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Microbiota of healthy dogs demonstrate a significant decrease in richness and changes in specific bacterial groups in response to supplementation with resistant starch, but not psyllium or methylcellulose, in a randomized cross-over trial.

Silke Salavati Schmitz[1$], Jorge Perez-Accino Salgado[1*], Laura Glendinning[2],

[1] Hospital for Small Animals, Royal (Dick) School of Veterinary Studies, College of Medicine and Veterinary Medicine, University of Edinburgh, Easter Bush Campus, Midlothian, EH25 9RG, UK

[2] The Roslin Institute, University of Edinburgh, Easter Bush Campus, Midlothian, EH25 9RG, UK

* This author's current affiliation is: Hospital Canis, Carrer Can Pau Birol 38, 17006 Girona, Spain

[$] corresponding author:

Prof. Silke Salavati Schmitz

University of Edinburgh, College of Medicine and Veterinary Medicine

Hospital for Small Animals, Royal (Dick) School of Veterinary Studies

Easter Bush Campus, Midlothian, EH25 9RG, UK

Telephone: +44(0)131-6507650

Fax: +44(0)131-6507672

Email: Silke.Salavati@ed.ac.uk

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Abstract

Even though dietary fibers are often used as prebiotic supplements in dogs, the effect of individual types of fibers on canine microbiota composition is unknown. The objective of this study was to assess changes in fecal microbiota richness, diversity and taxonomic abundance with 3 different fiber supplements in dogs. These were psyllium husk, resistant starch from banana flour and methylcellulose. They were administered to 17 healthy dogs in a cross-over trial after transition to the same complete feed. Fecal scores and clinical activity indices were recorded, and fecal samples collected before and at the end of supplementation, as well as 2 weeks after each supplement (washout). Illumina NovaSeq paired-end 16S rRNA gene sequencing was performed on all samples. After quality control and chimera removal, alpha diversity indices were calculated with QIIME. Differences in specific taxa between groups were identified using Metastats. Methylcellulose significantly increased fecal scores but had no effect on microbiota. Psyllium resulted in minor changes of specific taxa abundance, but with questionable biological significance. Resistant starch reduced microbiota richness and resulted in the most abundant changes in taxa, mostly a reduction in short-chain fatty acid producing genera of the Bacillota phylum, with an increase in genera within the Bacteroidota, Pseudomonadota, Actinomycetota and Saccharibacteria. In conclusion, while psyllium and methylcellulose led to few changes in the microbiota composition, the taxonomic changes seen with resistant starch may indicate a less favorable composition. Based on this, the type of resistant starch used here cannot be recommended as a prebiotic in dogs.

Data summary:

The paired-read fastq data that support the findings of this study have been deposited in the European Nucleotide Archive with the accession code PRJEB67805 (https://www.ebi.ac.uk/ena/browser/view/PRJEB67805). For the purpose of open access, the authors have applied a Creative Commons Attribution (CC BY) license to any Author Accepted Manuscript version arising from this submission.

Introduction

A number of health benefits are associated with the consumption of dietary fiber (DF), including compositional as well as functional changes of the intestinal microbiota (IM); for example plant and carbohydrate rich diets result in increased IM richness and bacterial short chain fatty acid (SCFA) production (1).

A low intake of DF does not only lead to reduced IM diversity, but also shifts the gut microbial metabolism away from SCFAs towards less favorable bacterial metabolites, often derived from amino acids (e.g. branched-chain fatty acids, ammonia, phenolic and indolic compounds and hydrogen sulfide), which can be detrimental to host health (1). The cytotoxic and pro-inflammatory nature of these metabolites contributes to the development of chronic diseases, particularly an increased prevalence of inflammatory bowel disease (IBD) and colorectal cancer (2).

A number of DFs are also considered prebiotics, defined as “a substrate that is selectively utilized by host microorganisms conferring a health benefit” (3). DFs are additionally classified by their physicochemical properties (water solubility and viscosity), as well as their fermentability. In most cases, soluble fiber types are fermented for example into short chain fatty acids (SCFAs) more quickly than insoluble types (1).

Chemically, DFs can be divided into 3 main classes: 1) non-starch polysaccharides (NSPs), which includes non-fermentable fibers like psyllium seed husk [*Plantago ovata*] (PSY), 2) resistant (non-digestible) oligosaccharides (for example fructo-oligosaccharides (4)); and 3) resistant starch (RS), for example granular starches from green bananas (5).

There is evidence that supplementation with specific DF restores some of the health benefits they can infer. Different types of RS (6), cellulose (7), and PSY have specific effects on the IM. For example, RS has been shown to enrich *Bifidobacteria*, *Ruminococcus* and other beneficial bacteria (1). PSY supplementation induces distinct IM community changes, including higher relative abundance of *Bacteroides* and *Parabacteroides* (fiber-digesting bacterial groups) up to 70% of the total IM bacteria, with a matching increase in SCFA production; and reduction of *Enterobacteriaceae* and *Pseudomonadaceae* (8). Even the nonfermentable fiber methylcellulose (MTC) has been shown to modulate IM composition and diversity as well as fecal bile acid metabolism and prevent weight gain in mice fed a high fat diet (9).

Not only do companion animals like dogs have a similar IM composition and richness to people (10,11), as they have co-evolved to be able to digest similar food (12) and can hence serve as appropriate model for IM related interventions; in “industrialized” populations, they also suffer from similar emerging diseases, including chronic inflammatory gut conditions like IBD (11,13). Different DFs (especially PSY) are commonly used in canine feed or given as supplements to individual pet dogs for a variety of spontaneously occurring intestinal conditions (e.g. “fiber-responsive” colitis (14), irritable bowel syndrome (15)), but very little is known about the effect of specific DF on dogs’ IM composition or function. One study showed that PSY results in a significant increase of the SCFAs propionate and n-butyrate in fecal samples of dogs after 15 days of supplementation (16). In addition, PSY might protect against colitis via activation of bile acid receptors in intestinal epithelial cells (17), which

could be particularly relevant, as severely altered fecal bile acid metabolism has recently been identified as a hallmark of canine IBD (18).

The goal of this study was to investigate the effect of 3 commonly used DF types on the IM composition and richness of healthy dogs to assess their potential health benefits and create evidence-base for their use as prebiotics in both dogs and potentially people.

Methods

Animals, feeding interventions and clinical scores

Dogs recruited for the study were privately owned pets and deemed healthy based on the absence of clinical signs and normal physical examination findings (table S1). They all had to be regularly wormed and treated for ectoparasites, and not have travelled abroad. Dogs on a prescription diet or any regular medication or supplements were excluded. Owners were asked to collect a freshly voided fecal sample (day -14) before transitioning all dogs to the same commercial complete dry dog food (Hill's Science Plan Advanced Fitness medium adult tuna and rice) at 1.4-1.8 RER depending on lifestyle for 2 weeks, after which another fecal sample was collected (day 0, baseline). After that, dogs were maintained on the same diet and randomized to receive each of the 3 study fiber supplements for a duration of 2 weeks, followed by a 2-week washout period before the next supplement (figure 1). Throughout the duration of the study, the dogs' diet was not permitted to be changed. Fresh fecal samples were collected on the last day of each supplementation and washout period (day 14, 28, 42, 56, 70, and 84). Owners were asked to keep a diary, that included daily confirmation of supplement administration (which were labelled A, B and C in otherwise plain dispensing bags), and scoring of each naturally voided fecal sample's consistency using a pictorial template of a well-established tool (Purina Fecal Score; PFS), where score 1 represents very hard and dry feces to score 7, which represents watery feces with no texture. In addition, owners were asked to fill in a validated questionnaire, the Canine IBD

Activity Index (CIBDAI (19)) at day -14 and 0, on the last day of each supplement (day 14, 42, and 70) and after a final washout of 2 weeks (end of study, day 84).

DF supplements used were commercially available food-grade additives, namely PSY husk (Colon Care Plus, Holland & Barrett, UK), a resistant starch (Green Banana Flour; Natural Evolution, UK) and MTC (Methocel, SpecialIngredients, UK). Dosing was identical for all 3 DFs, twice daily and based on individual dog's body weight: dogs < 5 kg received 2 g, dogs 5-10 kg 4 g, dogs >10-30 kg 8 g, dogs >30-50 kg 12 g and dogs > 50 kg 16 g of each supplement. This dosage was derived from empirically available doses for PSY (20). Supplements were advised to be given with the normal food ration and using a specific measuring spoon provided with specific dosing instructions for each supplement and dog to allow administration of the correct amount.

Fecal DNA extraction, 16S rRNA gene amplification, and sequencing

Fecal samples were aliquoted into sterile 5 ml Bijoux tubes within 60 minutes of receipt at the hospital and stored at -80° C until the time of analysis. DNA extraction was performed as described previously (21) using the DNeasy PowerLyzer PowerSoil Kit (Qiagen).

Extracted genomic DNA was sent to a commercial service provider (Novogene, Cambridge, UK; www.novogene.com) for 16S rRNA gene PCR amplification, DNA sample quality control, amplicon library preparation and Illumina NovaSeq paired-end sequencing with 30x coverage. Briefly, DNA concentration and purity was assessed on 1% agarose gels and DNA diluted to 1 ng/ml. The V4 region of the 16S rRNA was amplified using the primers GTGCCAGCMGCCGCGGTAA and GGACTACHVGGGTWTCTAAT with barcodes. All PCR reactions were carried out with Phusion High-Fidelity PCR Master Mix (New England Biolabs), resulting in an amplicon of ~300 bp in size. PCR products were mixed at equal density ratios, purified with Qiagen Gel Extraction kit, and the library generated with NEBNext® Ultra DNA Library Prep kit for Illumina (quantified via Qubit and q-PCR).

Sequence data processing, OTU clustering, taxonomic annotation and diversity analysis

Primer sequences were removed, then paired-end reads were merged using FLASH (v.1.2.7) (22). Quality filtering was used to obtain high-quality clean reads using the split_libraries_fastq.py command from Qiime (v.1.7.0) (23,24). Chimeras were detected using UCHIME (25) with the SILVA reference database (v.138.1) (26), then removed. OTU clustering at ≥ 97% similarity was performed using UPARSE (v.7.0.1090). A representative sequence for each OTU underwent taxonomic assignment, using the Qiime (v.1.7.0) command assign_taxonomy.py (mothur method) with the SILVA Database (26).

Statistical analysis

OTU counts were normalized by subsampling to the sample with the least counts (39,442 reads). Relative abundance values are reported throughout as a value between 0 and 1, where 0 indicated the complete absence of the taxa and 1 indicates that the taxa is 100% abundant. Subsequent alpha and beta diversity analyses were all performed on these normalized counts. Alpha diversity indices, including Observed-species (OTUs), Chao1 and Shannon, were calculated with QIIME (v.1.7.0). Significant differences in specific taxa between groups were identified using Metastats (27), with the Benjamini and Hochberg False Discovery Rate (27) used for correcting for multiple tests (adjusted p-value = q-value). NMDS graphs were constructed using values produced by metaMDS from the vegan (v.2.6.4) package, using Bray-Curtis dissimilarity values. PERMANOVA analyses were conducted using Bray-Curtis dissimilarity values, and the adonis2 command from the vegan (v.2.6.4) package. The significance of differences in abundance between groups of specific genera of interest was calculated using the Wilcoxon test.

Clinical data (CIBDAI and PFS) as well as alpha diversity indices were analyzed and compared using GraphPad Prism 9.5.1 for Windows, GraphPad Software, San Diego, California USA (www.graphpad.com). Data were tested for normality using Shapiro-Wilk tests and compared using Kruskall-Wallis tests with Dunn's multiple comparison test as post hoc analysis.

Results

Animal characteristics

A total of 24 dogs were initially recruited. Of those, 5 did not reach the end of their first supplementation stage due to palatability issues with the supplement. Two further dogs were removed from the study as they developed unrelated medical problems that prevented them from completing the study. Data from the remaining 17 dogs was included in the analysis. However, from 2 of those dogs, no fecal sample was collected at day 0 as they were accidentally transitioned to the first supplement too early. Another 2 dogs completed the first and second supplementation and washout phases but developed diarrhea with the third supplement (MTC), so they were taken off this supplement and a final sample collected at day 84. Hence, a complete dataset was available for 13/17 dogs.

For the 17 dogs, the order of DF supplements given can be found in table S2 and the available samples in table S3.

Methylcellulose, but not psyllium husk or resistant starch, causes an increase in fecal scores, which returned to normal at the end of washout periods:

Fecal scores at the start of the study were a median of 2 (range 1-4) and did not significantly change with the dietary transition (median of 2, range 1-3) ($p > 0.99$). There was also no difference between baseline samples and supplements with the exception of MTC, resulting in a median PFS of 5 (range 3-7) (figure 2A); and no significant change in fecal scores between the different washout periods ($p = 0.7$; figure 2B).

CIBDAI values were low at the start (day -14) with a median of 2 (range 1-4), and remained low throughout the supplementation periods, with no significant differences between DF supplements (see figure 3).

Dietary change did not significantly influence the intestinal microbiota:

Prior to quality filtering, samples contained 99,501 ± 17,945 (mean ± SD) OTUs. After quality filtering and clustering of OTUs, 1964 OTUs were identified, and samples contained OTU counts of 82,104 ± 14,848 (mean ± SD). All samples were then sub-sampled to 39,442 OTU counts prior to further analysis (table S4, S5). Rarefaction curves for samples plateaued, indicating that the sequencing depth was adequate. Based on these plots, observed OTU numbers reduced upon dietary change from baseline, but not significantly so (figure S1). Similarly, diversity indices remained unchanged (figure 4). Based on this, data from day 0 were considered the "baseline" for all subsequent analyses.

Resistant starch but not psyllium husk or methylcellulose reduce microbiota richness, but changes recovered during the washout period

Of the different supplements, only RS resulted in significant IM changes, as evidenced by a significant reduction in observed OTUs and Chao1, but unaltered Shannon diversity (figure 5).

When assessing relative abundances, there was no meaningful difference on the phylum level (figure S2). The average composition of the most abundant families and genera were also similar across groups (figure S3).

Baseline and DF treated samples did not cluster significantly separately by their overall community composition (PERMANOVA: $P > 0.05$, figure 6). Using Metastats to identify taxa that differed significantly between groups, 12 genera were found to be significantly differently abundant between baseline samples and PSY treated samples ($q < 0.05$, Sup_D-vs-P_metastats_genera.xls), with 8 that were more abundant in baseline samples (*Deinococcus*, *Hydrogenophilus*, *Anaerotruncus*, *IS-44*, *Kocuria*, *Exiguobacterium*, *Psychroglaciecola* and *A2*) and 4 that were more abundant in treated samples (*Psychrobacter*, *Anaerovibrio*, *Pseudoalteromonas*, *Lysinibacillus*). However, all of these genera were low in abundance, with the most abundant per group being *Psychrobacter,* at

0.00024 ± 0.00023 (mean ± SE). No genera were found to be significantly different between baseline and MTC treated samples (Sup_D-vs-M_metastats_genera.xls).

Thirty-seven genera were found to differ between baseline samples and samples after supplementation with RS (Sup_D-vs-R_metastats_genera.xls), with 21 being more abundant in baseline samples and 16 being more abundant in treated samples (table 1 and figures 7 and 8).

The relative abundance of specific bacterial groups of interest (e.g. part of the "dysbiosis index" [(28)] or associated with gut health in dogs in the literature) was also assessed. Using Wilcoxon rank test, there was a significant decrease in *Faecalibacterium* and *Peptoclostridium* with RS supplementation (figure 9), but this significance was not upheld after false discovery rate control. There was no difference in the abundance of any of those selected bacteria with the other DF supplements compared to baseline.

Discussion

This is the first study to assess and compare detailed fecal microbiota changes associated with supplementation of 3 types of commonly used DFs as specific supplements in dogs. While other studies have determined microbiota changes with high-fiber extruded diets (for example in comparison to hydrolyzed or high protein diets (29), for weight loss (30) or to modulate intestinal postbiotics (31)) or functional properties of certain types of fiber naturally occurring in raw ingredients (for example grains or cereals (32), miscanthus (33) or – recently – red ginseng (34)) or fiber blends (31), there is no study that compares single DFs from different broad fiber "categories" in the same dogs. This seems particularly surprising for psyllium husk, as this is one of the most commonly recommended DF for the use in intestinal disorders in dogs (35,36). For this reason, PSY was chosen as one of the supplements in the present study. Representatives of the other DF categories were chosen based on their easy availability (e.g. household ingredients), with green banana flour as a source of resistant

starch (RS) and pure methylcellulose (MTC; used as a gelling agent in baking, but also occasionally as a laxative (37)) as a non-digestible fiber. The latter effect of MTC was confirmed in this study, as it was the only one of the 3 supplements that caused a softer fecal consistency (as evidenced by significant increases in fecal scores). This did, however, not extend to any other unwanted adverse effects, as – based on the static CIBDAI indices – activity levels, appetite and other clinical parameters remained unchanged.

Overall, the effects of all 3 DFs on the microbiota richness and composition as assessed by 16S sequencing in these healthy dogs was mild, with most numerous changes found with RS supplementation, while there were no significant microbiota alterations with MTC. For PSY, while significant, differences found were in low abundance genera, hence these may not be biologically relevant. The most abundant change for PSY was for genus *Psychrobacter*, which belongs to the family Moraxellaceae, of the order Pseudomonadales within the class of Gammaproteobacteria. *Psychrobacter* is a widespread and evolutionarily successful group of bacteria and is likely to have a role as a commensal, degrading various dissolved organic carbon compounds other than sugars (38). Some species of the genus have been isolated as cause of human infections (38), and as it is a Gram-negative bacterium carrying hypoacylated lipopolysaccharides, it induces a TLR4-mediated inflammatory response (39). While this study does not dispute any clinical benefit seen with PSY supplementation in dogs with GI conditions, it does not support that any benefits are derived from significant microbiota changes. However, it is possible that changes would be more evident when giving PSY to dogs with specific GI conditions instead of healthy dogs. In addition, we did not assess microbiota function directly (e.g. measuring SCFA production). Prediction of metagenomic functions based on 16S data, for example using enrichment analysis (40) or bioinformatic tools like PICRUSt2 (41), is unlikely to be meaningful, as canine-specific databases are currently not of the desired quality, and tools are biased towards human microbiota taxa. It is possible that PSY administration was not of sufficient

duration or dose to detect related microbiota changes or that changes are related to taxa or species of very low abundance, which is difficult to capture with 16S sequencing alone.

Supplementation with RS led to the greatest number of taxa differing in comparison to baseline samples. This may indicate that RS supplementation has a greater effect on fecal microbiota composition. This is also supported by the significant differences that were seen in richness (but not diversity indices) for this DF. Interestingly, in another study that used RS as a supplement in healthy dogs, no changes of α- or β-diversity were observed (42).

Interpretation of the type of changes in microbiota abundance with RS supplementation and their meaning is challenging, as the majority of genera are not well described with regards to their microbiological niche, main physiological function or relevance in disease in dogs. The overall observation when assessing the phylogeny is that Firmicutes (renamed Bacillota) were generally reduced in their abundance (all of the ones belonging to the class of Clostridia, and some of the class of Bacilli; compare figure 7), while Proteobacteria (now Pseudomonata) and Bacteroidetes (now Bacteroidota) were increased (compare figure 8). Of the 21 taxa with lower abundance after RS supplementation, 8 have been associated with gut homeostasis, repair or SCFA production (*Sellimonas* (43), *Negativibacillus* (44,45), *Eubacterium* (46), *UCG-005* (47), *Intestinimonas* (48), *Rikenella* (49), *Anaerotruncus* (50,51), *UCG-009* (48)). Some of these findings are in line with Beloshapka et al. (2021), who also found *Anaerotruncus* to decrease with increased RS consumption. Furthermore, 7 of the 21 taxa are considered normal commensals of the oral microbiome (*Mogibacterium* (52)), GI tract or environmental (*Cellulomonas & Porphyromonas* (51), *Paludicola* (48) *Phenylobacterium* (53), *Pseudoxanthomonas* (54)), *family XIII UCG-001* (55), and 4 have been found to show differential abundance in human diseases or disease models compared to healthy controls (*Desulfovibrio* (56), *Candidatus stoquefichus* (45), and *Clostridium innocuum* (57) are associated with colitis, while *Peptostreptococcus* was shown to increase

298 in diabetic patients upon weight loss (58)). For 2 groups (A2 and *Kapabacteriales*) no
299 information could be found.

300 In contrast to the above, of the 16 taxa with increased abundance after RS treatment, 6
301 belonged to different classes of the phylum Pseudomonadota (previously Proteobacteria,
302 see figure 8), 5 to the phylum Actinomycetia (*Blastococcus, Microbacterium,*
303 *Saccharopolyspora, Brooklawnia* and *Propriociclava*), 3 to the phylum Bacillota/ Firmicutes,
304 1 to the phylum Bacteroidota (compare figure 7), and 1 to the phylum Saccharibacteria
305 (TM7X or *Nanosynbacter* (59)). Of these, the vast majority has been identified as being part
306 of the environment, e.g. soil dwelling, aquatic or part of plant root microbiota (60–62). Only 2
307 (*Vagococcus* and *Peptoniphilus* (63)) have been found as part of the human gut and
308 reproductive tract microbiota, some with possible pathogenic potential. While it is possible
309 that these samples had a contamination from the environment, it seems less likely that this
310 would only affect a specific subgroup of samples. Overall, the significance of these changes
311 remains unclear.

312 None of the DFs given showed any significant effect on bacterial groups comprising the
313 diagnostically used “dysbiosis index” (28), ie. there were no specific increases in “gut health”
314 markers like *Faecalibacterium* or *Clostridium sensu stricto1* (which contains *Clostridium*
315 *hiranonis*). Contrary to this, a significant increase in *Faecalibacterium* and *Roseburia* (both
316 Firmicutes) was seen with increased RS consumption in another study (42). These
317 differences might be due to different types and sources of RS. For example, Beloshapka et
318 al. (2021) used High-amylose maize cornstarch as source for RS.

319 Lastly, general limitations of next generation sequencing (NGS) workflows need to be
320 acknowledged. While we have strived to follow best practice in our methods, bias can be
321 introduced at all methodological stages during 16S rRNA analysis (64), and this can lead to
322 issues with reproducibility (65). This should be taken into account when interpreting results.

323

Conclusion

Overall, while MTC induced no discernable microbiota changes (but resulted in mild diarrhea), microbiota changes seen with PSY supplementation were mild and of questionable biological relevance. In contrast, microbiota changes induced by RS supplementation did not seem favorable, albeit being based on limited available information for the observed bacterial groups. Consequently, this particular RS would not be considered a desirable prebiotic and its use cannot be recommended in dogs.

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Author's contributions: SS contributed conceptualization, funding acquisition, formal analysis of parts of the data, project administration, supervision and writing of both the original draft and editing. JPAS contributed to conceptualization, investigation, methodology, project administration, parts of the formal analysis and review/ editing of the manuscript. LG provided data curation, formal analysis, supervision of parts of the methodology, validation and visualization as well as writing (reviewing/ editing) of the manuscript.

Sequencing data: The paired-read fastq data that support the findings of this study have been deposited in the European Nucleotide Archive with the accession code PRJEB67805.

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552 **Tables**

553 **Table 1:** Metastats for baseline samples vs samples after resistant starch (RS)

554 supplementation, showing differentially abundant bacterial taxa only. Relative abundance is

555 reported as a value between 0 and 1, where 0 indicated the complete absence of the taxa

556 and 1 indicates 100% abundance.SE = standard error.

Taxa	Mean baseline	SE baseline	Mean RS	SE RS	q value
Sellimonas	0.001674	0.000203	0.000978	0.000107	0.042502
Negativibacillus	0.001187	0.000185	0.000513	0.000115	0.042502
Eubacterium_brachy_group	0.000823	0.000175	0.000228	4.18E-05	0.042502
UCG-005	0.000749	9.37E-05	0.00027	4.31E-05	0.039722
Desulfovibrio	0.000194	8.41E-05	1.64E-05	1.02E-05	0.042502
Intestinimonas	0.000174	2.19E-05	8.39E-05	1.76E-05	0.042502
A2	0.000145	0.000145	0	0	0.024101
Candidatus_Stoquefichus	0.000129	2.40E-05	2.74E-05	1.32E-05	0.024101
Rikenellaceae_RC9_gut_group	0.000106	6.51E-05	0	0	0.042502
Mogibacterium	9.20E-05	3.75E-05	0	0	0.039722
Family_XIII_UCG-001	5.96E-05	1.72E-05	7.30E-06	5.64E-06	0.042502
Paludicola	3.07E-05	9.72E-06	0	0	0.042502
Porphyromonas	2.04E-05	1.39E-05	0	0	0.024101
Anaerotruncus	2.04E-05	2.04E-05	1.82E-06	1.82E-06	0.042502
Cellulomonas	1.70E-05	1.16E-05	0	0	0.039722
Kapabacteriales	1.53E-05	1.05E-05	0	0	0.042502
Clostridium_innocuum_group	1.53E-05	1.02E-05	0	0	0.042502
UCG-009	1.53E-05	1.36E-05	0	0	0.042502
Peptostreptococcus	1.53E-05	6.96E-06	0	0	0.042502
Phenylobacterium	1.53E-05	1.05E-05	0	0	0.042502
Pseudoxanthomonas	1.53E-05	1.53E-05	0	0	0.042502
Blastococcus	0	0	1.82E-05	1.12E-05	0.024101

Microbacterium	0	0	1.46E-05	7.91E-06	0.042502
Brooklawnia	0	0	1.64E-05	1.64E-05	0.032337
Propioniciclava	0	0	2.19E-05	2.00E-05	0.024101
Saccharopolyspora	0	0	2.92E-05	2.73E-05	0.024101
Segetibacter	0	0	0.00017	0.00015 3	0.024101
Lysinibacillus	0	0	3.65E-05	2.24E-05	0.024101
Vagococcus	0	0	4.20E-05	3.12E-05	0.024101
Peptoniphilus	0	0	1.82E-05	1.64E-05	0.024101
TM7x	0	0	0.000462	0.00037 7	0.024101
Pleomorphomonas	0	0	2.92E-05	2.25E-05	0.024101
Ochrobactrum	0	0	2.74E-05	2.74E-05	0.024101
Novosphingobium	0	0	1.46E-05	1.13E-05	0.042502
Pelomonas	0	0	6.39E-05	5.44E-05	0.024101
Schlegelella	0	0	4.38E-05	3.67E-05	0.024101
Alkanindiges	0	0	8.21E-05	8.21E-05	0.024101

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Figure captions

Figure 1. Illustration of the design and analysis of crossover fiber supplementation trial. Six separate supplementation sequences were designed (A-F). Different coloured supplement boxes represent the three different dietary fibers used. Filled stars indicate time points for fecal sampling and owner questionnaires; open stars indicate time points for fecal sampling only.

Figure 2. Fecal scores throughout the different supplementation phases (A) and at the end of each washout (WA) phase (B) for 17 dogs participating in the study. The higher the score the softer the stools. d -14 was before their change to a standardized diet at day 0, P = psyllium, M = methylcellulose, R = resistant starch, WA = washout phase.

Figure 3. Canine chronic enteropathy clinical activity index (CIBDAI) for the 17 dogs included in the feeding of different DF supplements. d -14 = before diet change, d 0 = after diet change, P = Psyllium husk, R = resistant starch, M = methylcellulose.

Figure 4. Observed OTUs, Chao1 and Shannon diversity index comparison between the baseline diet at day -14 and when all dogs were switched to a standardized diet at day 0. Wilcoxon Rank comparison revealed p-values of 0.45 for OTUs, 0.35 for Chao1 and 0.15 for Shannon.

Figure 5. Observed OTU numbers (A), Chao1 (B) and Shannon index (C) across all supplementations and washouts (WO).

Figure 6: NMDS clustering samples using Bray-Curtis dissimilarity values (stress=0.14). Samples originated from baseline (d 0) or from dogs that had received a DF supplement in their diet (M = methylcellulose, P = psyllium husk, R = resistant starch). Groups did not cluster significantly by composition (PERMANOVA: P =0.38).

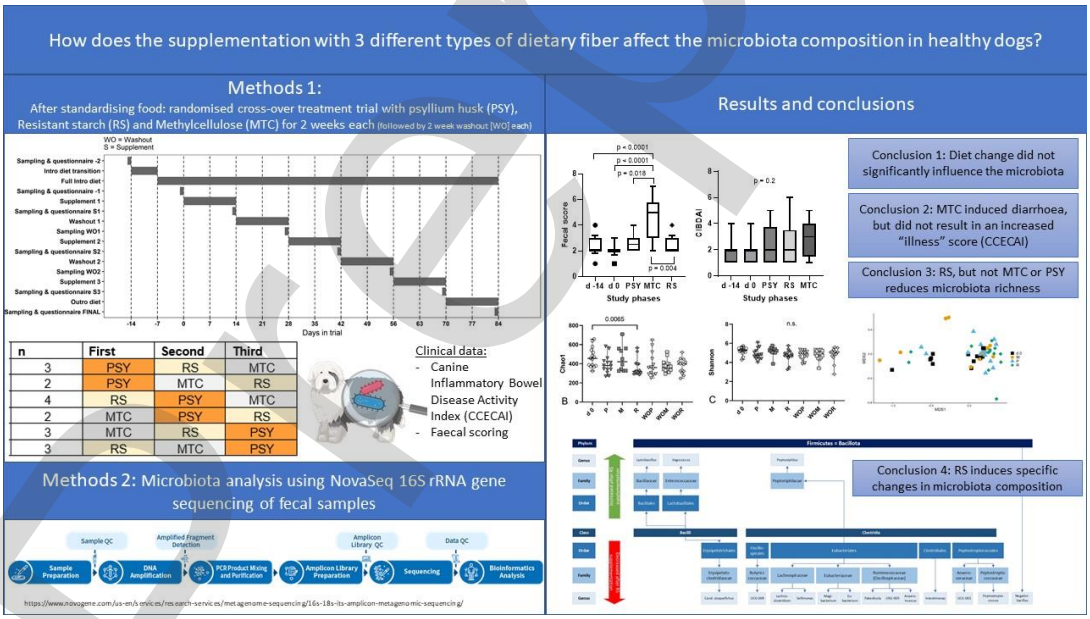
Figure 7: Difference in abundance of bacterial groups belonging to the phylum of Firmicutes/ Bacillota from samples after supplementation with resistant starch. The upper panel

(indicated by green arrow) shows groups increased after supplementation; the lower panel (indicated by red arrow) shows groups decreased.

Figure 8: Difference in abundance of bacterial groups belonging to the phyla Bacteroidetes/ Bacteroidota (left groups in shades of brown) and Pseudomonata/ Proteobacteria (right groups in shades of purple) from samples after supplementation with resistant starch. The upper panel (indicated by green arrow) shows groups increased after supplementation; the lower panel (indicated by red arrow) shows groups decreased.

Figure 9. Relative abundance of selected bacterial groups at baseline (day 0) and after supplementation with different DF (M = methylcellulose, P = psyllium husk, R = resistant starch).

Graphical abstract:



Graphical abstract legend: Microbiota of healthy dogs demonstrate significant changes in specific bacterial groups in response to supplementation with resistant starch (but not psyllium or methylcellulose) in this randomized cross-over trial.