

## Title

Gut microbiome changes related to fresh-cooked, bone and raw food and prey model diet in home-owned dogs. A 45-day feeding trial.

## Background

Freshly-cooked and raw diets are gaining popularity among dog owners as a healthier alternative to commercial dry foods (kibble) since they do not contain any preservatives and are better at retaining nutrients given the ingredients have not been processed at ultra-high temperatures.<sup>20</sup> There are two types of raw diets, Bone and Raw Food (BARF) and Prey Model (PM). Both are designed to resemble what the domestic dog's wild ancestors, the wolves, would eat in the wild but with distinct differences. The BARF diet contains 70-90% of raw meat, organs and bones and the rest are vegetables and fruits. The PM diet does not contain any vegetables as it believes that dogs in the wild are strictly carnivorous. Owing to the minimally processed nature of ingredients and the quality of the protein source in fresh-cooked and raw diets, they typically have higher fat and protein content, a wider essential amino acid profile but lower in carbohydrates than commercial dry foods.<sup>21</sup>

It is widely known that diet influences the composition and the function of the gut microbiome driven by the availability of nutrients passed down from the host's digestive system. The fermentation of these nutrients produces metabolites most notably short-chain fatty-acids (SCFA) which have been widely studied for their health-promoting effects.<sup>15,22</sup> Just like in humans, the largest immune system in dogs resides in the gut and with the rising incidents of skin allergies and stomach sensitivities, many have been looking to the gut microbiome as a potential therapeutic target.<sup>23</sup> A recently published study by Castañeda et al. showed that gut microbiome diversity was increased in BARF-fed dogs compared to those on commercial kibble.<sup>14</sup> Another study found that when dogs switched from a steamed cooked diet to commercial dry foods their skin microbiome diversity decreased.<sup>19</sup> Gut microbial diversity is considered a marker for health where a severe imbalance to the community structure opens the door for opportunistic pathogens.<sup>17</sup> In a normal healthy dog, the core gut microbial community consists of *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, *Fusobacterium* and *Proteobacteria* where an increase in *Fusobacterium* and decrease in *Firmicutes* is associated with raw diets.<sup>1</sup>

All the available studies to date have only compared one type of natural or raw diet to commercial dry food. There are distinct differences between freshly cooked, BARF and PM diets where the unique composition of each diet can have an effect on the gut microbiome. Therefore the aim of this study was to directly compare the impact of the three popular diets on the diversity, composition and predicted function of the gut microbiome of home-owned dogs using a 45-day feeding trial. The study also analysed clinical responses to the dietary intervention via the owners' reported symptoms survey.

# Methods

## Animals

The study had a broad recruitment portfolio to ensure the results from the study may be applicable to all dogs. The inclusion criteria were home-owned dogs of any breeds based in Singapore, 1+ years of age, had been on a kibble diet for at least 3 months and had no major health conditions prior to starting the trial. Owners were required to sign a consent form to participate. A baseline survey covering the biometrics, kibble protein type, dietary supplements, behaviour and health conditions of the dog was collected. At the end of the study, owners were asked to fill out a follow-up survey describing symptom changes in skin allergy/itchiness, stomach sensitivity, appetite, energy, behaviour and mobility.

## Diet

The study aimed to recruit 60 dogs with 10 dogs randomly assigned to a diet which were Premium Cooked (PC), Value Tray (VT), BARF and Prey Model (PM) plus 20 in a control group where participants stayed on the same kibble diet. Each participant was supplied with a minimum 45-day supply of Good Woof freshly-cooked (PC and VT) or fresh-raw diet (BARF and PM). The portion size matched the upper limit of daily recommended calories for each dog based on its weight. The feeding trial was conducted over 45 days (6 weeks) where two protein types were rotated every two weeks. See Appendix table 1 for details on the protein variants for each diet. A 7-day transition period was recommended for the freshly-cooked diets and 13 days for the raw diets.

## Sample collection

Owners were instructed to collect a sample before starting the trial and one after. Fresh faeces were collected into a sample tube filled with buffer solution and then placed inside a sealed plastic bag to be sent to the laboratory for sequencing.

## DNA extraction and 16S sequencing

The DNA was extracted from faecal suspension using QIAmp powerfecal DNA kit (Qiagen, USA). The DNA library for targeting the V3-V4 region on the 16S gene was prepared using the *Quick-16S Plus*<sup>TM</sup> NGS kit (Zymo Research, USA). Finally the DNA sequencing was performed on an Illumina Miseq system.

## Bioinformatics and Statistical analysis

All analyses were conducted in *R* version 4.0.2. An inhouse bioinformatic workflow was set up to perform quality control checks on the sequenced files and assign taxonomy to the sequence variants. Microbiome analysis was performed using the *Phyloseq* (1.32.0) and *Vegan* (2.6-4) libraries. The diversity scores were corrected for age. PICRUST2 was used for pathway prediction. The raw pathway abundances were converted to relative abundances and then

scaled to a percentile score between 1 to 10. A paired t-test was performed on normally distributed diversity scores and pathway scores.

## Results

### Study Demographics

Out of the 60 dogs recruited, 19 of them were not included in the final analysis. 11 of which were due to non-compliance for reasons such as the dog's disliking of the new diet and owner's health reasons meant unable to complete the study. The other 8 dogs were missing the post-intervention sample and therefore were also excluded. Table 1 shows the demographics of the remaining 41 dogs. The age range was from 1 to 15 years, with a median age of 4 years old. There were 23 females and 18 male. A total of 23 breeds were included in the study. The most common breed was singapore special (n=8).

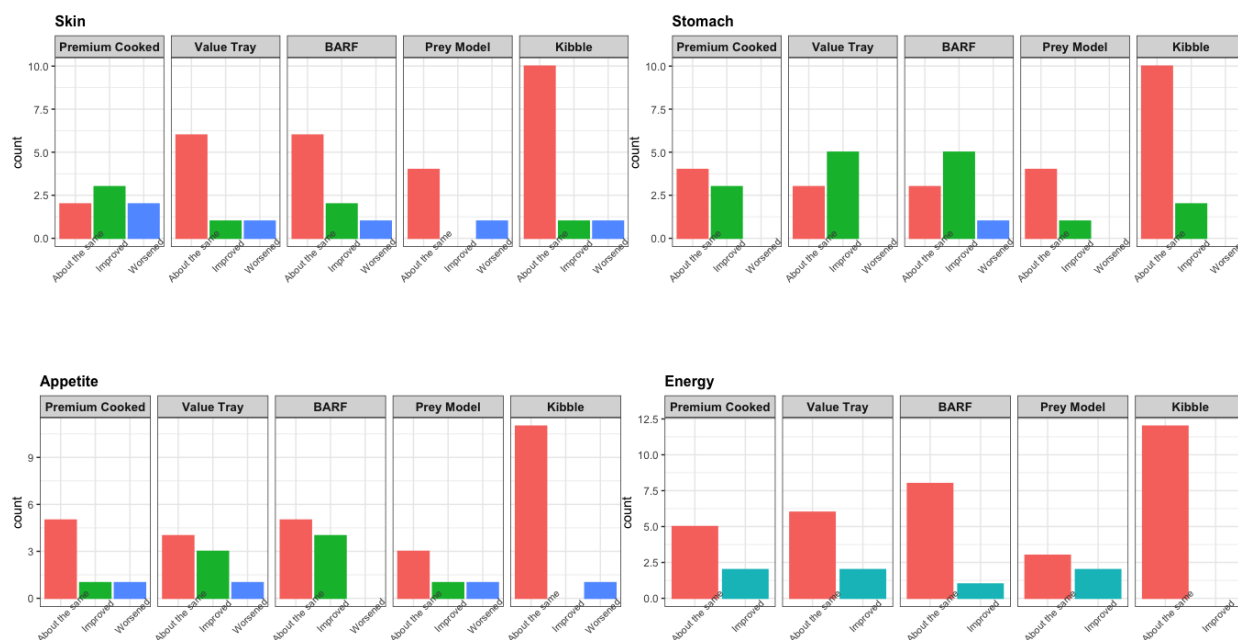
Diet	Number of dogs	Breed	Age (medium years, minimum-maximum)	Gender (Male : Female)
Premium Cooked (PC)	7	Mixed Breed 2 Cavalier King Charles Spaniel 1 Italian Greyhound 1 Labrador Retriever 1 Miniature Goldendoodle 1 Shih Tzu 1	3 (1-15)	4:3
Value Tray (VT)	8	Singapore Special 4 French Bulldog 1 Miniature Poodle 1 Pomsky 1 Border Collie 1	2 (1-9)	5:3
BARF	9	Border Collie 2 Bulldog 1 Cocker Spaniel 1 Jack Chi 1 Minnie Jack 1 Pomeranian 1 Pomsky 1 Shiba Inu 1	7 (1-10)	3:6
Prey Model (PM)	5	French Bulldog 3 Pomeranian 1 Singapore Special 1	6 (4-7)	0:5
Kibble	12	Singapore Special 3 Goldendoodle 2	3 (1-6)	6:6

		Boxer 1 Cavapoo 1 Doxiepool 1 French Bulldog 1 Pomsky 1 Miniature Schnauzer 1 Scottish terrier 1		
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Table 1. Table showing the number of dogs, breeds, age and gender ratio in each dietary group.

## Overview of self-reported outcomes

At the end of the study, dog owners were asked to fill-out a symptoms survey (Figure 1). The PC group recorded the most cases of both improvements and worsening of skin conditions. As for the stomach symptoms, both the VT and the BARF group had the highest proportion of dogs with improved stomach sensitivity followed by PC then PM. The control kibble group also saw two dogs in this category. It is worth noting that one of them switched to a home-cooked lean pork diet with vegetables during the trial. The other had reduced protein intake to help with his allergies. It is possible that the diet change had a positive impact on the dog's stomach and skin symptoms. The BARF group contained the only report of worsening stomach symptoms. The BARF group also had the highest number of dogs with an increased appetite. There was at least one record of improvement in energy level, mobility and behaviour in terms of anxiousness and reactivity for each diet with the exception of BARF where behaviour remained unchanged for all dogs.



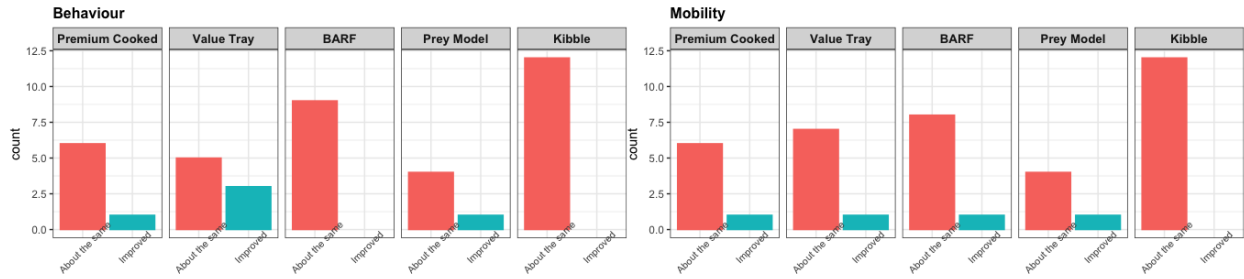


Figure 1. Follow-up symptoms survey showing number of responses for each symptom category.

In addition to the symptoms survey, owners were asked to record the weight of their dogs pre and post-intervention. Only dogs that experienced a weight change were shown in Figure 2. Dogs on the PC and the BARF diet gained the most weight. The dog on the BARF diet with a 25% weight gain also had an increased appetite. On the other hand, the highest weight loss was in the PC group at 17% where the owner reported that the dog was not enthusiastic about the food and seemed to be hungry all the time.

% Change in weight after the trial

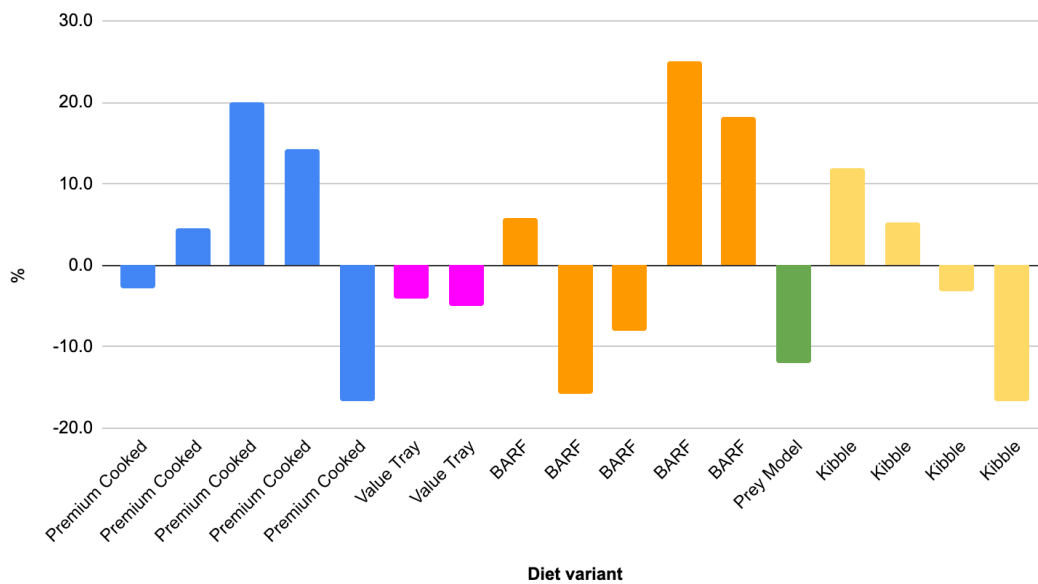


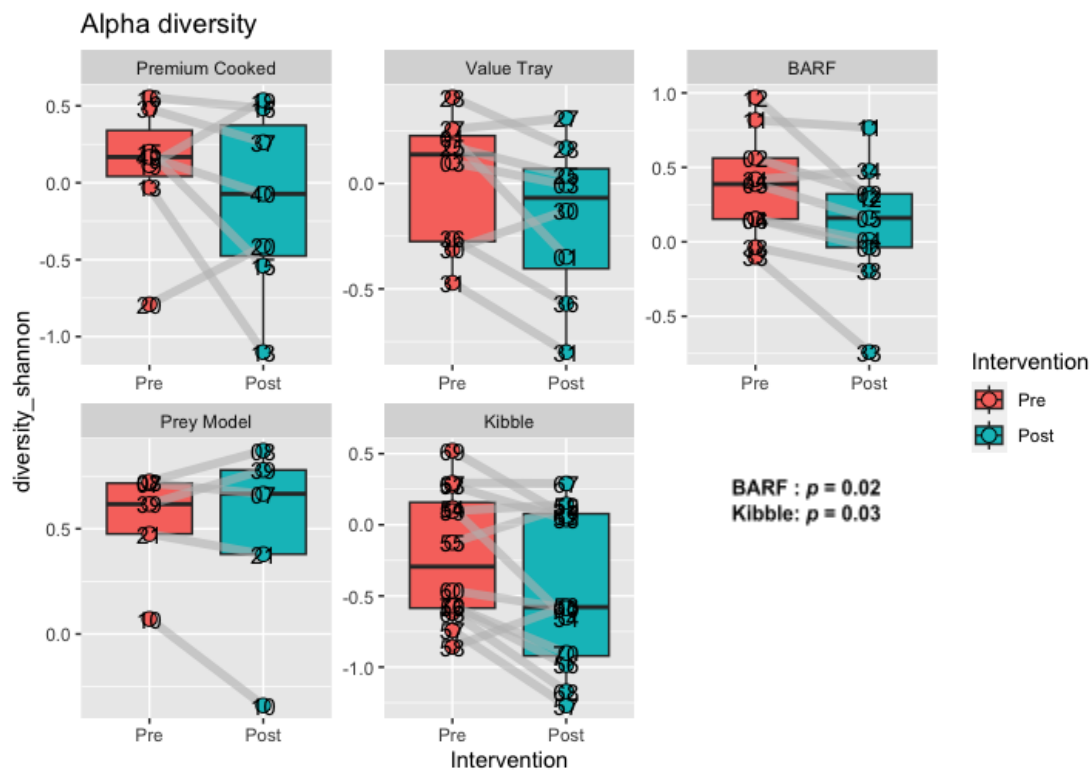
Figure 2. Bar charts showing the percentage of change in weight for dogs that had a weight change after the feeding trial.

# Gut microbiome changes associated with fresh-cooked and raw diet

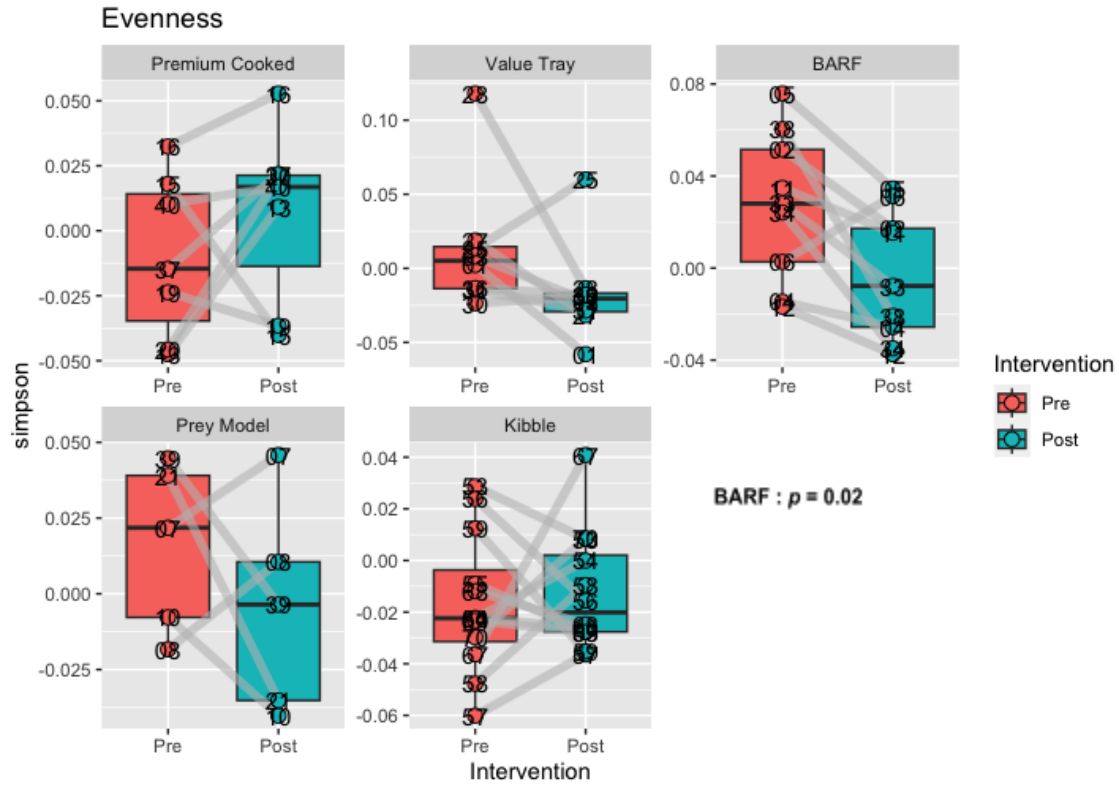
## Changes in measures of diversity (Alpha diversity, richness and evenness)

Alpha diversity, a measure of the number (richness) and the distribution (evenness) of microbial species in a sample, was significantly reduced in the BARF and the control kibble group (Figure 3A). Even though on a group level the difference for the PC and the VT diet was not significant; individually there were more dogs that had a lower diversity score post-intervention. The significant reduction in alpha diversity in the BARF group was mainly driven by reduced evenness (Figure 3B). A lower diversity score suggests that some bacterial species are better at adapting to the new diet than the others. Some dogs' richness scores changed more dramatically than the others. For example, post-intervention richness was significantly higher for subject 19 (PC) and subject 27 (VT) and lower for subject 12 (BARF) compared to the rest of the dogs in their respective group (Figure 3C). In the next section of this paper, the composition of the gut microbial community and their functionality will be explored.

A)



B)



C)

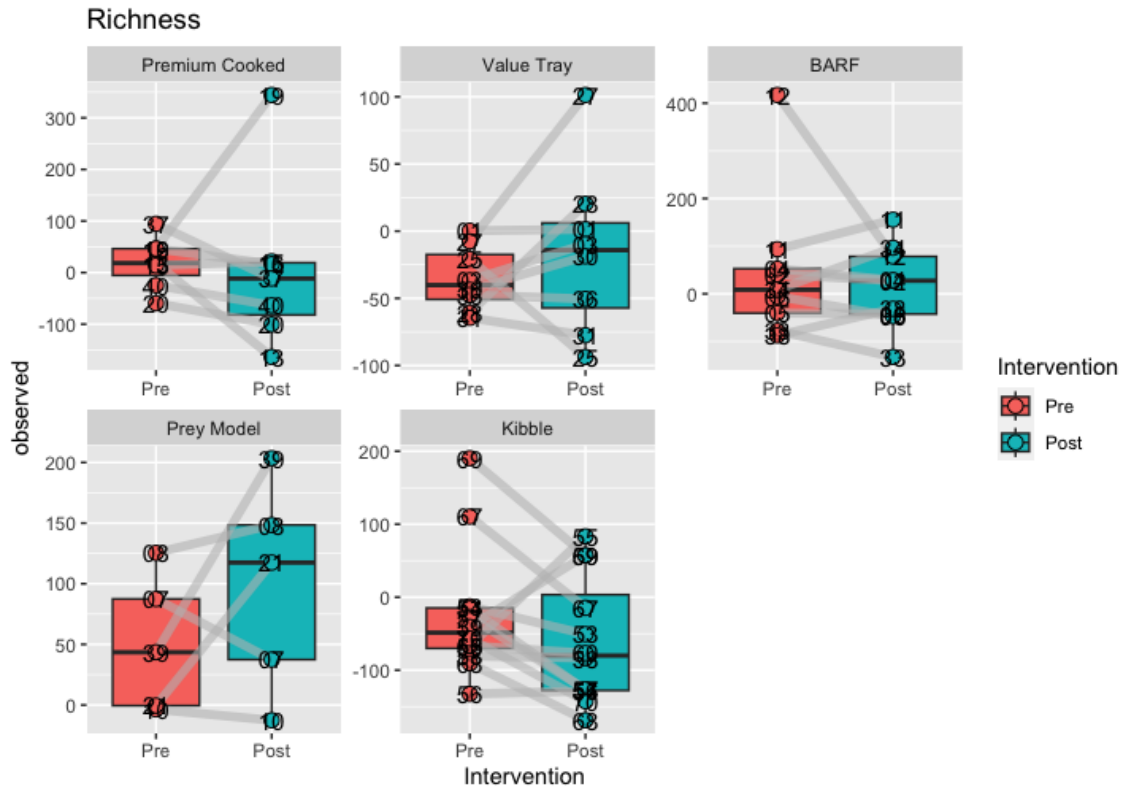
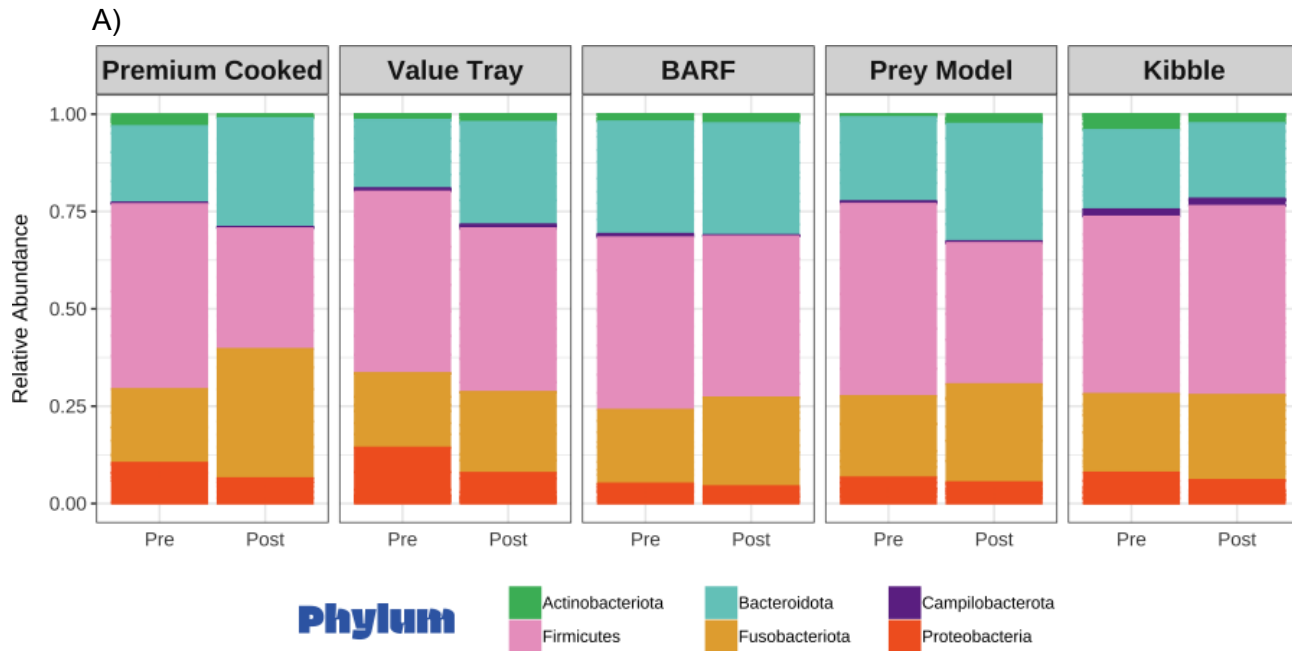


Figure 3. Box-Whiskers-plots showing A) Alpha diversity as measured by the Shannon's index; B) Evenness as measured by the Simpson's index; C) Richness as measured by the number of observed species. All scores were adjusted for age. Paired t-test ( $p < 0.05$ ).

### Phyla and genera level changes in bacterial composition

After the feeding trial, on average, the relative abundances of *Fusobacteria* and *Bacteroidetes* were increased in all fresh-cooked and raw diets. In contrast, there were proportionally less *Firmicutes* (Figure 4). This observation is in line with the existing literature from studying domestic dogs that switched from commercial kibbles to raw food to wolves living in the zoo whose diet consists of mostly meats with negligible amounts of vegetables. A consistent finding is that raw diets are associated with an increase in *Fusobacteria* responsible for the degradation of proteins and a reduction in the carbohydrate-associated phylum, *Firmicutes*.<sup>14</sup> In a normal dog's gut microbiome, *Fusobacteria* is one of the three most abundant phyla alongside *Firmicutes* and *Bacteroidetes* and its abundance can be severely impacted by antibiotic use<sup>5</sup> and GI diseases.<sup>6</sup>





B)

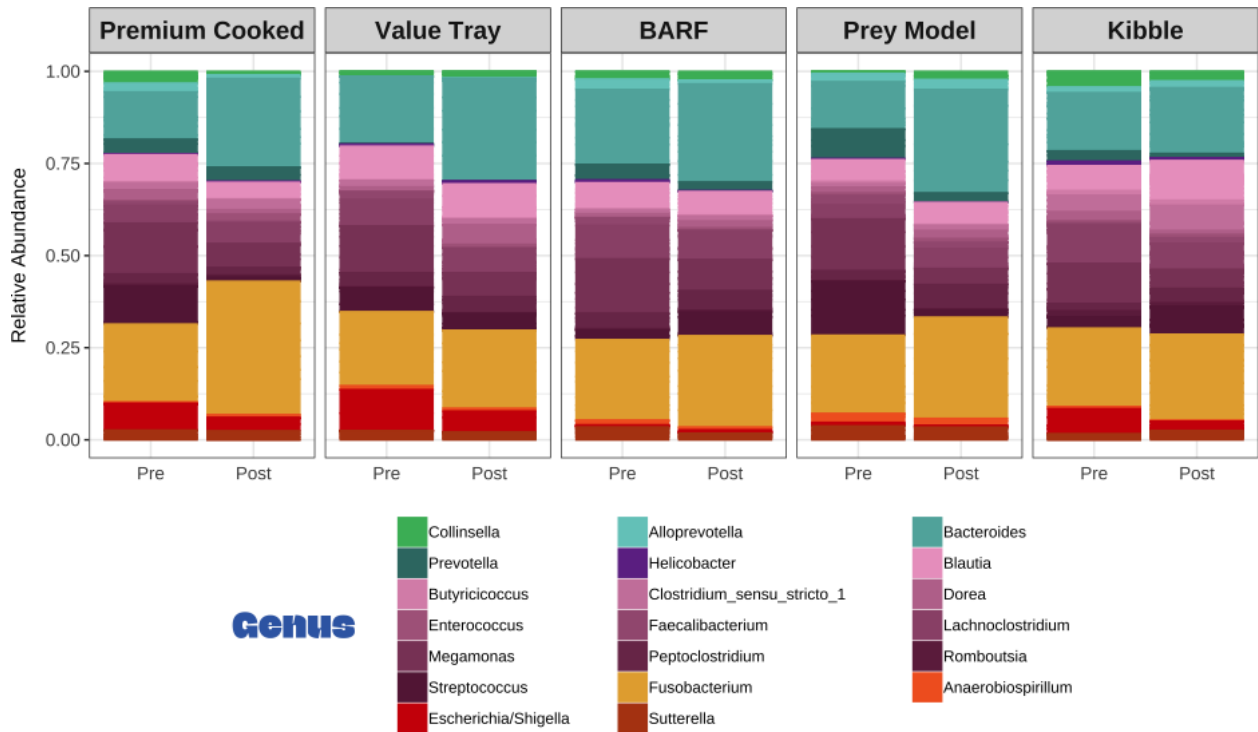


Figure 4. A) Average relative abundance of the top 6 phyla. B) Average relative abundance of the top 20 genera.

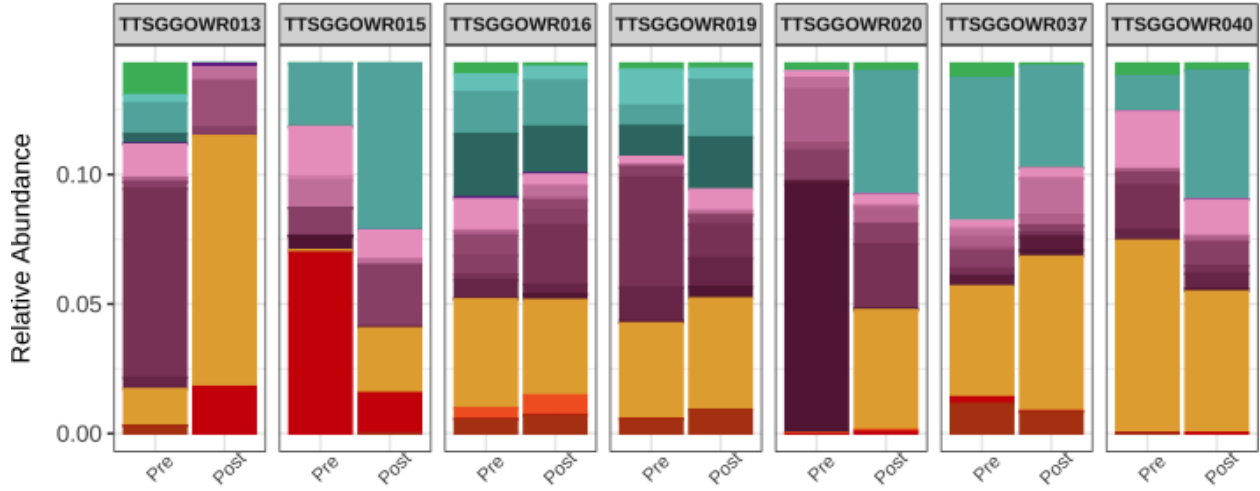
When examining individual cases, there was a fair amount of variation within each diet group (Figure 5 and Figure 6). Firstly, *Fusobacteria* and *Bacteroidetes* do not always increase together. The level of increase can be very different. For the raw diets, either one or both were increased in all samples apart from Subject 6, 12 and 33 in the BARF group where *Firmicutes* was more abundant (Figure 6C).

In Subject 13 (PC), after consuming a fresh-cooked diet *Fusobacteria* became the dominant genus whereas the rate of growth for *Bacteroidetes* was less prevalent (Figure 5A). In contrast for Subject 20, *Bacteroidetes* and *Fusobacteria* that were non-existent at baseline reappeared in the post-intervention sample and grew to a similar proportion (Figure 5A).

Subject 31 and 36 of the VT group present another interesting case. Both samples had close to or more than 50% of *Escherichia coli* (*E.coli*) (Figure 5B). An increase of *E.coli* is a hallmark of dysbiosis.<sup>17</sup> After the trial, the level of *E.coli* decreased where the reduction was more prominent in Subject 36. It is worth noting that Subject 36 was given cucumber and probiotics in addition to the main diet which explains the increase of *Firmicutes*. The addition of dietary fibre and probiotics together with the freshly-cooked diet might have helped rebalance the microbiome community and created a less favourable environment for *E.coli* as they are unable to efficiently extract nutrients from complex carbohydrates.<sup>17</sup>

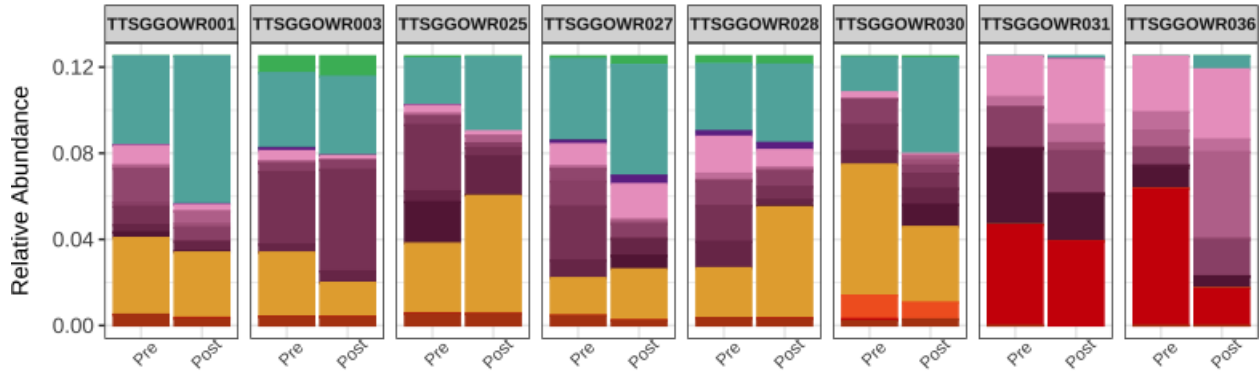
A)

### Premium Cooked

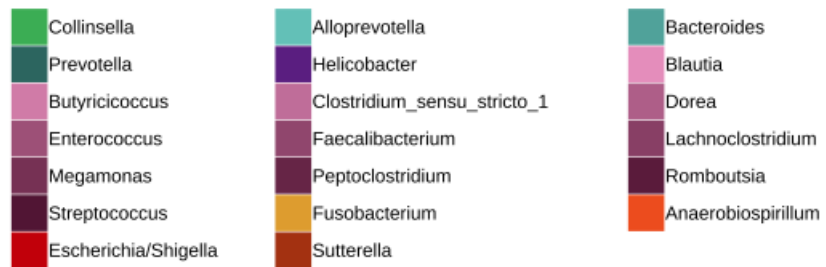


B)

### Value Tray

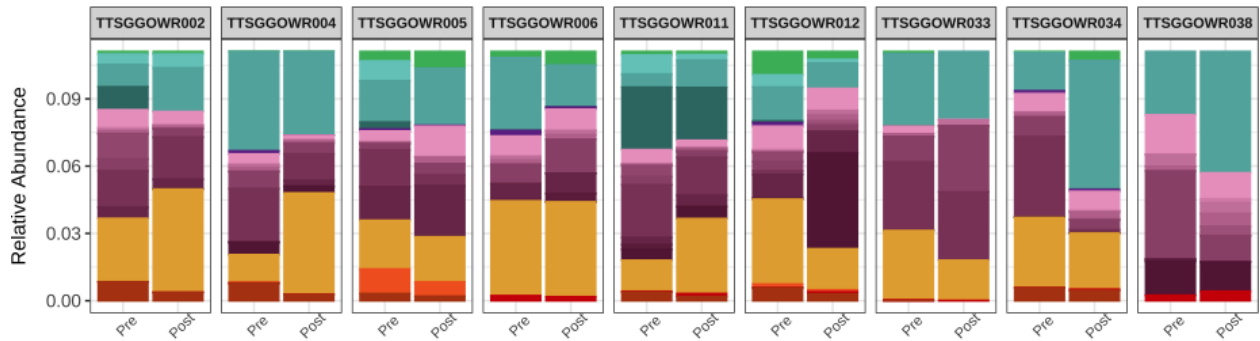


**Genus**



C)

**BARF**



D)

**Prey Model**

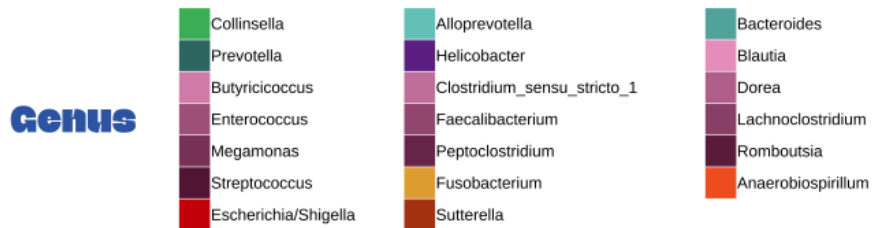
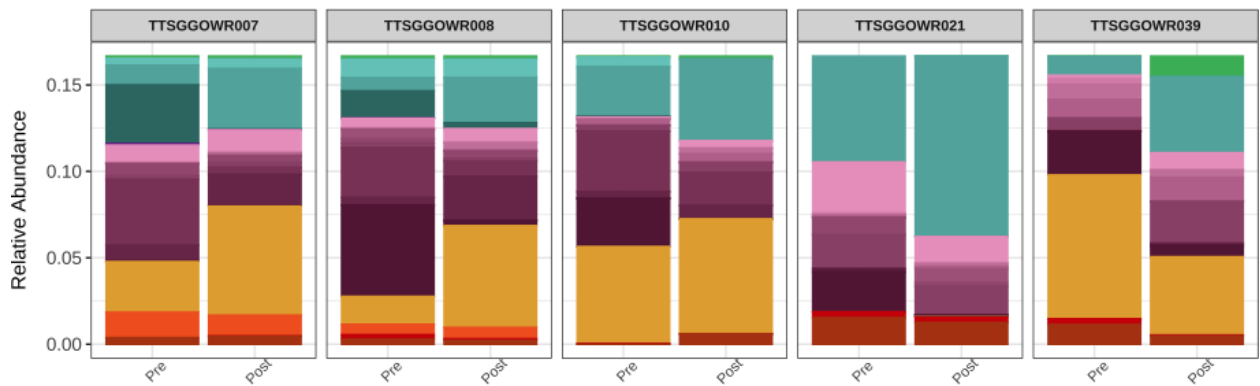
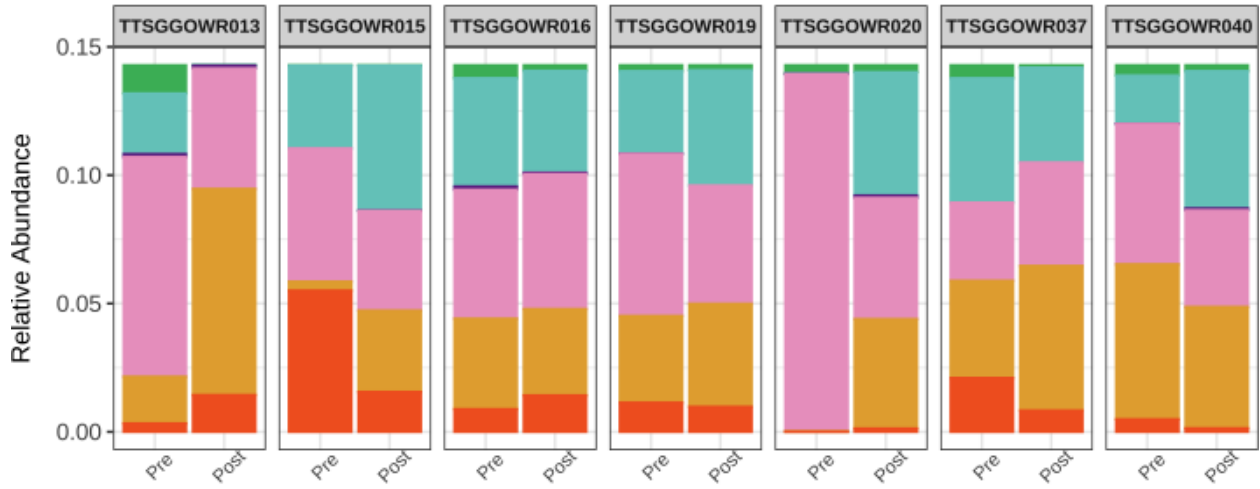


Figure 5. A) Relative abundance of the top 20 genera in dogs after a 45-days feeding trial of A) the Premium Cooked diet; B) the Value Tray diet; C) the BARF diet; D) the Prey Model diet.

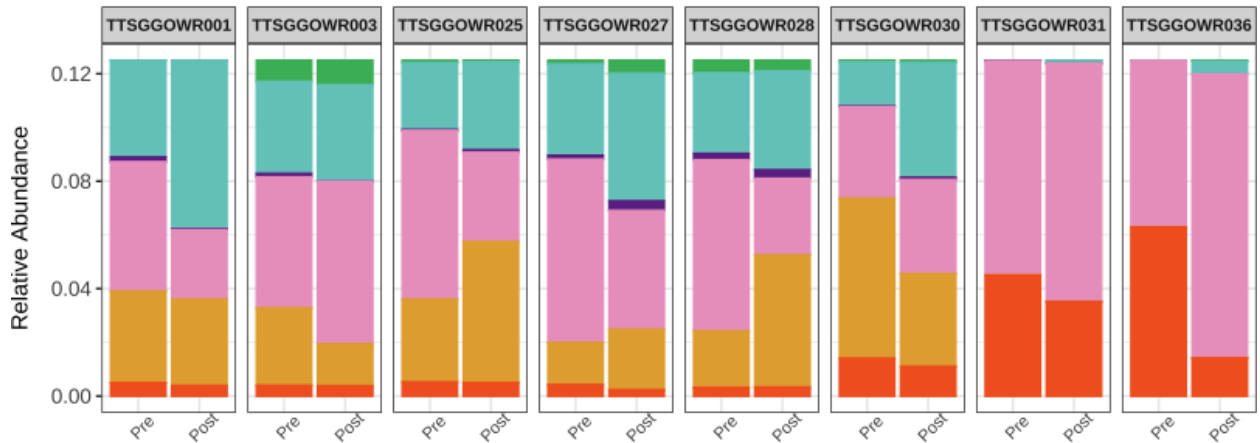
A)

### Premium Cooked

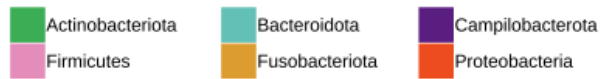


B)

### Value Tray

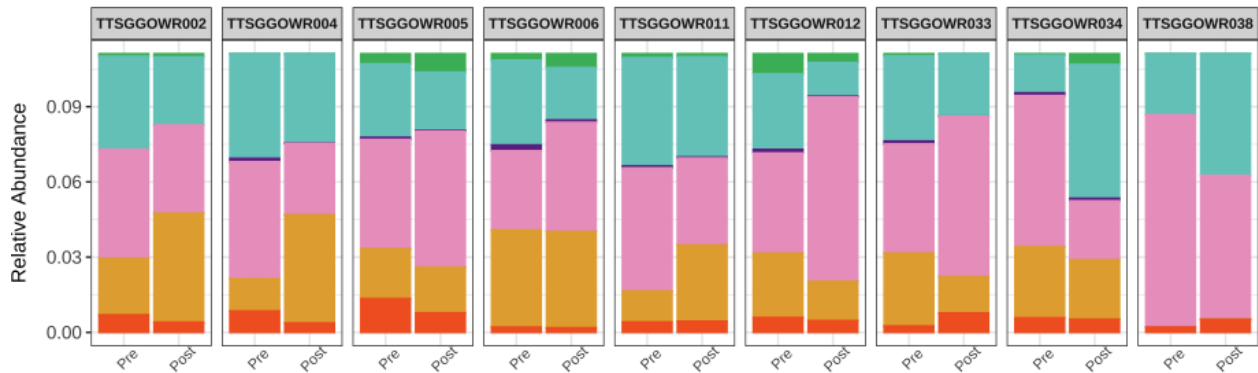


### Phylum



C)

### BARF



D)

### Prey Model

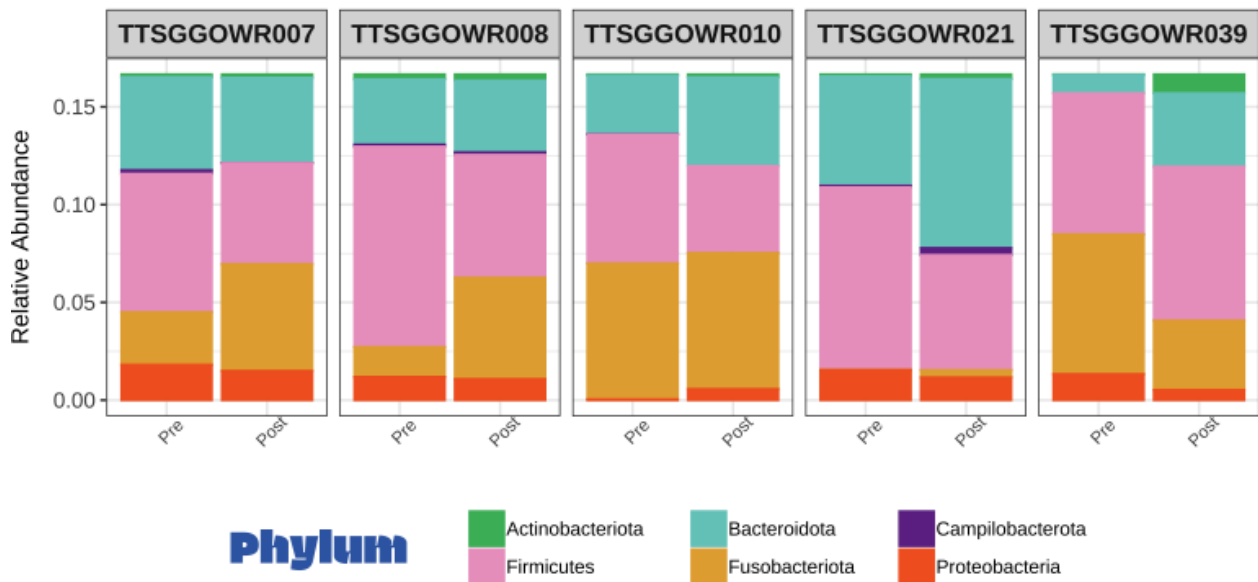


Figure 6. Relative abundance of the top 6 phyla in dogs after a 45-days feeding trial of A) the Premium Cooked diet; B) the Value Tray diet; C) the BARF diet; D) the Prey Model diet.

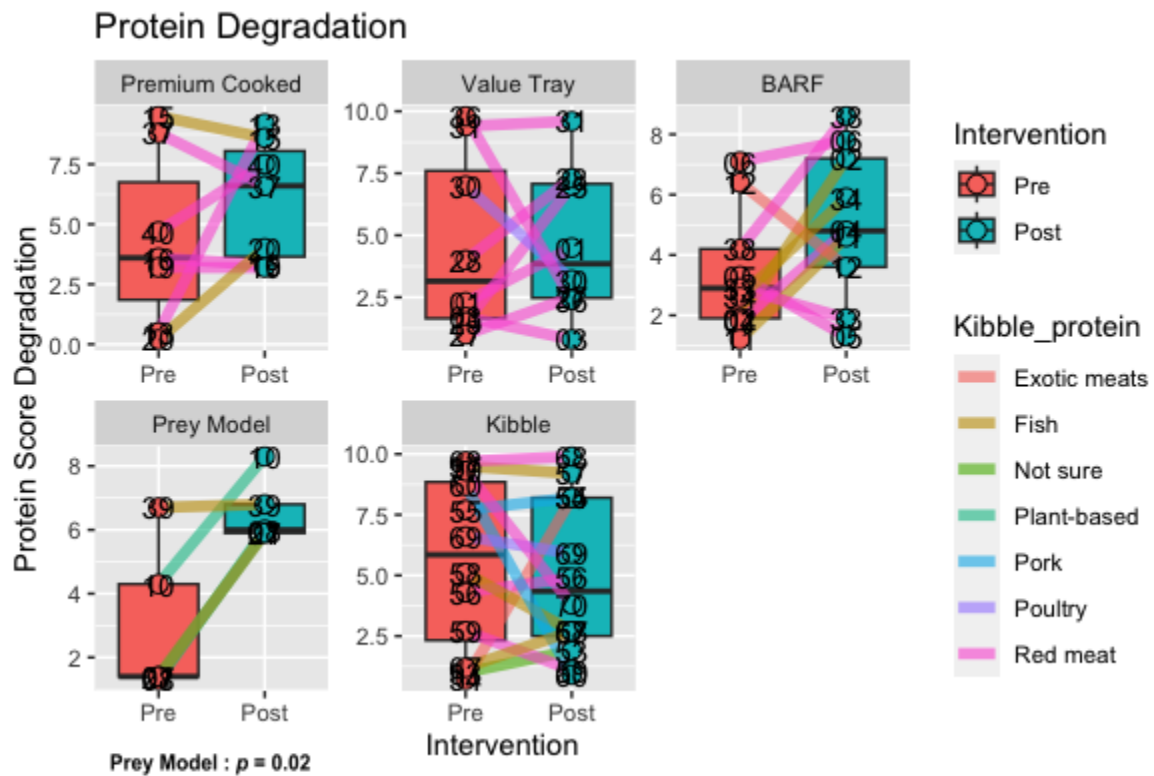
### Changes in Nutrient Functional Pathways

The gut microbiome plays an important role in breaking down nutrients and releasing metabolites that carry many health benefits. Simply knowing which bacteria are present (i.e. the composition of the microbiome) is not enough to appreciate the variety of functions they carry out as many species are capable of switching between different enzymes depending on the nutrient availability.<sup>8-9</sup> In order to understand which metabolic processes are taking place, a software called PICRUST2 is used to match microbial 16S sequences to a genome database of enzymatic reactions.<sup>10</sup> Predicted pathway abundances related to the degradation of protein,

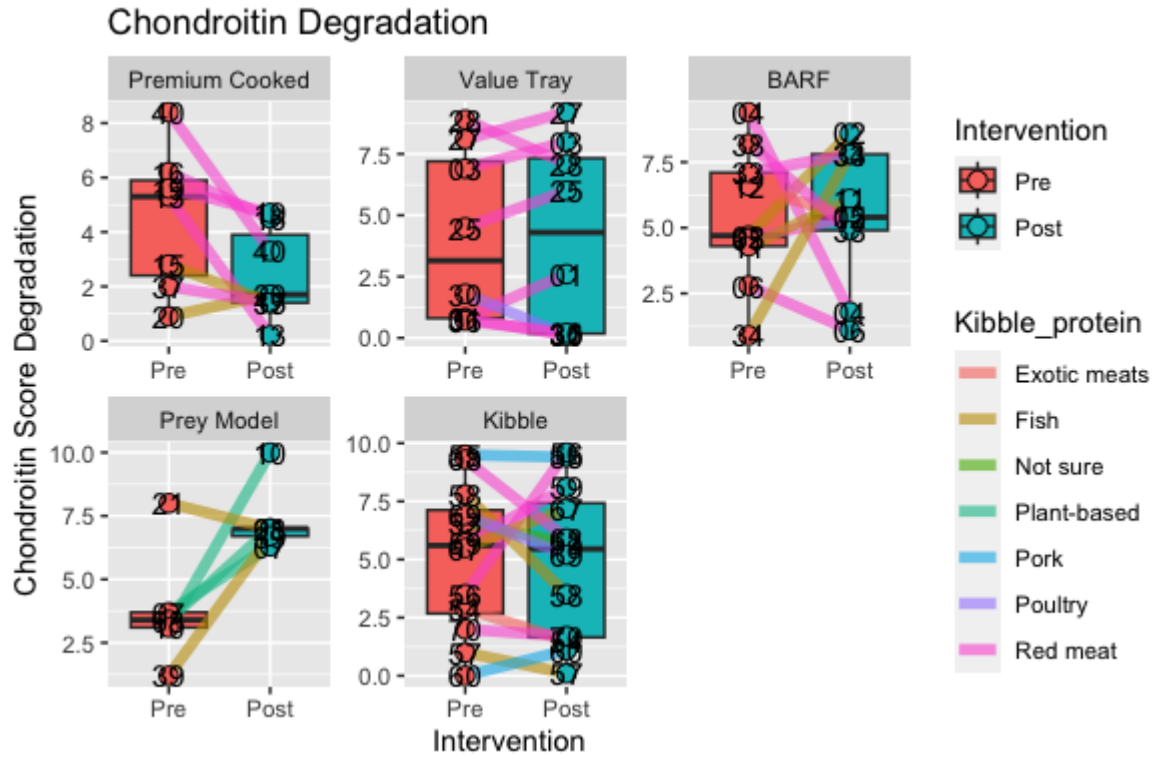
carbohydrates, fats and chondroitin as well as the fermentation processes for SCFA production were summarised at each nutrient level.

Pathways related to the degradation of protein and SCFA production were increased significantly in dogs fed the PM diet (Figure 7A and Figure 7E-G). Interestingly, all of the dogs in the PM group were on fish or plant-based kibbles before starting the trial. Thus the increase in protein degradation could be contributed by the differences in amino acid profiles between raw meats, fish and plant-based kibble.<sup>11</sup> Red meats typically have a higher lysine content and the fermentation of L-lysine is a prominent metabolic pathway in *Fusobacteria* which leads to the production of butyrate.<sup>12</sup> In addition to amino acids, the level of iron can also influence SCFA production.<sup>13</sup> There were three BARF-fed dogs who were on fish-based kibbles at baseline who also had enhanced protein degradation (Figure 7A). On the other hand, dogs fed red meat kibbles at baseline produced varied responses in terms of protein degradation (Figure 7A). The chondroitin degradation pathway followed a similar pattern where it was increased in dogs that transitioned from a fish or plant-based kibble diet to a raw-meat based diet (Figure 7B). This is most likely due to the presence of bones serving as a rich source of chondroitin. No significant differences were detected for other nutrients.

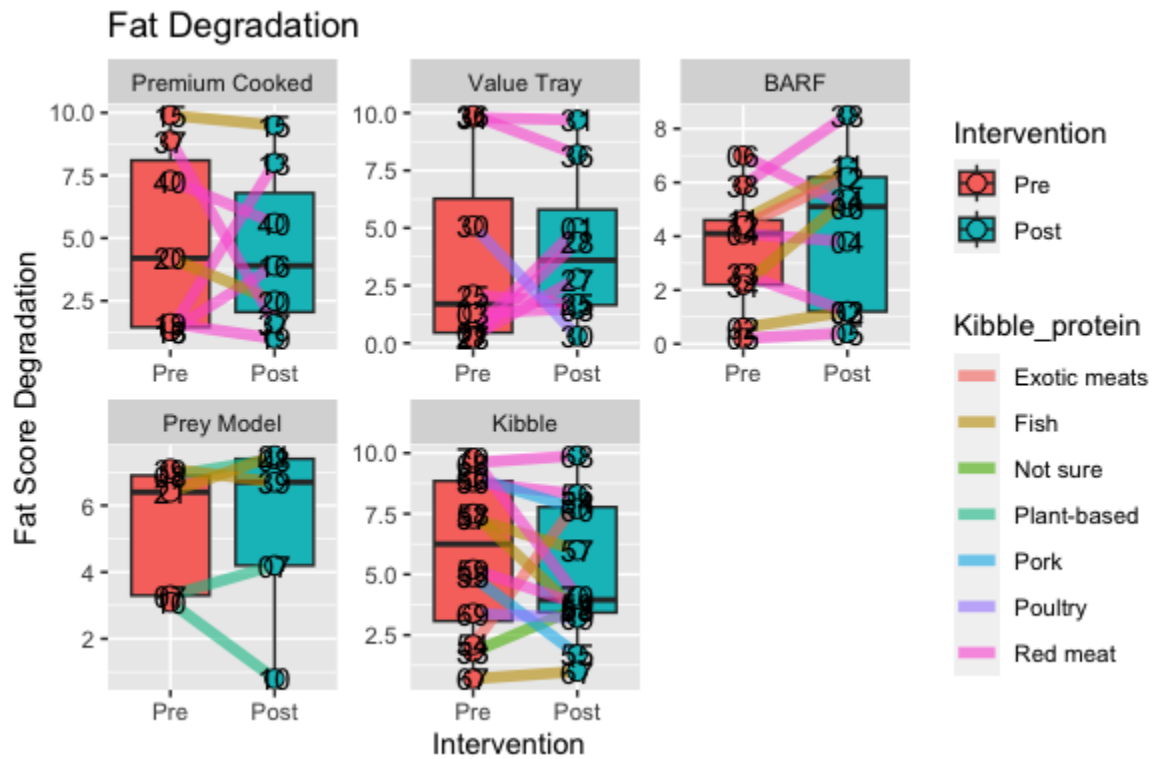
A)



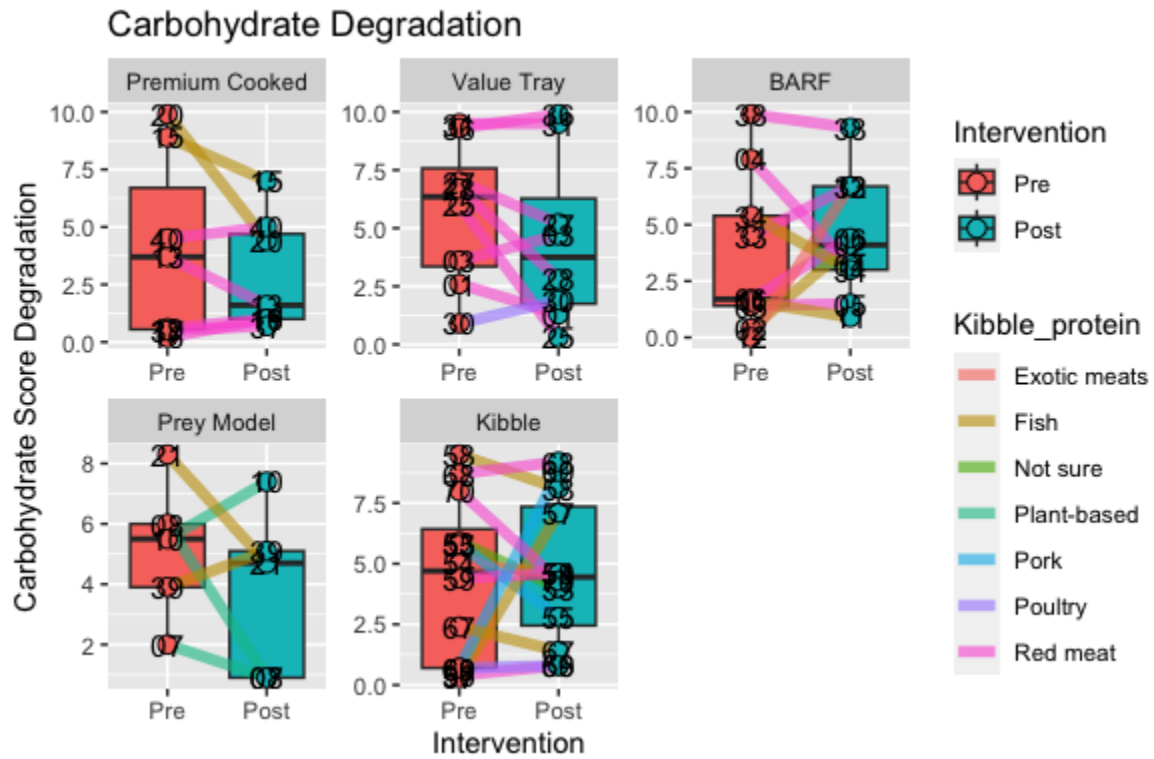
B)



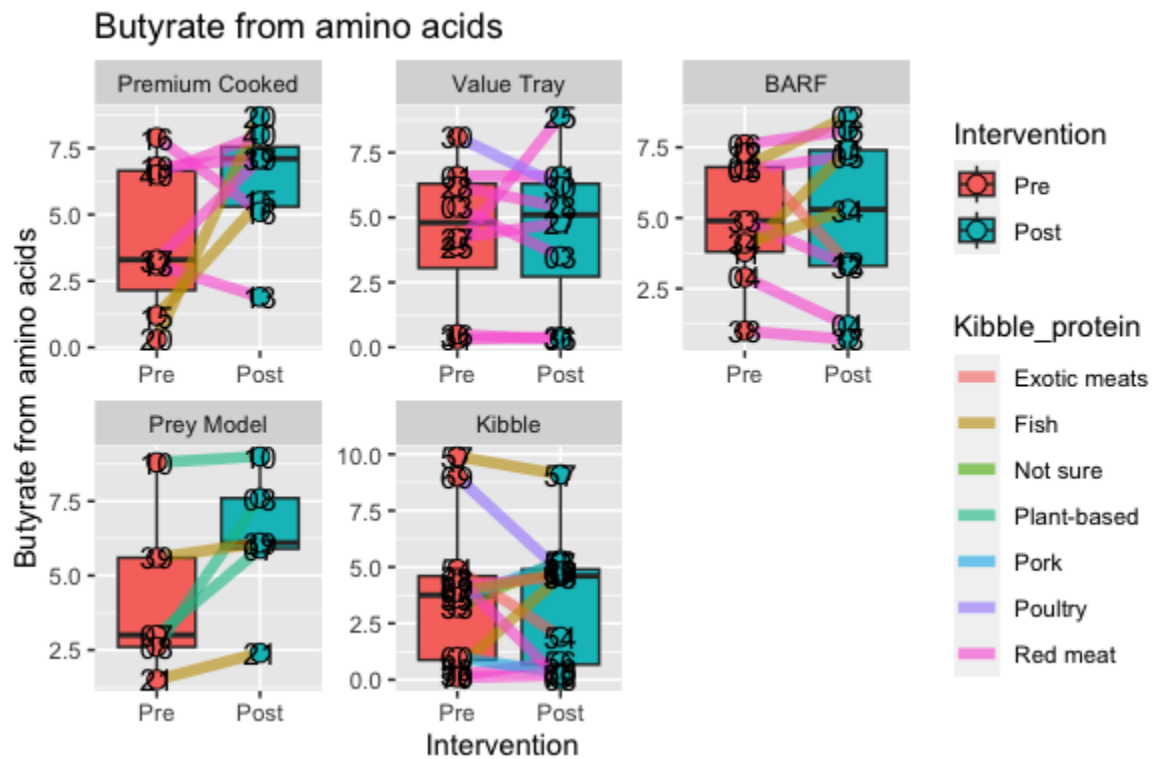
C)



D)



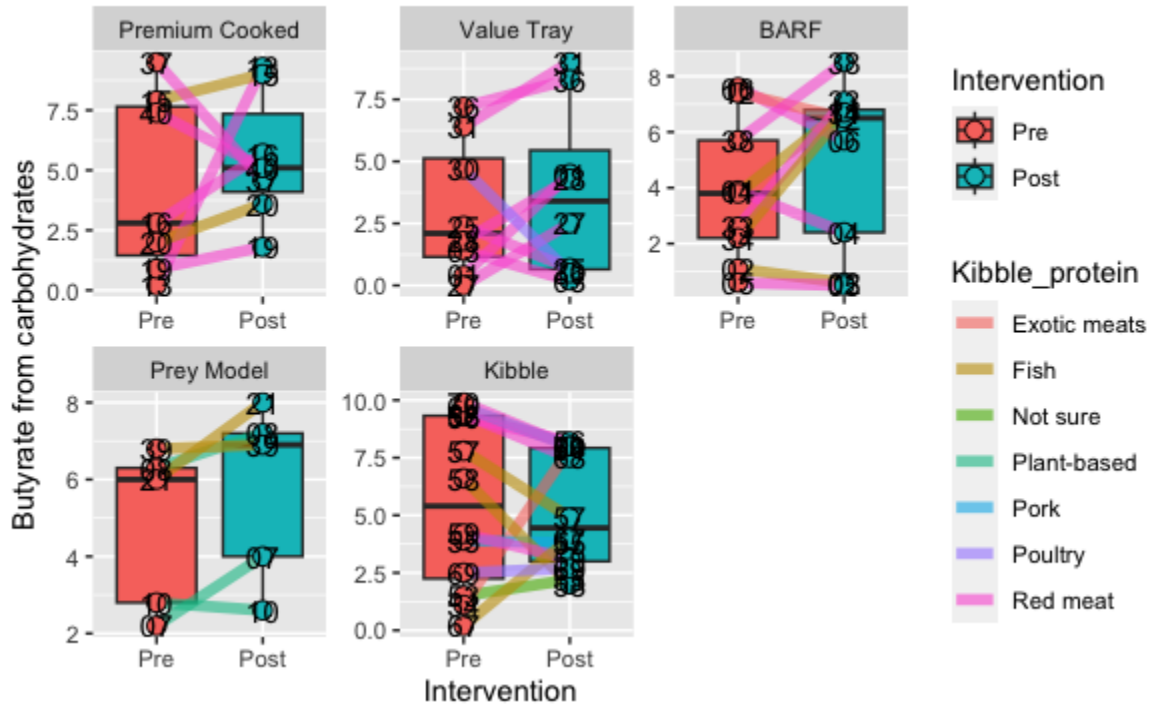
E)





F)

### Butyrate from pyruvate and succinate



G)

### Fermentation to SCFA

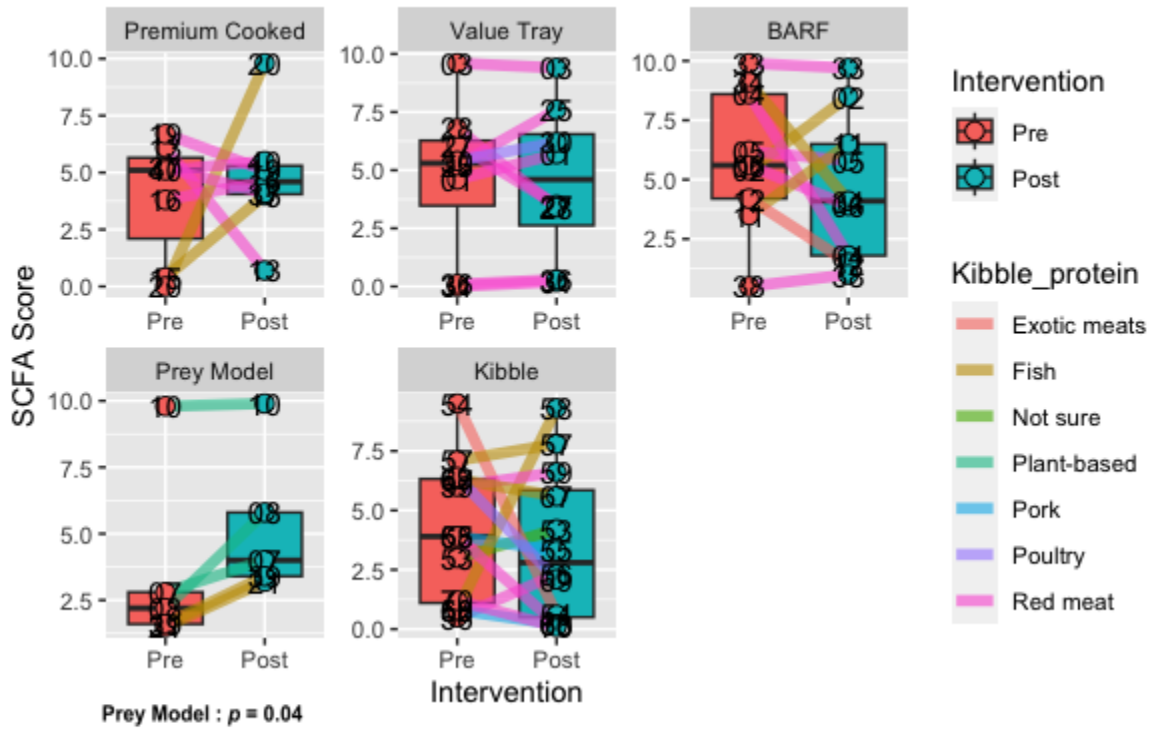


Figure 7. Box-plots showing predicted pathway scores (1 to 10) between pre and post intervention samples for the degradation of A) Protein; B) Chondroitin; C) Fats; D) Carbohydrates; E) Fermentation of pyruvate and succinate to butyrate; F) Fermentation of amino acids to butyrate and G) Total SCFA production. Paired t-test ( $p < 0.05$ ). Lines connecting each paired-sample were coloured according to their baseline kibble protein. Pathway prediction was performed using PICRUST2 combined with an internal reference database. Relative abundances for the degradation pathways were aggregated at the macronutrient level.

## Conclusion

A 45-day feeding trial was conducted in home-owned dogs to investigate microbiome changes associated with freshly-cooked, BARF and Prey Model diets. To our knowledge, this is the first time the three diets were being compared in the same study. Overall, dogs on the fresh-cooked and raw diets recorded more improvements in skin itchiness, stomach sensitivity and appetite compared to those on kibbles. The dog with the highest weight loss was in the PC group, this was associated with an observed disinterest in the diet, recorded at -17%. The dog with the most weight gain was in the BARF group, recorded at 25%, this was associated with an observed increased appetite. There were no withdrawals due to adverse reactions.

At the end of the trial, alpha diversity and evenness were significantly reduced in dogs fed the BARF diet which is likely due to the fact that raw-meat based diets contain less carbohydrates than kibbles, therefore a significant number of carbohydrate-associated species in *Firmicutes* is lost as a result. However, there is conflicting evidence in the current literature where alpha diversity was increased in dogs that switched from kibbles to raw in one study<sup>4</sup> and no change was observed by Schmidt et al.<sup>3</sup>. The study by Kim et al. found lower evenness but an overall increased alpha diversity driven by the significantly higher richness score for dogs on a high protein natural diet versus commercial diet.<sup>18</sup> A more recent study found that BARF-fed dogs had higher diversity and richness in their microbiome compared to those on commercial diets.<sup>14</sup> The study by Schmidt et al. sampled two independent groups of dogs where the BARF group had been on the diet for at least 6 months (0.6 - 9 years). In contrast, dogs in this study were on the BARF diet for only 1.5 months. It is possible that the gut microbiome community, after being subjected to such drastic change in nutrient availability, takes time to rebalance itself and diversify in order to fully adapt to the new environment. Due to the short-term nature of this intervention it is unknown at this stage whether a longer feeding period would eventually yield a higher alpha diversity score. However, an advantage this study has over the others by comparing pre versus post intervention samples is that each dog acts as its own control which minimises variation arising from different breeds, living environments, behaviours and activity levels, all of which can have an impact on the microbiome and are difficult to account for based on household surveys.<sup>15</sup>

Even though there are conflicting results between this study and the existing literature with regard to the diversity score, one consistent finding is that raw diet feeding is associated with an increase in *Fusobacteria* and decrease in *Firmicutes*. In addition to the diversity and

composition of the microbiome, when evaluating the health benefits of raw diets it is also important to consider how efficient the gut microbiota is at breaking down nutrients and what other downstream metabolic processes are taking place in a protein-rich environment. The degradation of protein was increased in the Prey Model diet where there were more microbial activities related to the fermentation of amino acids to SCFA. An interesting observation was that all of the dogs that were fed fish or plant-based kibbles at baseline showed an increase in protein degradation after transitioning to raw-diets. Those on red meat based kibbles produced a more varied response. It has been known that the source of protein and its ratio with carbohydrates have an influence on the microbiome.<sup>16</sup> Hence this might partially explain why dogs fed the same kibble protein type i.e. fish and plant-based showed similar responses to raw diets. Chondroitin degradation and SCFA production were also increased in these dogs. This suggests that dogs on fish or plant-based kibbles are lacking important nutrients for bone development and essential amino acids commonly found in animal meats such as L-lysine which is a precursor of butyrate. It is important to keep in mind that the degradation pathways are based on prediction using bacterial gene markers rather than direct measurements of metabolites. For future studies, we suggest using metabolomic profiling to provide a more complete picture on the functional capacities of the gut microbiome.

Three dogs fed a freshly-cooked diet (1 in PC and 2 in VT) had close to 50% or more of their gut microbiome made up of *E.coli* at baseline which was reduced after the trial. Although a majority of the *E.coli* strains are harmless, their pathogenic potential increases if they start to proliferate and show dominance over the other phylum.<sup>17</sup> Perhaps a freshly-cooked diet has the potential to limit the growth of *E.coli* and may therefore help to reduce the likelihood of pathogenic infection.

It is clear that switching from a kibble to a freshly-cooked or a raw-meat based diet has a significant impact on the gut microbiome of home-owned dogs. Each dog's gut microbiome adapts differently to the same diet which reiterates the importance of using microbiome profiling to guide nutritional intervention. For pet food companies, understanding how different sources of protein impact the gut microbiome can help maximising the health benefit of home-cooked and raw-meat based meals for the masses or developing special feeds tailored to dogs with skin allergies and stomach sensitivities.

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