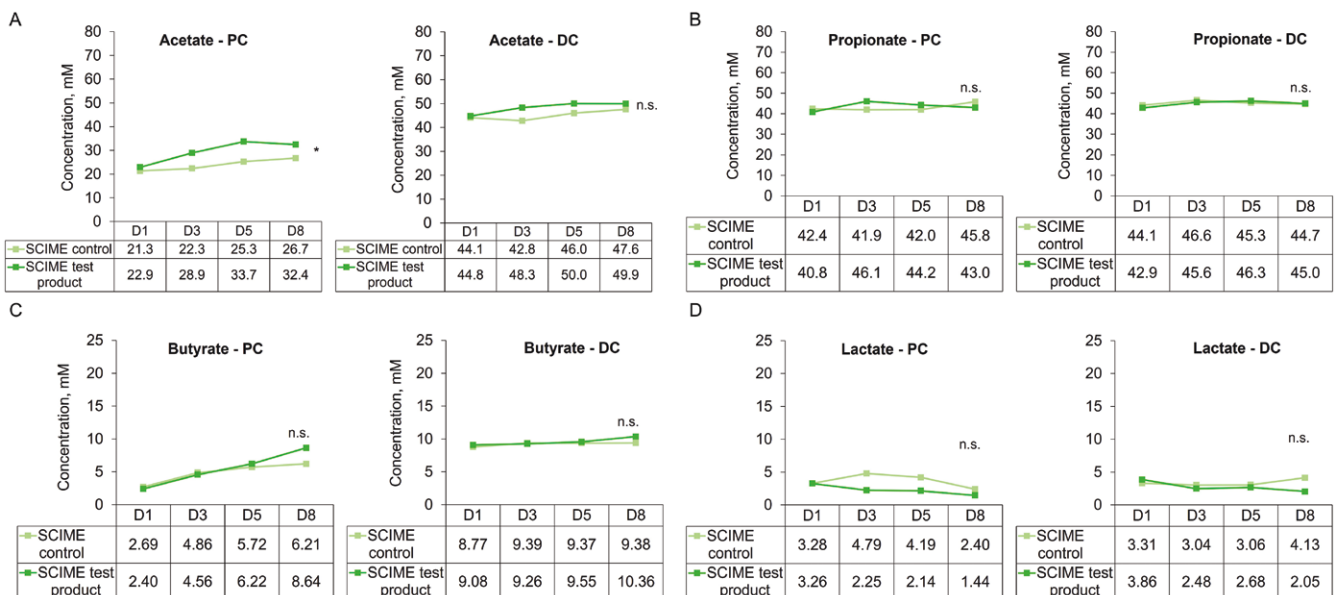


Figure 2. Microbial metabolic activity over time (D1, D3, D5, D8) in the proximal (PC) and distal colon (DC) of the SCIME is shown as changes in (A) acetate, (B) propionate, (C) butyrate, and (D) lactate concentrations. Each measurement was performed in a single repetition. Data for average values were derived using data from 3 canine donors. A 2-way ANOVA for repeated measures was used to compare the effect of repeated administration between control and treatment (interaction). * $P < 0.05$. D, day; DC, distal colon; PC, proximal colon; SCIME, Simulator of the Canine Intestinal Microbial Ecosystem.

DC they went from 8.8 (7.7) to 9.4 (8.4) mM in the control arm (+7%) and from 9.1 (7.3) to 10.4 (7.7) mM in the test arm (+14%), respectively. There was however a significant difference between donors in both control and treated groups. Butyrate levels were not affected by the test product for donor A in either the PC or DC (Figure S1C). Donor B had low butyrate levels overall. Donor C had a strong butyrogenic response with test product supplementation at D8 in the PC while the analysis over the whole period did not show any statistical significance. Butyrate levels were unaffected by supplementation in the DC for this donor.

Finally, lactate levels seemed to decrease over time in both groups, especially in the treated group, but there was no difference between time points and groups in the PC and DC (Figure 2D). In the PC, the means (SD) went from 3.3 (2.9) to 2.4 (2.2) mM in the control arm (−27%) and from 3.3 (5.1) to 1.4 (2.0) mM in the test arm (−56%), on days 1 and 8, respectively. In the DC, they went from 3.3 (2.9) to 4.1 (6.6) mM in the control arm (+25%) and from 3.9 (4.2) to 2.0 (3.4) mM in the test arm (−47%), respectively. There was however a significant difference between donors in all groups, in the PC and DC. For donor A, lactate production was low throughout the study and regardless of test conditions (Figure S1D). Donors B and C seemed to have reduced lactate levels relative to control in both the PC and DC, but significant differences were only observed for donor C in the PC ($P = 0.008$).

Markers of proteolytic fermentation

bSCFA levels seemed to increase in both groups but there was no difference between time points and groups, in the PC and DC (Figure 3A). Mean (SD) values went from 0.8 (1.3) to 1.5 (2.6) mM in the control arm (+87%) and from 0.8 (1.4) to 1.5 (2.4) mM in the test arm (+74%) in the PC, on days 1 and 8 respectively. In the DC, mean values went from 1.8 (2.1) to 2.5 (2.2) mM (+35%) and from 2.6 (1.9) to 2.7 (1.8) mM (+7%), respectively. There was again a significant difference between donors in both compartments. A significant increase in bSCFA was observed following product supplementation vs the control in the DC of donor B ($P = 0.015$; Figure S2A).

Ammonium levels significantly increased in both conditions in the PC, particularly on day 8 ($P = 0.036$ and 0.006 in the control and test conditions, respectively) with no difference between groups (Figure 3B). Mean (SD) values went from 81.3 (26.7) to 170.7 (88.6) mM in the control arm (+110%) and from 85.7 (17.6) to 201.7 (63.1) mM in the test arm (+135%) in the PC, on days 1 and 8, respectively. No

difference between time points and conditions was observed in the DC. Mean (SD) values in the DC went from 342.0 (65.1) to 356.3 (69.2) mM in the control arm (+4%) and from 359.7 (1.9) to 368.0 (32.0) mM in the test arm (+2%); respectively. While donors A (in the PC and DC) and C (in the DC) seemed to have reduced ammonium levels with test product supplementation vs control, the level was significantly increased with test product supplementation in both the PC and DC of donor B ($P = 0.019$ and $P = 0.001$, respectively; Figure S2B).

Microbial community composition

In the DAPC plots for the SCIME model, there was a clear shift with test product treatment vs control in both the PC and DC (Figure 4). Absolute levels of phyla and families for each single donor are shown in Tables S1, S2, and S3.

Firmicutes were the most abundant phylum in both colon regions (Figure 5). During test product supplementation vs the control, the abundance of Bacteroidota and Fusobacteriota increased, while that of Proteobacteria decreased in the PC, and the abundance of Firmicutes increased in the DC.

treeclimbR analysis (i.e., differential abundance analysis) was used to identify which bacterial groups were responsible for the significant and/or biologically relevant changes observed in microbial community composition. Across the different donors tested, bacterial groups involved in primary substrate degradation were enriched in the PC following test product supplementation compared with the control. *Bifidobacterium* was enriched at D1 and D3, reaching levels close to biological relevance. *Prevotella* abundance was enriched with test product supplementation towards the end of the treatment period (reaching levels close to biological significance) mainly at the expense of the biologically reduced *Bacteroides*. Furthermore, *Sutterella*, a member of the Proteobacteria phylum, had a biologically significant lower abundance at D1 following product supplementation (Figure 6). In the DC, the butyrate-producing genus *Fusobacterium* was consistently enriched, reaching biological relevance at D1, D3, and D5, and the butyrate-producing *Ruminococcaceae* family was enriched, reaching levels close to biological relevance at D5 and D8, with test product supplementation vs control (Figure 6).

Discussion

Overall, this study suggested an increase in microbial fermentation in the gut microbiome of canine donors with soft

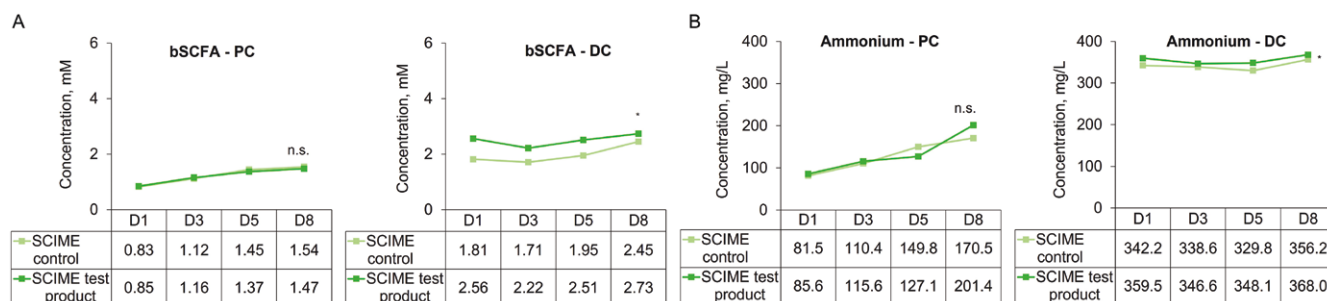


Figure 3. Microbial metabolic activity over time (D1, D3, D5, D8) in the proximal (PC) and distal colon (DC) of the SCIME is shown as changes in (A) bSCFA and (B) ammonium concentrations. Each measurement was performed in a single repetition. Data for average values were derived using data from 3 canine donors. A 2-way ANOVA for repeated measures were used to compare the effect of repeated administration between control and treatment (interaction). * $P < 0.05$.

bSCFA, branched short-chain fatty acid; D, day; DC, distal colon; PC, proximal colon; SCIME, Simulator of the Canine Intestinal Microbial Ecosystem.

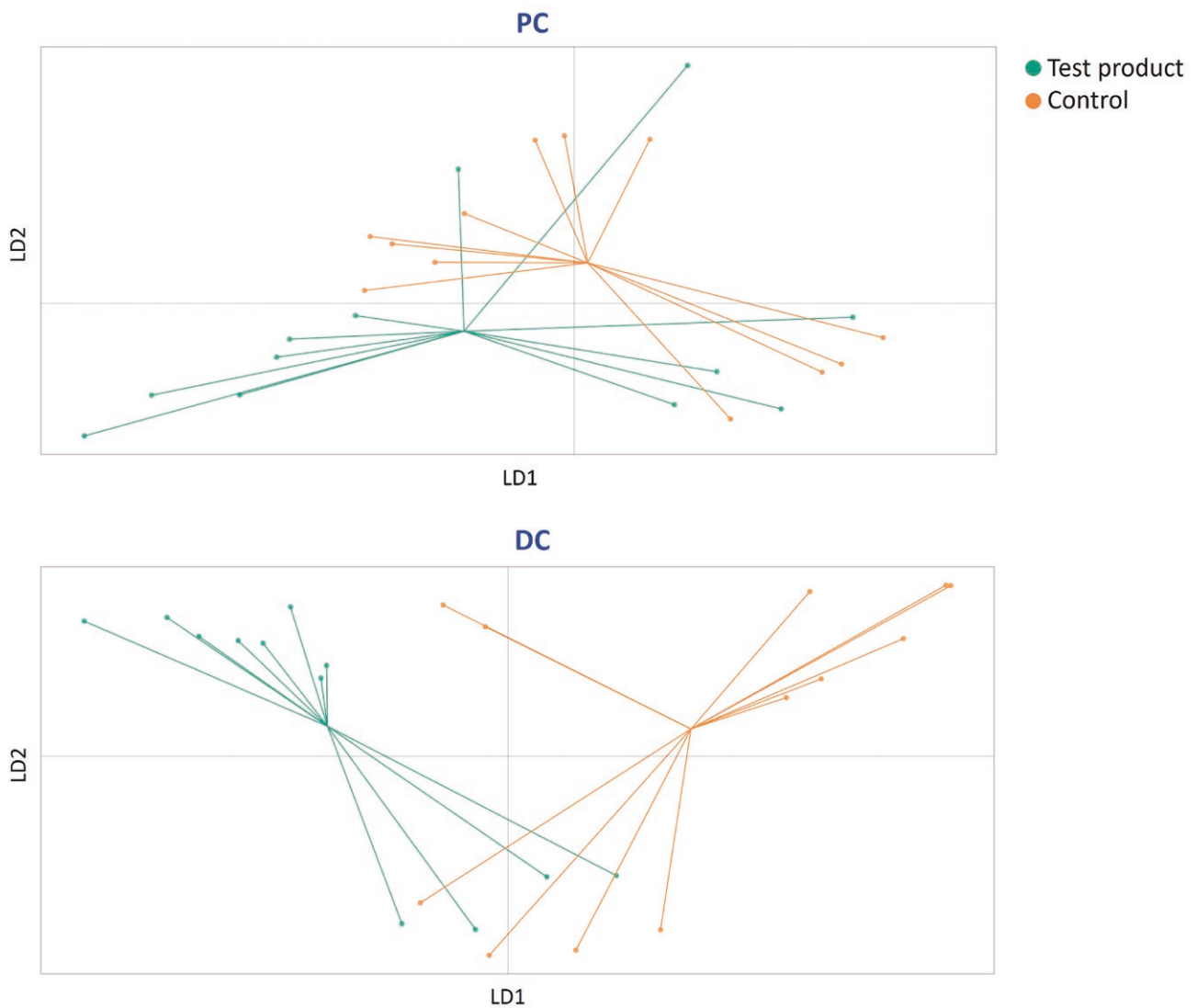


Figure 4. Discriminant analysis of principal components showing differences in community composition following treatment with the test product or control for the 3 canine donors in the SCIME model (days 1, 3, 5, and 8). Control, $n = 12$; test product, $n = 12$. Each dot represents one sample. DC, distal colon; PC, proximal colon; SCIME, Simulator of the Canine Intestinal Microbial Ecosystem

stools following supplementation with the test product. Propionate levels increased in the PC early after supplementation. Acetate and butyrate were also increased, although more in a donor-dependent manner while lactate levels tended to be decreased with supplementation compared to control. The health-promoting *Bifidobacterium* and *Prevotella* genera were mainly responsible for the observed increase in microbial fermentation.

Lactate and SCFAs, including acetate, propionate, and butyrate are byproducts of saccharolytic fermentation in the colon; their levels reflect overall fermentation by the gut microbiota. Lactate can be converted to butyrate and/or propionate by lactogenic microbes (Culp and Goodman, 2023). Lower levels of lactate following treatment can be considered as an indicator of efficient cross-feeding interactions within the microbial community. Supplementation with the test product resulted in increased propionate and acetate production, although not to a significant level for the latter. The increase in both SCFAs was mainly observed in the PC indicating that the test product can be easily fermented. Butyrate levels appeared

to increase later in the supplementation period, particularly in the PC, but the changes were not significant upon considering the entire treatment period. Butyrate is an important product of microbial fermentation, as it is a primary source of energy for epithelial cells and helps to maintain a healthy intestinal barrier (Martin-Gallausiaux et al., 2021).

The effect on SCFA production varied a lot, depending on the gut microbiota of the selected donors. Individual donor results indicated that there may be potential for test product supplementation to have a propiogenic and butyrogenic effect in some specific canine donors. It has been shown that dogs with serious GI problems (i.e., inflammatory bowel disease) have significantly lower levels of fecal acetate, propionate, and butyrate compared with healthy dogs (Minamoto et al., 2019). Thus, the test product-dependent increase in propionate and butyrate production, for some individual donors, can be considered a beneficial effect that must be further investigated with a higher number of donors.

bSCFAs and ammonium are indicators of proteolytic fermentation, which is generally considered less desirable

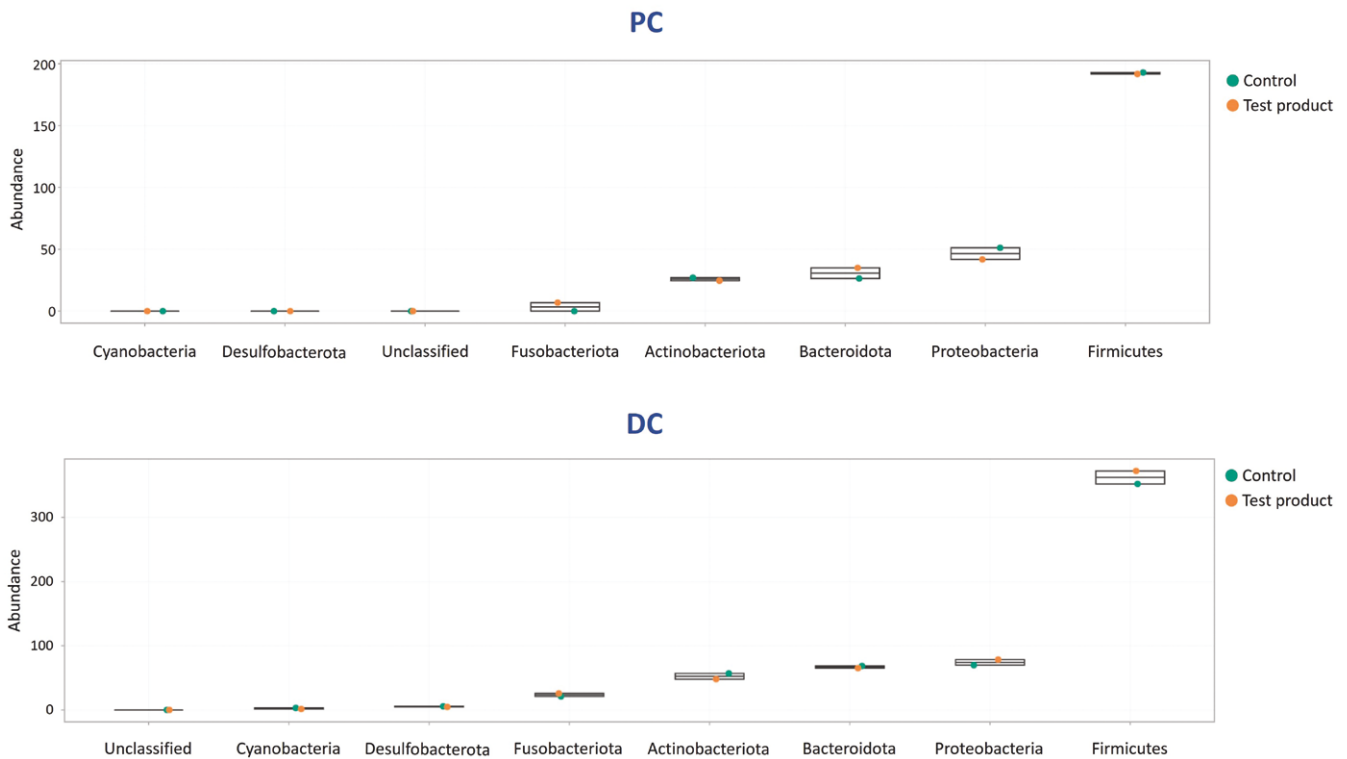


Figure 5. Jitter plots showing average abundances (log2 abundances) across all donors at the phylum level in the proximal (PC) and distal colon (DC). Data for average values were derived using data from 3 canine donors across all timepoints (D1, D3, D5, and D8). DC, distal colon; PC, proximal colon; SCIME, Simulator of the Canine Intestinal Microbial Ecosystem.

because the process involves the production of highly toxic compounds (Diether and Willing, 2019). In the PC for the SCIME model, changes in bSCFA and ammonium levels were similar to the test product and control. Significant increases vs control were observed for both parameters (in the DC mainly) in only one donor (B) and the levels of both bSCFA and ammonium remained within the normal physiological range (Duysburgh et al., 2020; Van den Abbeele et al., 2020).

DAPC plots evaluating beta diversity showed distinct clustering of the test product and control samples, indicating changes in the microbial community composition with repeated administration of the test product. Indeed, microbial community composition analysis demonstrated that primary substrate degraders involved in saccharolytic fermentation were enriched following repeated administration of the test product and provided insight into the mechanisms behind the increases in specific SCFAs. The increased abundance of the Bacteroidota phylum with test product supplementation vs control likely explains the increased propionate and acetate production (donor-dependent), as both SCFAs are mainly produced by bacteria in this phylum (Garcia-Lopez et al., 2019). The treatment with the test product also increased the concentration of Firmicutes and of Fusobacteriota phylum (the latter especially for Donor C). Most butyrate-producing bacteria are members of the Firmicutes phylum (Singh et al., 2022) and members of the Fusobacteriota phylum are also butyrate-producers. A positive effect on butyrate production was only observed with donor C, thus again highlighting the importance of inter-individual variability in assessing the modulation of the gut microbiota. In dogs, Fusobacteriota are severely impacted by gastrointestinal diseases and their recovery is slower than

other phyla (Pilla and Suchodolski, 2019; Pilla et al., 2020). Attempting to increase Fusobacteriota using specific food ingredients, such as those found in the test product, maybe a useful strategy for improving gastrointestinal diseases. Furthermore, the strong enrichment of *Bifidobacterium* early in the SCIME study reached levels close to biological relevance. *Bifidobacterium* are considered beneficial saccharolytic bacteria with versatile fiber-degrading potential. They mainly produce acetate and lactate, which are important drivers of cross-feeding interactions with other bacteria that result in the production of health-promoting metabolites such as propionate and butyrate (Riviere et al., 2016). Thus, the enrichment of *Bifidobacterium* is associated with a potential health benefit.

The Proteobacteria phylum is mostly linked with enteric diseases as it contains several opportunistic pathogenic microorganisms, as well as commensal bacteria involved in proteolytic fermentation (Rizzatti et al., 2017). The reduced abundance of Proteobacteria observed with test product administration strengthens the conclusion that primary substrate degraders involved in saccharolytic fermentation were enriched following repeated administration of the test product, thereby reducing proteolytic fermentation as indicated by the reduction in ammonium production in some donors.

As with any in vitro study, the study findings are limited in that they cannot be linked directly to in vivo effects. Future studies of the test product in dogs with a defined dysbiosis should therefore be conducted to confirm these in vitro findings. Considering the individual donor data, the study findings indicate that there were some donor-dependent differences in the test product effects. This suggests that the effects may be dependent on the level of specific microbiome composition

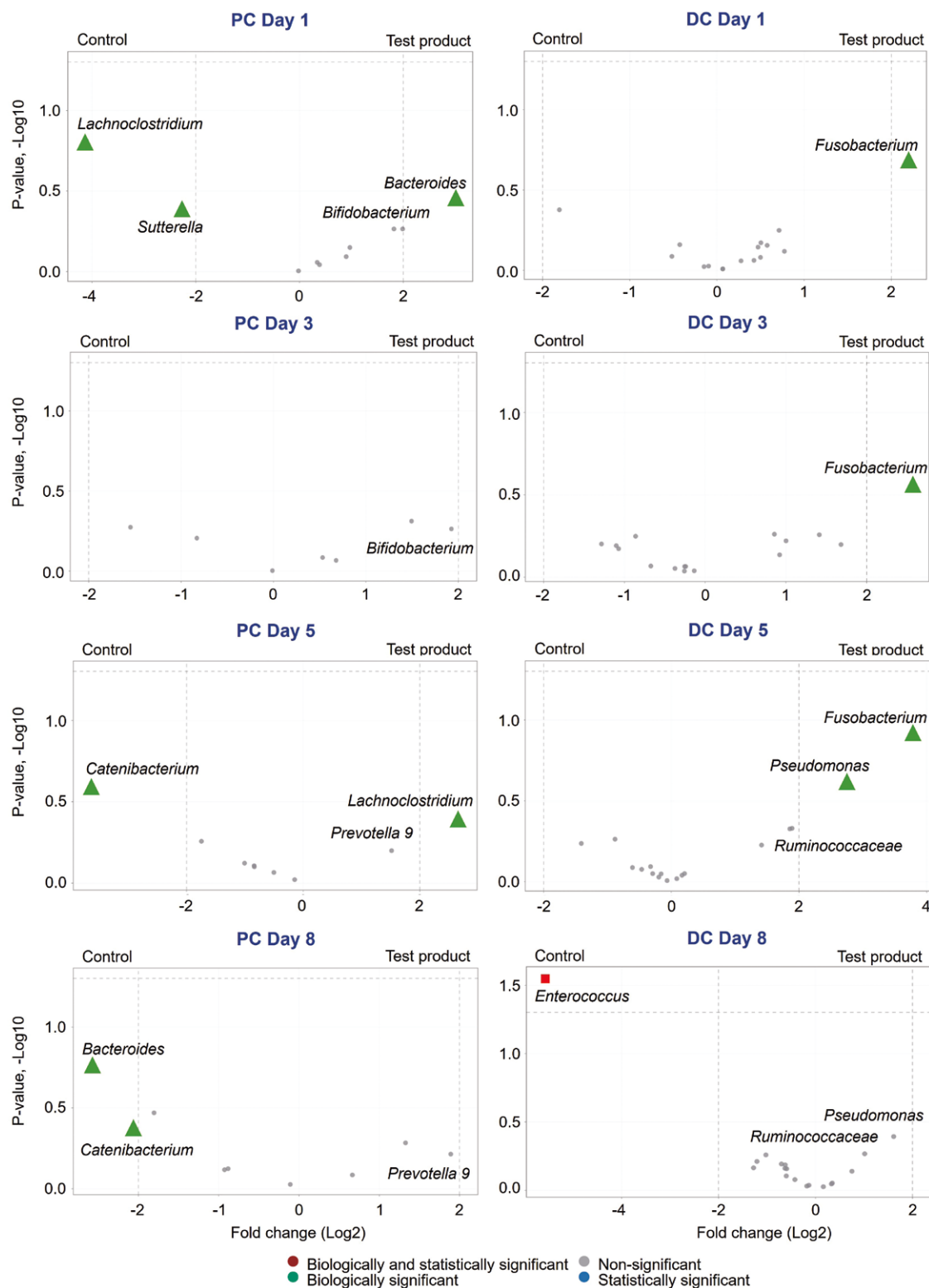


Figure 6. Differential abundance analysis at days 1, 3, 5, and 8 in the proximal (PC) and distal colon (DC) across 3 canine donors using treeclimbR analysis. The analysis is based on relative abundance data (total sum scaling). The scatter plot classifies taxa into 4 categories based on abundances in the compared conditions: biologically and statistically significant (square), biologically significant but not statistically significant (triangle), neither biologically nor statistically significant (round), or statistically significant but not biologically significant (star) for the difference between test product and control. DC, distal colon; PC, proximal colon; SCIME, Simulator of the Canine Intestinal Microbial Ecosystem.

of an individual dog and as such may not be generalizable to all dogs.

In conclusion, using *in vitro* models inoculated with the fecal microbiota of dogs with soft stools, it was demonstrated

that test product supplementation improved SCFA production, increased gut microbiome diversity, and enhanced the growth of several gut bacteria associated with health benefits to the host. Changes in SCFA production could be linked

with changes in specific microbial abundance. The changes observed in this pilot study are indicative of the fact that repeated administrations of the test product may have the potential to reduce dysbiosis-associated symptoms in dogs with moderate digestive problems, such as soft stools, and that this potential must be further investigated in terms of interindividual variability following repeated administration.

Supplementary Data

Supplementary data are available at *Journal of Animal Science* online.

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Conflict of interest statement

C.N., F.L., P.M., and C.R. are employees of Virbac SA.

Author Contributions

Cindy Duysburgh (Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Validation, Visualization, Writing—original draft, Writing—review & editing), Celine Nicolas (Conceptualization, Project administration, Supervision, Writing—review & editing), Mattia Van den Broeck (Data curation, Formal analysis, Investigation, Writing—review & editing), Fanny Lloret (Writing—review & editing), Patricia Monginoux (Writing—review & editing), Christophe Rème (Supervision, Writing—review & editing), and Massimo Marzorati (Conceptualization, Methodology, Supervision, Writing—review & editing)

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