



# Rapid Reconstitution of the Fecal Microbiome after Extended Diet-Induced Changes Indicates a Stable Gut Microbiome in Healthy Adult Dogs

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**ABSTRACT** The gut microbiome has an important role in health, and diet represents a key lever for shaping the gut microbiome across all stages of life. Maternal milk consumption in neonates leads to long-term health effects, indicating that pliability in the infant gut microbiome in response to diet can drive enduring change. The ability of diet to drive lasting changes in the adult gut microbiome is less understood. We studied the effect of an extreme dietary shift on the fecal microbiome of 46 Labrador retriever dogs (mean age, 4.6 years) over 11 months. Dogs were fed a nutritionally complete, commercially available complex diet (CD) for a minimum of 5 weeks, followed by highly purified diets (PDs) for 36 weeks, and the initial CD for at least a further 4 weeks. Fecal samples were collected at regular intervals for DNA extraction. By analyzing 16S rRNA genes and the metagenomes, we observed minor effects on microbial diversity but significant changes in bacterial taxa and genetic potential when a PD was fed. Specifically, metagenomics identified an enrichment of quinone- and GABA-related pathways on PD, providing insights into dietary effects on cross-feeding strategies impacting community structure. When dogs returned to the CD, no significant differences were found with the initial time point. These findings are consistent with the gut microbiome being rapidly adaptable but capable of being reconstituted when provided with similar diets. These data highlight that long-term changes in the adult dog gut microbiome may only be achieved through long-term maintenance on a specified diet, rather than through feeding a transitional diet.

**IMPORTANCE** Diet can influence the adult gut microbiome (the community of bacteria) and health outcomes, but the ability to make changes persisting beyond feeding of a particular diet is poorly understood. We investigated whether feeding highly purified diets to adult dogs for 36 weeks would alter bacterial populations sufficiently to result in a persistent change following the dogs' return to a commercial diet. As expected, the microbiome changed when the purified diet was fed, but the original microbiome was reconstituted within weeks of the dogs returning to the commercial diet. The significance of these findings is in identifying an intrinsic stability of the host microbiome in healthy dogs, suggesting that dietary changes to support adult dog health through modifying the gut microbiome may be achieved only through maintenance on a specified diet, rather than through feeding transitional diets.

**KEYWORDS** canine, diet, fecal metagenome, fecal microbiota

In humans, gut bacterial communities and their genetic potential (the gut microbiome) begin to establish immediately after birth and are influenced by mode of birth (caesarean versus natural birth), environment, and early life nutrition (1, 2). Postweaning, divergent diets in different geographical communities can drive the establishment

**Citation** Allaway D, Haydock R, Lonsdale ZN, Deusch OD, O'Flynn C, Hughes KR. 2020. Rapid reconstitution of the fecal microbiome after extended diet-induced changes indicates a stable gut microbiome in healthy adult dogs. *Appl Environ Microbiol* 86:e00562-20. <https://doi.org/10.1128/AEM.00562-20>.

**Editor** Andrew J. McBain, University of Manchester

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**Received** 5 March 2020

**Accepted** 7 April 2020

**Accepted manuscript posted online** 17 April 2020

**Published** 17 June 2020

of distinct adult gut microbiome profiles (3, 4). Microbial perturbations following antibiotic exposure also shape the gut microbiome and can lead to lasting changes (5, 6). These changes may have important consequences for the host, as the gut microbiome has associations with a number of conditions, including obesity, cardiovascular disease, inflammatory bowel disease, and diabetes (7–9).

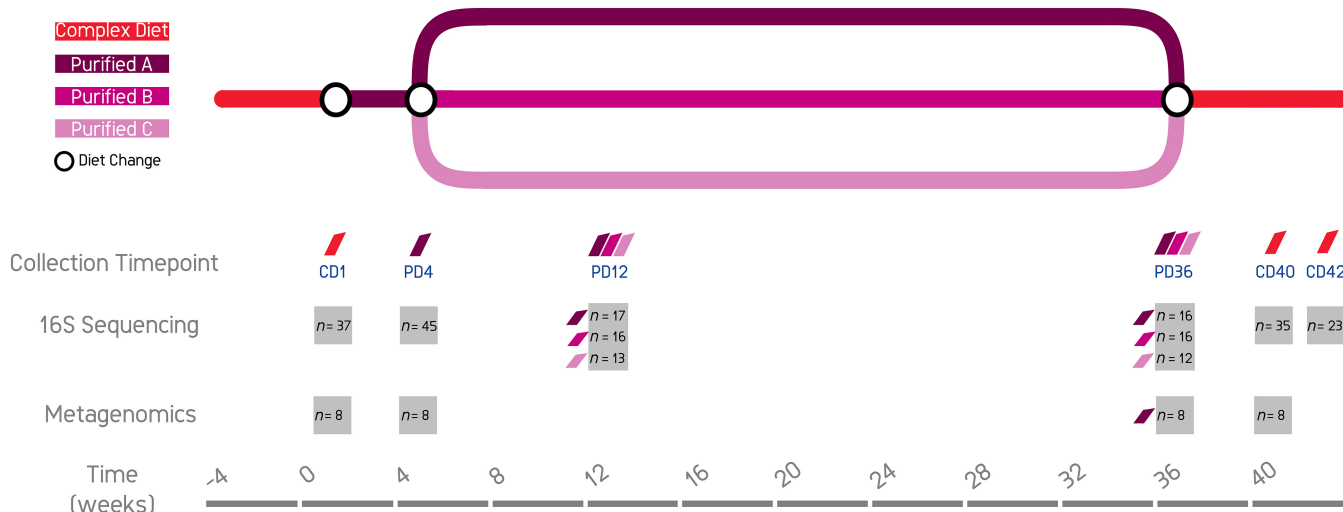
Improving health through dietary management of the microbiome is an attractive proposition. While temporary change of the adult gut microbiome through diet is well established (10), the ability of diet interventions to induce significant, long-term change in the adult microbiota remain relatively unexplored. Research studies could provide insights into the development of transitional diets to establish new microbiomes to improve health. However, controlled dietary intervention studies in humans are often hampered by an inability to exert sufficient dietary control to observe meaningful effects on health mediated through the microbiome. Rodent models have offered some insights into potential fecal microbial extinction during transitional diet feeding (11), but dogs have a close nutritional relation to humans and experience similar environmental stimuli and disease pathologies. To date, canine research has shown that early life represents an important time for the shaping of the gut microbiome (12), that manipulation of dietary macronutrients plays key roles in controlling the fecal microbiome (13, 14), and that the gut microbiome has an association with health and disease in dogs and cats (14).

The purpose of this study was to investigate whether, through feeding of a diet substantially different in composition for 36 weeks, it may be possible to alter the gut microbiome of healthy adult dogs sufficiently to enable the establishment of new microbiome community structures following return to the initial diet. The study took advantage of a nutrition intervention study using a nutritionally complete, highly purified diet (PD) (15), fed between periods of feeding a nutritionally complete, commercially available complex diet (CD). PDs have been used since the 1920s (16, 17) to meet the needs of individual studies by offering complete control over nutrient compositions. Such diets are commonly used in research as background diets to investigate the addition of novel raw materials/functional foods (18, 19) because they are much less complex and less variable than CDs. The stepwise breakdown of fibrous components of CDs is expected to require complex cross-feeding strategies and the establishment of diverse niches for a range of gut bacteria compared with those that may exploit other environments where nutrients are supplied in their more basic or purified form. As such, we hypothesized that an extremely different, highly purified diet fed for 36 weeks could be offered as a transitional diet to alter the gut microbiome sufficiently to disrupt the host microbiome and enable the establishment of different, stable microbiomes when returned to CD. We tested this hypothesis using both taxonomic and metagenomic analyses of fecal samples.

## RESULTS

**Detection of fecal microbiota composition by 16S rRNA gene sequence analysis.** Of the 276 samples proposed for collection pretrial (Fig. 1), a total of 230 fecal samples from 46 dogs were successfully collected and analyzed. Before rare count (see Materials and Methods) and noise removal, the mean read count per sample was 54,175 (median read count per sample of 51,940). After rare count and noise removal, the mean read count per sample was reduced to 52,160 (median read count per sample, 50,057). A total of 35,398 operational taxonomic units (OTUs) were detected from 16S rRNA gene sequence analysis, which reduced to 238 OTUs after rare count and noise removal. There was no significant difference in read depth between the 6 sample time points.

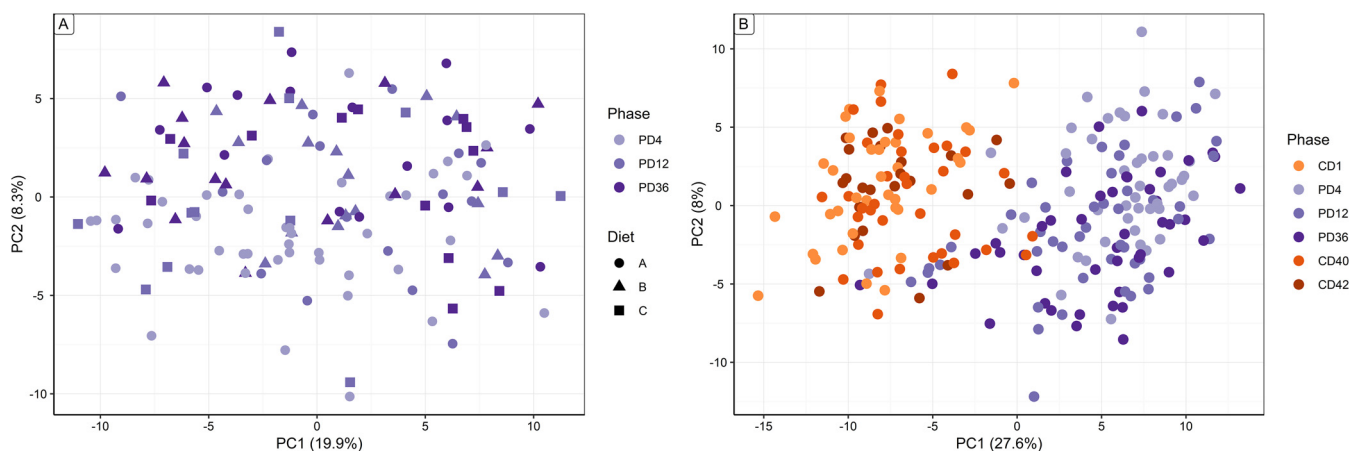
**Methionine:cystine ratio had no observable effect on microbial community structure.** The three PDs differed in the methionine:cystine ratio. Previous reports indicated that methionine may affect discrete populations of bacteria (20, 21). However, principal-component analysis (PCA) and heatmap analysis comparing PD and CD indicated that the three PDs fostered similar fecal microbial community composition



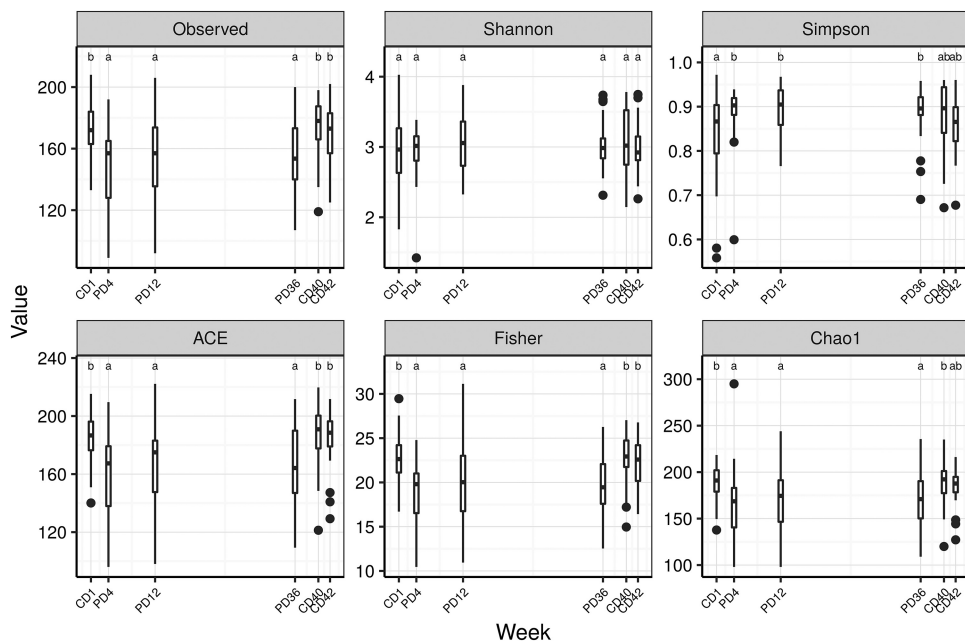
**FIG 1** Study design showing timelines, diet changes, and fecal sample collection points, with numbers analyzed for each group, for 16S sequencing and metagenomics.

with the methionine:cystine ratio and did not induce a detectable impact on fecal bacterial representation (Fig. 2A). Based on a lack of observed influence of PDs on bacterial phyla, microbiota data from the 3 diets at the 4-week (PD4), 12-week (PD12), and 36-week (PD36) time points (i.e., the initial PD time point and 8 and 32 weeks thereafter, respectively) were classified as PD for statistical analyses to compare the effects of long-term PD exposure across the cohort.

**Feeding of complex versus purified diets differentiates fecal microbial communities at the taxonomic level.** Principal-component analysis of the OTU data identified variance between CD and PD samples, observable in PC1 (Fig. 2B), indicating that the microbiota composition was affected by diet within 4 weeks of diet transition. We had hypothesized that a prolonged period of consuming PD would not only alter the microbial community structure but also reduce the diversity and species richness. Using observed diversity, the mean values were significantly lower on PD (Fig. 3); however, using the Shannon index as our primary measure of diversity, no significant difference between microbial community diversity across sample points was observed (Fig. 3). The data were analyzed using four additional indices, and except for Shannon, a significant reduction in diversity/richness of the fecal bacterial community composi-



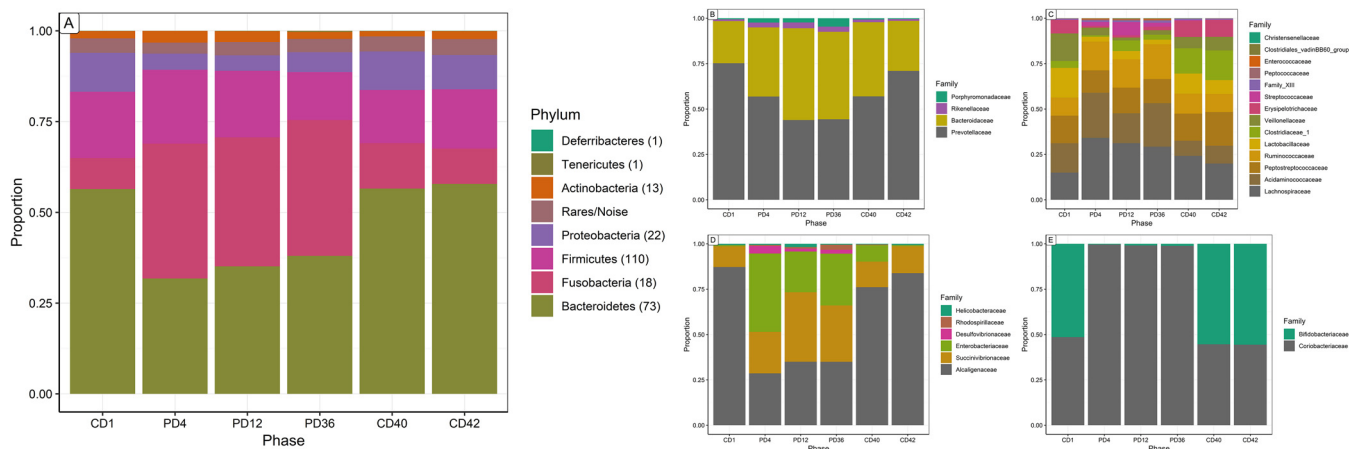
**FIG 2** Principal-component analysis (PCA) score plots of OTU data. Following Illumina sequencing analysis of fecal DNA, PCA was used to assess the variance of samples when fed one of three purified diets (PDs) only, where all dogs were fed diet A at PD4 (A), and variance when also including phases when fed complete diets (CDs) (B). Numbers represent week of study.



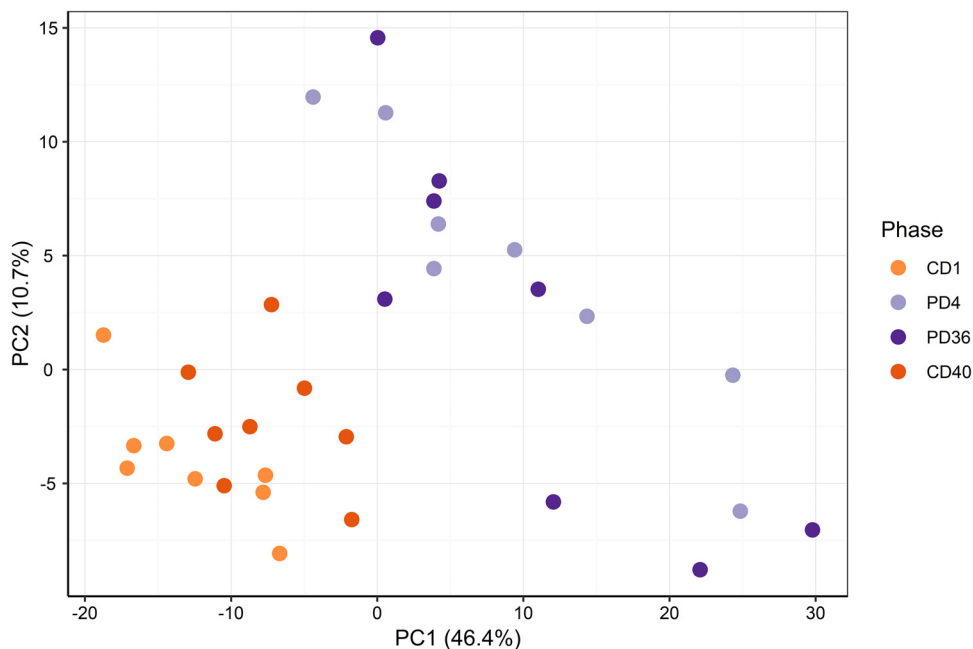
**FIG 3** The diversity of fecal microbiota was determined at each sampling phase using 6 different approaches. Box plots represent quartiles. Time points that do not share a letter are significantly different by Tukey tests ( $P < 0.05$ ).

tion was detected when dogs consumed PDs compared with those observed at the initial baseline fecal sample (CD1) time point (Fig. 3).

When considering the distribution of bacteria at different taxonomic levels, distinct shifts in abundance were also observed. *Bacteroidetes*, *Fusobacteria*, and *Firmicutes* were the dominant phyla across the entire sample cohort (Fig. 4A). *Fusobacteria* showed the largest changes in proportional representation, increasing by around 2.5-fold during PD feeding. This phylum was represented by a single genus, *Fusobacterium*. *Bacteroidetes* decreased in proportion ~2-fold (Fig. 4A), and at the family level, an increased proportion of *Bacteroidaceae* and a decreased proportion of *Prevotellaceae* were observed (Fig. 4B). Within the *Firmicutes* family, large proportional increases in *Lachnospiraceae* and decreases in *Lactobacillaceae* were observed when dogs transitioned from CD to PD (Fig. 4C). When considering changes within individual phyla, *Deferribacteres* showed the most consistent and statistically significant shift with diet. This phylum, represented by a single genus, *Mucisprillum*, was consistently present on CD



**FIG 4** Taxonomic relative abundance distribution of fecal microbiota at each sampling phase, at the phylum level (A), with number of OTUs in each phylum listed in the legend, and at the family level for *Bacteroidetes* (B), *Firmicutes* (C), *Proteobacteria* (D), and *Actinobacteria* (E).



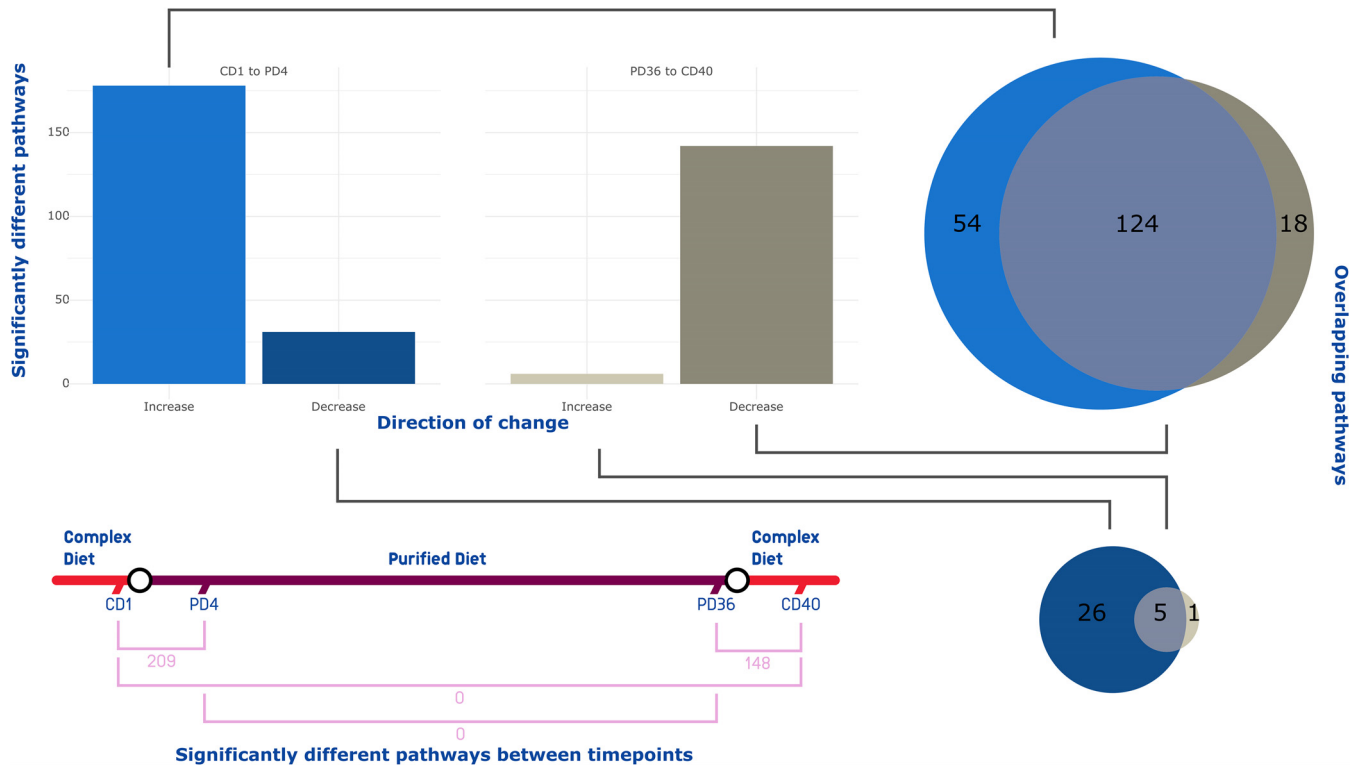
**FIG 5** Multigroup PCA scores of metagenome data at the pathway level, for four time points (data for 8 dogs fed PD "A").

and largely absent on the PD diet (see Fig. S1 in the supplemental material). Although overall representation of the *Proteobacteria* remained relatively unchanged, large proportional increases in *Enterobacteriaceae* and concurrent decreases in *Alcaligenaceae* were observed following the transition from CD to PD (Fig. 4D). Similar observations were also made within the *Actinobacteria* phylum where large proportional changes at the family level (increases in *Coriobacteriaceae* and decreases in *Bifidobacteriaceae*) following the transition from CD to PD were observed (Fig. 4E). Despite these changes, PCA analysis and taxonomic profiles pre- and post-PD showed that CD1, CD40, and CD42 communities are similar (Fig. 2B, Fig. 4A to E), suggesting the reconstitution of the pretrial microbial communities occurred within 4 to 6 weeks of changing the diet.

**Metagenomics identifies significant changes in genetic potential of the gut microbiome associated with diet.** Metagenomic analysis of a subset of the fecal samples was used to interrogate the data set. Prior to filtering for quality and host DNA contamination, there was a median and mean read count per sample of  $56,045,949 \times 2$  and  $55,601,432 \times 2$ , respectively. After trimming and host contaminant DNA removal the median read count per sample was  $41,864,532 \times 2$  and the mean read count per sample was  $42,534,357 \times 2$ .

PCA analysis of the functional pathway relative abundances showed separation between PD and CD samples in PC1/PC2 (Fig. 5). Subsequent univariate analysis showed no significant differences in any of the pathways comparing CD1 and CD40 time points (Fig. 6). Together, these data suggested that feeding dogs PDs for 36 weeks did not result in long-lasting alterations to the fecal microbial composition or its genetic potential.

**Diet-induced changes in the metagenome are stable over 32 weeks but are reversible within 4 weeks.** To compare metagenomics data, abundance differences in the genetic potential from a total of 354 pathways were analyzed. Following dietary transition from CD1 to PD4, the relative abundance (RA) of 209 pathways differed significantly ( $P < 0.05$ ). Of these, 178 pathways increased in RA and 31 decreased in RA after transition to a purified diet. Conversely, dietary transition back from PD36 to CD40 resulted in 148 pathways that significantly differed in RA ( $P < 0.05$ ). Of these, 142 pathways decreased in RA, while only 6 increased on return to CD. Of the 178 pathways



**FIG 6** A comparison of significant changes in pathway abundance between different time points. Following the transition to PD, 178 pathways increased and 31 decreased in abundance (bar chart, left-hand side; CD1 to PD4). Following the transition back to CD, 142 pathways decreased and 6 increased on the return to CD (bar chart, right-hand side; PD36 to CD40). Of the 178 pathways that increased from CD to PD, 124 decreased following the return to CD (top Venn diagram), while 5 of the 31 that decreased from CD to PD increased on return to CD (bottom Venn diagram). The total number of significant changes between time points (described in the time point bar at the bottom of the figure) shows that no pathway had a significant difference between PD4 and PD36 or between CD1 and CD40.

that increased from CD to PD, 124 decreased following return to CD, while 5 of the 31 that decreased from CD to PD increased on return to CD. No pathway differed significantly in the same direction at the two transitions (e.g., was elevated following transition at both time points). In addition, no pathway had a significant difference between PD4 and PD36 nor between CD1 and CD40, suggesting that the genetic potential was stable on these diets within 4 weeks of feeding a diet and was restored within 4 weeks of feeding CD (Fig. 6).

An analysis of the 129 pathways identified as significantly different following both diet transitions identified pathways related to metabolic functions, such as energy metabolism, vitamin B and other cofactor syntheses, nucleotide syntheses, and bioenergetics, as well as bacterial membrane component synthesis (see Table S2 in the supplemental material).

Pathway enrichment analysis was used to aid context and provide an objective analysis of the 129 pathways that changed significantly following both dietary transitions. The 129 metabolic pathways are members of 28 superpathways, of which four superpathways met our enrichment inclusion criteria for further interpretation, namely, cofactor biosynthesis (33 pathways), tricarboxylic acid cycle (TCA) variants (6 pathways), amine degradation (5 pathways), and metabolic regulators (2 pathways) (Table 1).

## DISCUSSION

The objective was to investigate whether long-term (36-week) feeding of PD to adult dogs would alter the gut microbial community sufficiently to enable new, stable bacterial community compositions to develop following a return to the original diet. If so, transitional diets might be an approach to modify the gut microbiome to support host health in dogs with, or predisposed to, chronic conditions. Our observations were

**TABLE 1** Superpathway members meeting enrichment set analysis criteria

Superpathway	Pathway name	Pathway code
Amine degradation	4-Aminobutanoate degradation V	PWY-5022
	Allantoin degradation to glyoxylate III	PWY-5705
	GABA shunt	GLUDEG-I-PWY
	Superpathway of <i>N</i> -acetylglucosamine, <i>N</i> -acetylmannosamine, and <i>N</i> -acetylneuraminic acid degradation	GLCMANNANAUT-PWY
Cofactor biosynthesis	Superpathway of ornithine degradation	ORNDEG-PWY
	2-Carboxy-1,4-naphthoquinol biosynthesis	PWY-5837
	Molybdenum cofactor biosynthesis	PWY-6823
	NAD <i>de novo</i> biosynthesis I (from aspartate)	PYRIDNUCSYN-PWY
	NAD salvage pathway I (PNC VI cycle)	PYRIDNUCSAL-PWY
	NAD salvage pathway III (to nicotinamide riboside)	NAD-BIOSYNTHESIS-II
	NAD/NADH phosphorylation and dephosphorylation	PWY-5083
	NAD/NADP-NADH/NADPH mitochondrial interconversion (yeast)	PWY-7269
	Polyisoprenoid biosynthesis ( <i>Escherichia coli</i> )	POLYISOPRENSYN-PWY
	Pyridoxal 5'-phosphate biosynthesis I	PYRIDOXSYN-PWY
	Pyridoxal 5'-phosphate salvage II (plants)	PWY-7204
	Superpathway of demethylmenaquinol-9 biosynthesis	PWY-5862
	Superpathway of menaquinol-10 biosynthesis	PWY-5896
	Superpathway of menaquinol-11 biosynthesis	PWY-5897
	Superpathway of menaquinol-12 biosynthesis	PWY-5898
	Superpathway of menaquinol-13 biosynthesis	PWY-5899
	Superpathway of menaquinol-6 biosynthesis I	PWY-5850
	Superpathway of menaquinol-7 biosynthesis	PWY-5840
	Superpathway of menaquinol-8 biosynthesis I	PWY-5838
	Superpathway of menaquinol-9 biosynthesis	PWY-5845
	Superpathway of phyloquinol biosynthesis	PWY-5863
	Superpathway of tetrahydrofolate biosynthesis	PWY-6612
	Superpathway of tetrahydrofolate biosynthesis and salvage	FOLSYN-PWY
	Superpathway of thiamine diphosphate biosynthesis I	THISYN-PWY
	Superpathway of thiamine diphosphate biosynthesis II	PWY-6895
	Superpathway of ubiquinol-8 biosynthesis (prokaryotic)	UBISYN-PWY
	Tetrapyrrole biosynthesis I (from glutamate)	PWY-5188
	Tetrapyrrole biosynthesis II (from glycine)	PWY-5189
	Ubiquinol-10 biosynthesis (prokaryotic)	PWY-5857
	Ubiquinol-7 biosynthesis (prokaryotic)	PWY-5855
Ubiquinol-8 biosynthesis (prokaryotic)	PWY-6708	
Ubiquinol-9 biosynthesis (prokaryotic)	PWY-5856	
Pantothenate and coenzyme A biosynthesis III	PWY-4242	
1,4-Dihydroxy-2-naphthoate biosynthesis II (plants)	PWY-5791	
Metabolic regulators	Mannosylglycerate biosynthesis I	PWY-5656
	ppGpp biosynthesis	PPGPPMET-PWY
TCA variants	Superpathway of glyoxylate bypass and TCA	TCA-GLYOX-BYPASS
	TCA cycle I (prokaryotic)	TCA
	TCA cycle IV (2-oxoglutarate decarboxylase)	P105-PWY
	TCA cycle V (2-oxoglutarate:ferredoxin oxidoreductase)	PWY-6969
	TCA cycle VII (acetate producers)	PWY-7254
	TCA cycle VIII ( <i>Helicobacter</i> )	REDCITCYC

consistent with reports that diet change significantly alters the taxonomic structure of the gut microbial communities (13, 22). However, we also demonstrated that changes induced by long-term feeding were transitory because fecal microbial communities pre- and posttrial showed similar microbial taxonomic structure and genetic potential. Considering the extreme nature of the diet manipulation used, we suggest that long-term manipulation of the adult microbiome may be achieved only by maintaining feeding of a specified diet and not by deployment of transitional diets.

We surmised that the CD microbiome would be more diverse, with different microbes specializing in niches required for the stepwise breakdown and utilization of complex substrates (including polysaccharides and fermentable fibers present in complex diets), than the PD microbiome, with limited nutrient diversity (21). This would be consistent with observations in human populations differing in fiber content with fecal microbiota (4) and gene richness (23). It was, therefore, unexpected when long-term feeding on PD had no detectable impact on our primary measure of fecal microbial

diversity, the Shannon index. Further analysis at the taxonomic level identified significant changes in the fecal microbial community profiles between CD to PD (Fig. 3), consistent with reports that diet, particularly those differing macronutrient profiles, can have a profound impact on fecal microbial composition in dogs (13, 22). Notably, 4 weeks after an extreme diet transition, it was possible to have a distinct fecal microbiome community structure, but with a similar diversity, that remained stable over a further 32 weeks.

Notable changes in bacterial communities between the two diets were observed, particularly in the two most dominant phyla. *Fusobacteria* showed approximately 2.5-fold increased prevalence during PD feeding. These OTUs were, in general, represented by *Fusobacterium* at the genus level. *Fusobacteria* are non-spore-forming, Gram-negative, obligate anaerobes which are asaccharolytic (incapable of breaking down carbohydrates for energy). *Fusobacteria* have been reported in dog feces and were higher when dogs were fed a “natural” diet (bone, raw meat, and vegetable) than two commercial feeds (24). Reduced incidence of members of the *Bacteroidetes* phylum (2-fold decrease) was observed on PD (Fig. 3A). *Bacteroidetes* are composed of three large classes of Gram-negative, non-spore-forming, anaerobic or aerobic, rod-shaped bacteria, described to be prevalent in dogs (13, 22). We also observed significant reductions in *Deferribacteres* on PD, although the proportional representation of this group was small. *Deferribacteres* are a group of fast-growing opportunists, many of which do not have a sequenced genome, but which have been described in dogs (25). *Deferribacteres* were represented by *Mucispirillum* at the genus level, which are bacteria inhabiting the mucous layer and the prevalence of which has correlated with the thickness of the intestinal mucous barrier (26).

To determine whether long-term PD feeding altered the gut microbiome sufficiently to result in a novel fecal microbial community after dogs were offered a complex diet again, we compared CD1 to CD40/CD42 time points. Dogs returned to a bacterial community structure similar to that seen at CD1, suggesting that the intrinsic microbial composition in these dogs was resilient. In other words, bacteria unable to flourish in the lumen on the PD regime were able to reestablish within weeks of hosts being fed a diet similar to the original one. Individual taxonomic changes can be difficult to interpret, because at various taxonomic levels, bacteria are metabolically diverse (27). Thus, qualitative descriptions of these diet-induced alterations are of limited value and further insights were sought through comparing changes to the metagenome.

To investigate the genetic potential induced by long-term feeding of PD, a subset of samples were analyzed using metagenomic sequencing. A group of 32 samples from 8 dogs provided a representative perspective of the microbiome pretrial (CD1) and posttrial (CD40) alongside those from early (PD4) and late (PD36) stages during the dietary intervention. Analysis of the metagenomic data showed a broad effect of diet on the pathway level (357 pathways representing 28 of 40 superpathways affected). This effect was stable between 4 and 36 weeks of feeding the PD but did not persist, with a significant but transitory effect for many pathways reverting within 4 weeks of transitioning back to CD. These data support the findings of the 16S rRNA composition analysis regarding a broad-ranging and stable effect of diet that was transient, with no significant change between CD1 and CD40, returning to a similar diversity profile within 4 weeks. However, unlike taxonomic diversity that reduced on PD, the genetic potential was elevated when dogs were fed PD compared with being fed CD. This observation is based on the number of pathways that increased on PD and decreased on CD (124 pathways) compared with those that decreased on PD and then increased on CD (5 pathways). This highlights that bacterial diversity did not reflect the metabolic functional potential of the microbiome, nor did it conform to our expectation that those genes required to exploit the complex raw materials would represent increased genetic potential on CD.

An interpretation of the areas of metabolism most significantly changing with diet highlighted four superpathways related to energy metabolism, specifically related to amine degradation, TCA cycle, and electron transfer systems. As PDs containing purified



amino acids are readily absorbed by the host (reflected in the high fecal crude protein apparent digestibility of 96% [15]) we suggest that enrichment of the *N*-acetylglucosamine, *N*-acetylmannosamine, and *N*-acetylneuraminate degradation pathway reflects the ability of bacteria to scavenge from intestinal sources, including host cell mucus and cell membranes, and bacterial cell wall structures (28). The other amine degradation pathways relate to carbon and nitrogen metabolism via the GABA shunt. The GABA shunt provides a mechanism for generating energy from glutamate without losing nitrogen as ammonia (29), which may be particularly important when amino acid availability is low. In addition, GABA has a role in cross-feeding, with transcription of GABA-producing pathways reported in *Bacteroides*, *Parabacteroides*, and *Escherichia* species in stool samples and GABA being identified as the sole growth factor produced by *Bacteroides fragilis* required to support growth of a previously only coculturable bacterium KLE1738 (30). We suggest that, under conditions of sufficient nutrient supply, the glutamate decarboxylase (GAD) system, induced under anaerobic conditions, provides a mechanism for some bacteria to manage acid stress through a glutamate/GABA antiporter, releasing extracellular GABA with an additional proton from glutamate decarboxylation (29). GABA export then supports the growth of other, luminal bacteria able to utilize the GABA as C and N sources. However, the relatively nutrient-deprived digesta provided by highly digestible dietary PD resulted in donor bacteria using strategies other than GABA export. Subsequently, those bacteria catabolizing GABA had fewer resources and were less able to replicate. This resulted in their decreased relative abundance in the fecal microbiome, while bacteria scavenging closer to the host mucosa under more microaerobic conditions increased in relative abundance.

Other evidence supports the increased relative abundance of mucosa-associated bacteria, with enrichment within the superpathway representing the largest number of pathways ( $n = 33$ ), namely, that of cofactor biosynthesis. Of 33 pathways, at least 24 were related to the synthesis of specific menaquinones and ubiquinones, which provide the electron transfer link to cytochrome oxidases and are known to have different redox optima (31). Increased abundance suggests that bacteria capable of adapting to more varied microaerobic conditions, such as in close proximity to host cells, were more prevalent, an interpretation that is also consistent with enrichment of the TCA-variant superpathway. It is also notable that, similar to GABA, quinone biosynthesis by donor bacteria was identified as an essential growth factor for unculturable gut microbiota (32). Menaquinone biosynthetic pathways are missing from many unculturable OTUs from human stool samples (32, 33), and anaerobic respiration by some bacteria require donor-derived menaquinone biosynthesis (e.g., members of the *Faecalibacterium* genus [32, 34]). The loss of quinone biosynthesis has occurred independently across lineages and been suggested as a “cheater” strategy, taking advantage of quinones produced and released by other organisms into the environment (32). Although speculative, enrichment for quinone biosynthesis pathways in the metagenomes on PD may simply reflect a reduced relative abundance of those bacteria reliant on quinone extracellular transport from donor bacteria, which is reduced under these conditions. Future work could include *in vitro* cross-feeding studies to determine the relative abundance of GABA and quinone utilizers with conditions suitable for GABA and quinone production.

Other cofactor pathways enriched on PD are those for biosynthesis and salvage of folate, vitamin B6, and NAD. Other nutrient-related pathways (in purine and pyrimidine synthesis) were also increased on the PD, consistent with the very restricted provision of nucleotides from the ingredients used. We suggest that these pathways reflect the increased demand for bacterial biosynthesis of nucleotides for DNA and RNA synthesis rather than scavenging them from the diet. The other superpathway, metabolic regulators, included the guanosine pentaphosphate (ppGppp) pathway, representing that the stringent stress response can be induced by amino acid starvation, due to stalled ribosomes. This is consistent with PD resulting in a nutrient-deprived lumen, where bacteria living in closer proximity to the host were capable of tolerating stress or

scavenging from fewer resources and were relatively more abundant than those that benefit from a more nutrient-diverse environment provided by CD. An important consideration from this is the confounding role complex diets have in driving population changes in the lumen, resulting in a relatively low representation of those members of the gut microbiome more commonly inhabiting the microhabitats of the inner and outer intestinal mucus layers and crypts (35) that may better reflect host-microbe interactions.

Restoration of the CD fecal microbiome within 4 weeks suggests a stable resident gut microbiome that is capable of recolonization of the lumen from reservoirs in response to diet change. Some bacteria, for example *B. fragilis* strains, routinely exchange between the intestinal crypts and the lumen (36), while regional structural variations, such as the cecum or appendix, may similarly serve as microbial reservoirs (37). Together, these reservoirs may explain the transitory effect of feeding a purified diet over such an extended period of time and explain why transitional diets may not provide an effective approach to modify the adult microbiome. Our findings support the view that intentional manipulation of the microbiome may be more effective in early life, when perturbations of the microbiota may shape the adult microbiota (38), and may establish desired microbial species and/or community structures to support health benefits (6, 39, 40). As many dogs obtain nutrition predominantly from nutritionally complete, commercial diets throughout life, there are opportunities to support appropriate host-microbe interactions that are unmanageable in normal, healthy human populations. Dogs experience similar stimuli and disease pathologies as humans (41), and a recent diet intervention study involving dogs, humans, mice, and pigs observed the greatest phylogenetic similarity and genetic potential between the dog and human microbiome (42). Therefore, canine-based studies may also highlight the potential for dietary influences in supporting beneficial microbe-human interactions.

In conclusion, our work has provided detailed insights into the changes that occur in the adult dog fecal microbial communities following long-term diet change and suggests that they are dynamic and fully reversible. Consequently, our findings are consistent with the proposition that nutritional interventions aiming to modulate health through the microbiome are likely to require maintained feeding to persist.

## MATERIALS AND METHODS

**Animals.** Adult Labrador retriever dogs ( $n = 46$ ) with an average age of 4.6 years (range, 2.0 to 7.8 years) were enrolled. Veterinary checks (physical and blood analysis) prior to and during the course of the study indicated they were healthy throughout. Dogs were housed and exercised with other dogs in their dietary group. Studies were approved by the Waltham Animal Welfare and Ethical Review Body and performed under the UK Regulation of Animals (Scientific Procedures) Act of 1986.

**Diets.** Diets included a standard, commercially available, nutritionally complete, and balanced kibble-type diet (Pedigree Adult Complete, dry; referred to as CD) and three highly purified, nutritionally complete diets (Ssniff Spezialdiäten GmbH; referred to as PD). PDs contained crystalline amino acids instead of intact protein and no complex carbohydrates, except for pure corn starch, maltodextrin, and refined cellulose powder. The three PDs differed in the ratio of methionine to cystine (0.98, 0.34, and 0.24), with total sulfur-containing amino acids being maintained at 2.68 g/1,000 kcal. PDs were isocaloric and isonitrogenous (via alanine). The complete dietary analysis of PDs is described in Table S1 in the supplemental material. CD was offered in specific amounts to maintain an individuals' ideal body condition score (43). PDs were initially offered at individually determined maintenance energy requirements (MERs), estimated by energy intake equivalent to the CD phase, but intakes were adjusted as required to maintain individuals' ideal body condition score (43).

**Study design.** Details of the experimental design and sample collection time points are shown in Fig. 1. Dogs were initially fed CD for a minimum of 5 weeks. Baseline fecal samples (CD1) were collected from dogs prior to transition onto the purified diet (PD, "A"). Fecal samples were collected again after 4 weeks (PD4) on PD A. The dogs, randomized into 3 groups, were then fed either PD A ( $n = 17$ ), PD "B" ( $n = 16$ ), or PD "C" ( $n = 13$ ). Dogs in these dietary groups were balanced for age and sex. Diets were then fed for a further 32 weeks, and fecal samples were collected at 8 weeks (PD12) and 32 weeks (PD36) after entry onto the individual PD treatment arms. Dogs were transitioned back onto feeding the CD, and fecal samples were collected at 4 weeks (CD40) and 6 weeks (CD42) posttransfer onto this diet. Data are missing for two reasons, namely, either fecal samples were not collected or data were of low sequence data quality (see Fig. 1 for data sampled).

**Fecal microbiota and microbiome analysis.** Samples of fecal material from the core of freshly evacuated feces were obtained using sterile metal spatulas by opening the sample longitudinally and

subsampling from the core. Samples (200 mg wet weight) were stored in Eppendorf tubes at  $-80^{\circ}\text{C}$  within 60 min of defecation.

**DNA preparation.** DNA was extracted from fecal samples (100 mg) using the QIAamp 96 PowerFecal QIAcube high-throughput (HT) kit (Qiagen; according to the manufacturer's instructions) and an epMotion 5075 robot (Eppendorf). In brief, material was homogenized using a tissue lyser (Qiagen) (5 minutes at 30 Hz, room temperature), followed by digestion with proteinase K (Qiagen) (10 minutes, room temperature). Lysates were applied to a QIAamp 96-well plate under vacuum at 90 kPa. Remaining bound impurities were removed by washing, and pure DNA was eluted under centrifugation in buffer ATE (100  $\mu\text{l}$ ). Purified DNA was analyzed by Nanodrop spectrophotometry. Only samples with a 260/280 nm ratio of  $>1.8$  were used.

**Dual indexing barcoding for microbiota analysis.** The 16S rRNA PCR amplicons were prepared for sequencing using the 319F/806R dual-indexed PCR approach (44). In brief, primers were arrayed into 96-well plates at a final concentration of 0.1  $\mu\text{M}$ . Master mix containing the Phusion high-fidelity (HF) enzyme (New England BioLabs) and HF buffer including genomic DNA (10 ng) was then applied to each well and cycling performed as follows:  $98^{\circ}\text{C}$  for 30 s;  $98^{\circ}\text{C}$  for 15 s,  $58^{\circ}\text{C}$  for 15 s, and  $72^{\circ}\text{C}$  for 15 s (30 cycles); and  $72^{\circ}\text{C}$  for 60 s. Agarose gel electrophoresis confirmed successful amplification of the samples.

**Sequencing and bioinformatic analyses of fecal bacterial community composition.** The 16S rRNA amplicons for sequencing on the Illumina MiSeq platform (Eurofins Genomics, Germany) were prepared according to standard protocols.

Forward and reverse reads were assembled into contiguous sequences spanning the entire 16S rRNA gene V3-V4 regions using FLASH assembler (45). Tags were removed using TagCleaner (46), and sequences were demultiplexed in QIIME using `split_libraries_fastq.py`. Chimeric sequences were removed using `userarch61` (47). Sequences were clustered at  $>98\%$  identity using `uclust` (48) to generate operational taxonomic units (OTUs), and the most abundant sequences were chosen as cluster representatives.

The relative abundance and distribution across samples was assessed for each OTU to separate noise from consistent but rare OTUs (latter defined as mean proportion of  $<0.05\%$  in at least two dogs in any study group). Representative sequences of all OTUs that passed the filtering criteria were searched against the Silva small subunit (SSU) database release 128 (49) using `blastall 2.2.25` (50). If the alignment did not meet the cutoff criteria of  $\geq 98\%$  sequence identity and  $\geq 98\%$  query sequence coverage, genus or higher level annotations were used.

**Statistical analyses of bacterial diversity and community composition.** Diversity and richness indices (observed, Shannon, Simpson, abundance-based coverage estimator [ACE], Fisher, and Chao1) were calculated for all samples and modeled using a linear mixed effects model. The response of this model was the diversity, with phase included as a fixed effect, and a random effect of dog was included to account for the repeat measurements of the sequence composition within each dog. From this model, the average diversity of the microbiota at each phase was estimated for each diversity calculation, with 95% confidence intervals, and a Tukey test was performed to compare all pairs of phases while maintaining a false-positive rate of 5%. An R package (`phyloseq v1.16.2`) calculated the other alpha diversity metrics (see detailed methods in reference 32).

Multigroup principal-component analysis (PCA) was performed on the relative abundance of rare/noise-filtered OTUs with group set as dog to account for the repeat measurements, and OTUs were scaled to unit variance to allow changes in the smaller proportion OTUs to show. Scores and loadings were extracted from these results, and the scores were visualized by study phase to investigate changes over time and individual purified diet to assess whether this level of granularity was necessary.

All analyses were performed using R v3.3.3 using the `lme4`, `multcomp`, `mixOmics`, `FactoMineR`, and multigroup libraries (42).

**Sequencing and bioinformatic approaches for fecal metagenome analysis.** DNA samples ( $n = 32$ ) from eight dogs (5 female [F], 3 male [M]; average age of 5.0 years; range, 3.7 to 7.5 years) all receiving PD A were taken forward for a pilot study to assess metagenomic content. Only CD1, PD4, PD36, and CD40 time points were selected to provide representative profiles pre- and posttrial alongside short- and long-term PD exposure. Aliquots from the same purified DNA samples were used for both 16S rRNA and metagenomic sequencing. Standard genomic library preparation, DNA fragmentation, adapter ligation, amplification, and size selection were performed (Eurofins, Germany). Sequencing was completed using a  $2 \times 150\text{-bp}$  paired-end approach using the Illumina HiSeq 4000 platform to generate a total of 50 million reads per sample.

A dog reference genome database was created using `bowtie2-build` with the *Canis lupus familiaris* reference genome CanFam3.1 (NCBI accession number [GCA\\_000002285.3](https://.ncbi.nlm.nih.gov/assembly/GCA_000002285.3)) (51). Paired-end FASTQ files were processed using the dog reference genome database and `KneadData` (<https://bitbucket.org/biobakery/kneaddata/wiki/Home>) to perform quality control and remove contaminant host DNA. The composition of the microbial communities was profiled using `MetaPhlan2` (52). `HUMAnN2` was used to profile the abundance of microbial pathways against the `ChocoPhlan` nucleotide reference database and the `Uniref90` protein reference database (53, 54). Relative abundances of `MetaCyc` pathways were calculated using `humann2_renorm_table` with the option “-units relab.”

**Statistical analyses of fecal metagenome composition.** Comparisons of fecal metagenome composition were made between CD1 and PD4, CD1 and CD40, PD4 and PD36, and PD36 and CD40 for all pathway relative abundances using a Wilcoxon signed-rank test. This test was used as it makes no assumptions about the data distribution and is also unaffected by zero inflation. Within each contrast, Benjamini-Hochberg correction was applied to the *P* values to maintain a 5% false-positive rate.

To perform enrichment analysis for an assessment of diet effects on fecal metagenomes, each pathway was matched to all upstream level 2 pathways, referred to herein as superpathways, using the MetaCyc v23.0 (55) database. Level 1 pathways from the database were excluded, as they provided little discrimination in the subsequent analysis. Prior to enrichment analysis, each individual pathway was marked as being significant if it met the criteria of having significant differences between CD1 versus PD4 and PD36 versus CD40 in the univariate analysis by the Wilcoxon signed-rank test. Enrichment analysis was then performed by calculating the ratio of the total number of pathways to the number of significant pathways within each superpathway. This was then divided by the ratio of the total number of pathways to the total number of significant pathways in the whole data set to give an enrichment value. An enrichment value greater than 1 indicates that a superpathway contains more significant pathways than the data average. Permutation testing was also performed by randomly assigning the significance tag to the pathways 1,000 times to give a *P* value for each enrichment value.

Following the enrichment analysis, the following *a priori* criteria were implemented for inclusion: enrichment >1; at least 2 pathways detected, if 2 both must be significant; if 3 to 5 pathways are detected, then ≥50% must be significant; and if >5 are detected, then ≥25% must be significant and the overall *P* value is <0.05.

**Data availability.** Metagenomics and 16S sequencing data were submitted to ENA under primary accession number [PRJEB34360](https://doi.org/10.1093/bioinformatics/btj036).

## SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

**SUPPLEMENTAL FILE 1**, PDF file, 0.1 MB.

**SUPPLEMENTAL FILE 2**, XLSX file, 0.01 MB.

**SUPPLEMENTAL FILE 3**, XLSX file, 0.01 MB.

## ACKNOWLEDGMENTS

We thank all of our colleagues at the WALTHAM Centre for Pet Nutrition for their care and expertise in the training, welfare and sample collection of dogs used in the study and those in the research team who supported us, specifically Nicola Kirkwood for lab support and Matt Harrison who managed the purified diet study.

The work was funded by Mars Petcare UK, and the funders had no role in the design, analysis or writing of this article.

All authors were employed by Mars Petcare UK at the time of the study.

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