The Journal of Nutrition
Nutrient Physiology, Metabolism, and Nutrient-Nutrient Interactions

Dietary Protein and Carbohydrate Levels Affect the Gut Microbiota and Clinical Assessment in Healthy Adult Cats

Dayakar V Badri,[1] Matthew I Jackson,[1] and Dennis E Jewell[2]

[1]Hill's Pet Nutrition, Inc, Topeka, KS, USA; and [2]Department of Grain Science and Industry, Kansas State University, Manhattan, KS, USA

ABSTRACT

Background: Relative levels of dietary protein and carbohydrate intake influence microbiota and their functional capabilities, but the effect has not been well documented in cats.

Objectives: The impact of 3 foods with different protein:carbohydrate ratios on the gut microbiota and functional attributes in healthy adult cats was evaluated.

Methods: Male and female cats ($n = 30$; mean age: 5.1 y; mean body weight: 5.26 kg) were fed 1 of 3 foods [P28 (28.3% protein, dry matter basis), P35 (35.1%), and P55 (54.8%)] for 90 d in a Williams Latin Square design. Each food had a 1:1 ratio of animal (dried chicken) to plant (pea) protein; protein replaced carbohydrate as protein level increased. Fecal microbiota and their functional capability were assessed with 16S sequencing and the Kyoto Encyclopedia of Genes and Genomes database, respectively.

Results: Fecal pH, ammonia, and branched-chain fatty acids (BCFAs) were higher when cats consumed P55 food than when they consumed P28 and P35. Clear separation of samples between P28 and P55 based on bacterial genera was observed, with partitioning into saccharolytic and proteolytic functions, respectively. Significantly higher α diversity was seen with P55 than with P28 and P35. Amino acid metabolism, mucin foraging pathways, and urea metabolism were higher with P55 than with P28, whereas feces from cats fed P28 had higher concentrations of carbohydrate-active enzymes and enzymes involved in SCFA pathways than with P55. Bacterial genera that showed positive associations with amino acid catabolism also showed positive associations with mucin degradation.

Conclusions: Despite higher protein digestibility and less protein arriving to the colon, when healthy adult cats consumed the highest level of protein (P55), their gut microbiota exhibited higher mucin glycan foraging and amino acid metabolism, leading to higher fecal pH, ammonia, and BCFAs. This is likely due to lower availability of carbohydrate substrates and dietary fiber as protein replaced carbohydrate in the food. *J Nutr* 2021;151:3637–3650.

Keywords: feline, protein concentrations, feces, microbiota, fecal pH, proteolysis, mucin foraging

Introduction

The intestinal microbiota plays an important role in host development and health. Previous studies have demonstrated the impact of intestinal microbiota on maintaining the overall well-being of pets and its role in various disease conditions (1–4). Dietary factors play a critical role in driving the intestinal microbiota composition and function (1, 5–7), to a greater degree than genetic factors (8). The effects of these dietary factors on the functions and metabolites of the intestinal microbiota have been associated with several disease conditions (9–13). Further, the gastrointestinal (GI) microbiota and its functional products influence host health (14, 15).

Several studies have demonstrated the importance of determining appropriate dietary protein levels in cats, which are obligate carnivores (16–20). Dietary protein levels can affect the production of microbially derived toxic metabolites in the large intestine that may serve as contributing factors for renal insufficiency and cardiovascular disorders in dogs and cats (1, 21, 22). In humans, increased dietary protein or decreased carbohydrates may also reduce saccharolytic fermentation (23, 24). It appears that the presence of dietary fiber is protective against detrimental health outcomes associated with excess colonic proteolysis (25), although additional factors such as parasite burden may contribute.

Only a few studies have examined the impact of dietary protein levels on the gut microbiota of adult cats (26) or growing kittens (27, 28). These studies used isocaloric foods; however, in addition to differing protein levels, they also had different levels of fat and total dietary fiber. Further, the protein level in the high-protein food was adjusted without correcting

Manuscript received February 1, 2021. Initial review completed June 16, 2021. Revision accepted August 19, 2021.
First published online September 29, 2021; doi: https://doi.org/10.1093/jn/nxab308.

for animal compared with plant protein, and amino acid compositions in tested foods were not reported.

To our knowledge, other studies have not systematically analyzed the effects of feline foods formulated by maintaining ratios of individual amino acids as well as animal/plant protein sources, and compensating for protein with carbohydrates without changing levels of fat and total fiber. Thus, the objective of the present study was to examine the impact of foods formulated in this manner with 3 dietary protein levels on blood and serum chemistry, urinalysis, and the gut microbiota of healthy adult cats followed by analysis of functional attributes.

Methods

Animals and experimental design

Thirty clinically healthy adult cats (15 male, 15 female) with mean age 5.1 y (range: 2.1–8.9 y) and mean body weight (BW) 5.26 kg (range: 3.1–7.5 kg), all spayed or neutered and owned by Hill's Pet Nutrition Inc., were included in the study. Cats had no evidence of chronic systemic disease as determined by physical examination, blood and serum analyses, urinalysis, and absence of fecal parasites. Exclusion criteria were compromised renal function based on serum symmetric dimethylarginine (SDMA) concentrations (>14 μg/dL), known GI abnormalities, or history of food allergies and antibiotics. All cats were individually housed with regular access to socialization without restrictions. All animal usage protocols were approved by Hill's Institutional Animal Care and Use Committee and Animal Welfare Committee (CP753a.0.0.0-A-F-D-ADH-MULTI-319-MULTI) in compliance with the NIH guide (29).

All cats were fed Hill's Science Diet® Feline Adult for 14 d in the prefeed period. Cats were then randomly assigned based on age and sex to 1 of 6 different groups of 5 cats each in a Williams Latin Square design (30), such that each group was fed 1 of the P28 (28.3% crude protein, dry matter basis), P35 (35.1%), or P55 (54.8%) foods for 90 d in a distinct sequence (**Supplemental Figure 1**). Each cat was fed based on caloric requirements, calculated based on BW, and had access to food for 23 h/d. No adverse events were reported, no cats were removed from the study, and all returned healthy to the colony at the end of the study.

Study foods

Supplemental Table 1 lists the formulations of the 3 isocaloric foods used in this study. All met the Association of American Feed Control Officials' (AAFCO's) maintenance nutrition requirements (31). The same protein and carbohydrate source ingredients were represented in all 3 foods and their inclusion levels varied depending upon their crude protein levels (**Supplemental Table 2**). Dietary protein levels were adjusted by changing carbohydrate [nitrogen-free extract (NFE)] levels without altering dietary fat levels in all 3 foods. All 3 foods were made in dry form at the experimental foods pilot plant at Hill's Pet Nutrition Inc. Complete nutrient profiles of all 3 foods were analyzed by a commercial laboratory (Eurofins Scientific, Inc.) followed by AOAC methods (32).

Sample collection and processing

Blood, urine, and fecal samples were collected at the end of the prefeed period and on days 45 and 90 of each treatment period. For each treatment, some samples were missing (P28: 6 cats missed 1 time point, n = 54; P35: 8 cats missed 1 time point, n = 52; and P55: 4 cats' samples missed; 2 missed both time points and 2 missed 1 time point, n = 54). Fecal samples were collected immediately after defecation. Stool quality was assessed on a 5-point scale (33). Feces were homogenized (ThinkyMixer, Thinky USA Inc.), their pH was measured, and they were frozen as aliquots within 45 min of collection. Samples were snap-frozen immediately in liquid nitrogen followed by storage at −80°C until further processing. Fecal ammonia was measured by the indophenol blue method (34). Fecal SCFAs were analyzed by Metabolon Inc. and fecal IgA by MD Biosciences Inc. Cats were sedated before phlebotomy, and blood was separated using serum separator tubes. Blood count profiles (Sysmex XN 1000-V, Sysmex America, Inc.) and serum chemistry (Cobas c501, Roche Diagnostics) were analyzed according to manufacturers' instructions. Urine samples were collected via cystocentesis, and urine specific gravity was measured with a temperature-compensated handheld refractometer (Reichert Technologies). Urine pH and other urinalysis measures were assessed using standard clinical protocols.

Digestibility analyses

Digestibility tests were run according to the AAFCO digestibility protocols (31). True protein digestibility was calculated as follows, with integumental protein calculated as in Kendall et al. (35): true protein digestibility = [protein intake − (fecal protein − integumental protein)]/(protein intake). Digestibility = (nutrient intake − fecal excretion)/(nutrient intake). Bypass protein was calculated as: bypass protein (grams/BW $kg^{0.75}$) = protein intake grams × (100 − % true protein digestibility) × 0.01/BW $kg^{0.75}$.

Fecal microbiota analysis and bioinformatics processing

The 16S fecal microbiota analysis was performed as previously described (21, 36). For every Miseq run, 1 mock community sample (positive control) and water (negative control) were included to monitor the sequence run parameters and bioinformatics processing. Sequence runs with a quality score (Q30) >80% were processed for further analysis. The sequences were de-multiplexed to obtain FASTQ files, which were processed into contigs from pairs of reads, and chimeras were removed using standard parameters (UCHIME) of Mothur, version 1.39.5 (37, 38). All retained sequences were aligned to the GreenGenes reference database (gg.13.5.99). Sequences were classified against the GreenGenes database using the naïve Bayesian classifier (39) within Mothur with a minimum confidence of 80% for each assignment. Operational taxonomic units were identified based on taxonomic hierarchy and further processed using the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) protocol (40) to correct for copy numbers of the 16S genes in their respective taxa, followed by predicting functional attributes using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database.

Statistical analysis

For the microbiota analyses, the abundance data at the phyla, family, and genera levels, and PiCRUSt-predicted functional data were first filtered based upon prevalence (>50%) in the data set and the percentage of reads retained per sample (>99.9%). Similarly, the PiCRUSt-predicted functional KEGG data represented at >50% prevalence in the data set and >90% reads per sample were further analyzed. After data set filtering, the count data were transformed into centered log-ratio (CLR) values to enable appropriate statistical analysis using the ALDEx2 R package (41). α Diversity was evaluated as the Shannon index and inverse Simpson index using unfiltered data at the genera level.

Principal component analysis (PCA) was performed as an exploratory analysis to examine the differences by food treatment for both microbiota composition and PICRUSt-predicted functional data

Supported by Hill's Pet Nutrition, Inc. (to DVB). The funder was not involved in the study design; collection, analysis, and interpretation of the data; the writing of this article; or the decision to submit it for publication.
Author disclosures: DVB and MIJ are current employees of and DEJ is a former employee of Hill's Pet Nutrition, Inc.
Supplemental Figures 1–4 and Supplemental Tables 1–9 are available from the "Supplementary data" link in the online posting of the article and from the same link in the online table of contents at https://academic.oup.com/jn/.
Address correspondence to DVB (e-mail: dayakar_badri@hillspet.com).
Abbreviations used: AAFCO, Association of American Feed Control Officials; BCFA, branched-chain fatty acid; BUN, blood urea nitrogen; BW, body weight; CLR, centered log-ratio; ES, effect size; FDR, false discovery rate; GI, gastrointestinal; KEGG, Kyoto Encyclopedia of Genes and Genomes; NFE, nitrogen-free extract; PCA, principal component analysis; PICRUSt, Phylogenetic Investigation of Communities by Reconstruction of Unobserved States; SDMA, symmetric dimethylarginine.

sets. For the microbiota abundance, 59 genera and PICRUSt-predicted functional KEGG data and 5554 KEGG orthologs were included after filtering for the analysis. CLR-transformed values were used for both data sets to perform PCA.

As a crossover design study, mixed-model full factorial analysis was performed for fecal, blood/serum, and urinary analytes independently by using food type, food feed order, and time as fixed effects with all accompanying interaction terms and animal identification as a random effect with false discovery rate (FDR)-corrected P values (**Supplemental Table 3**). This analysis revealed that only 1 of the 4 interaction terms in the full factorial, food type × food feed order, affected a meaningful number of analytes (defined as ≥5% within matrix in accordance with $\alpha = 0.05$). Further, food feed order as a singular main effect showed no impact on analytes from feces, blood/serum, or urine and time showed no impact on fecal analytes. Thus, the full factorial model for feces was reduced to food type as a main effect and food type × food feed order as an interaction, whereas the model for blood/serum and urine was reduced to food type and time as main effects and food type × food feed order as an interaction. The resultant reduced model P values were FDR-adjusted with the Benjamini–Hochberg correction. Tukey's post hoc test was used to assess pairwise differences across diet types for each analyte.

Significant KEGG functions affected by food treatment were identified by paired-sample Wilcoxon signed-rank test and the resultant P values were adjusted for FDR with the Benjamini–Hochberg correction. Two-way hierarchical clustering analyses of bacterial genera and KEGG functions by food type were carried out using the Ward method. The relations among genera, KEGG functions, blood and serum chemistry, urinalysis data, and macronutrient intakes adjusted by metabolic BW were assessed by linear regression analysis and only those with R^2 values > 0.20 and P values < 0.001 were reported. All statistical analysis was performed using JMP Pro software version 15.0 (SAS Institute).

Results

Proximate analysis of foods and digestibility analyses

Three isocaloric foods with mean ± SD of 3940 ± 29 kcal/kg were made by varying protein levels to 28.3% (P28), 35.1% (P35), and 54.8% (P55) by replacing carbohydrate (NFE) levels on a dry matter basis (**Supplemental Table 2**). The ratio of animal (dried chicken) to plant protein (pea protein) was 1:1 in each of the 3 foods. With the exception of methionine (added as a supplement), all amino acid levels were proportional to total protein levels (**Supplemental Table 2**). True protein digestibility was significantly higher in P55 than in P28, with no difference between P35 and P55 (**Supplemental Table 4**). Similarly, calculated bypass protein was significantly different in all pairwise comparisons (P28 > P55 > P35).

No significant differences ($P = 0.86$) in food intake were observed in cats fed the test foods, with mean ± SD intakes of 63.2 ± 9.24, 62.4 ± 7.75, and 62.8 ± 7.74 kcal/kg $BW^{0.75}$, respectively. However, mean ± SD intakes of carbohydrates, protein, and total dietary fiber (all $P < 0.001$) but not fat ($P = 0.18$) were significantly different, with protein intakes when cats consumed P28, P35, and P55 of 17.9 ± 2.00, 21.7 ± 2.72, and 34.8 ± 4.32 kcal/kg $BW^{0.75}$, carbohydrate intakes of 32.4 ± 4.73, 27.0 ± 3.38, and 14.6 ± 1.80 kcal/kg $BW^{0.75}$, and total dietary fiber intakes of 1.07 ± 0.15, 0.94 ± 0.11, and 0.72 ± 0.08 g/BW $kg^{0.75}$, respectively. The mean ± SD BWs for the cats at the end of the treatment periods were 5.2 ± 1.1 (P28), 5.2 ± 1.1 (P35), and 5.4 ± 1.1 kg (P55). These differences only reached statistical significance for P55 compared with both P28 and P35, although Cohen's effect size (ES) (42) indicated that the clinical significance of these differences was either nonexistent or at the lowest limit of the range defined as small (P28 compared with P55, $P = 0.006$, ES = −0.2; P28 compared with P35, $P = 0.85$, ES = −0.03; P35 compared with P55, $P = 0.022$, ES = −0.18).

Clinical analysis of blood and urine

Blood and serum chemistry values after consumption of the test foods were all within normal reference ranges. However, within those ranges, blood urea nitrogen (BUN), BUN:creatinine ratio, mean corpuscular volume, phosphorus, globulins, and triglycerides were significantly higher when cats consumed P55 than when they consumed P28 or P35 (**Table 1**). Lipemic status was also increased with P55 compared with both P28 and P35. In contrast, blood calcium, creatinine, and SDMA concentrations were significantly higher when cats consumed P28 than when they consumed P55.

Similarly, urine values after consumption of the test foods were all within normal reference ranges established from the internal colony (Table 1). Within these ranges, however, specific gravity and creatinine concentrations significantly differed across the foods (Table 1); when cats consumed P55, they had significantly lower urinary creatinine and pH than with P28 or P35. Further, when fed P55, cats had significantly higher urine specific gravity than with P28.

Fecal analysis

Fecal scores were within the subjective acceptable score range (>3), although within this range the scores were significantly different. Inclusion of more protein resulted in a dose-dependent increase in stool firmness, with P35 and P55 significantly firmer than with P28 (Table 1). Higher protein in the food dose-dependently led to higher fecal ammonia and pH, with all pairwise differences reaching significance. Fecal IgA concentrations were significantly higher when cats consumed P28 than when they consumed P55.

Fecal SCFA analysis showed that all 3 measured putrefactive branched-chain fatty acids (BCFAs) were dose-dependently higher with increasing dietary protein, with all pairwise differences significant (**Table 2**). In contrast, the putrefactive SCFA valerate was higher with P28 and P35 than with P55. The saccharolytic SCFA butyrate was dose-dependently lower with increasing protein, whereas propionate, which may be derived from microbial catabolism of either dietary bypass protein or carbohydrates, was significantly higher when cats consumed P55 than when they consumed P28 and P35. Ratios of acetate to propionate were significantly greater when cats consumed P28 than when they consumed P35 and P55.

Fecal microbiota

For fecal microbiota abundance data, 7 phyla, 32 families, and 59 genera were considered for statistical analyses after meeting filtration criteria. Of these, 7 phyla (100%), 18 families (56.3%), and 31 (52.5%) genera were significantly affected by food (**Table 3**). The phyla Actinobacteria and Bacteroidetes were significantly greater when cats consumed P28 than when they consumed P35 and P55. In contrast, Firmicutes, Fusobacteria, and TM7 were significantly higher in feces when cats consumed P55 than when they consumed P28 and P35. However, no significant differences were observed in these phyla when cats consumed P28 or P35. Eleven families were significantly higher in the feces when cats consumed P55 than when they consumed P28 and/or P35, including Bacteroidaceae, Clostridiaceae, Streptococcaceae, Fusobacteriaceae, and Enterococaceae. Other families, such as Lachnospiraceae, Ruminococcaceae, Erysipelotrichaceae, and

TABLE 1 Serum/blood, urine, and fecal analyses of cats fed P28, P35, and P55 foods each for 90 d in this study[1]

	Test food			Overall mixed model FDR-adjusted *P* value			
Analytes	P28	P35	P55	Treatment	Time	Treatment × food fed order	Reference range[2]
Serum							
Albumin, g/dL	3.49 ± 0.043[a]	3.47 ± 0.053[a]	3.48 ± 0.052[a]	0.749	<0.001	<0.001	2.8–3.8
Albumin/globulin	1.13 ± 0.030[a]	1.08 ± 0.034[b]	1.03 ± 0.033[c]	<0.001	<0.001	0.417	0.6–1.4
BUN, mg/dL	18.9 ± 0.531[c]	20.1 ± 0.420[b]	25.1 ± 0.449[a]	<0.001	0.486	0.486	14.8–29.4
BUN:creatinine	13.6 ± 0.588[c]	14.9 ± 0.510[b]	19.4 ± 0.504[a]	<0.001	0.775	0.005	11.1–25.0
Calcium, mg/dL	9.98 ± 0.160[a]	9.67 ± 0.108[b]	9.61 ± 0.071[b]	<0.001	0.693	<0.001	8.9–11.4
Creatinine, mg/dL	1.43 ± 0.045[a]	1.38 ± 0.041[b]	1.31 ± 0.038[c]	<0.001	0.101	<0.001	0.8–1.7
Globulin, g/dL	3.13 ± 0.068[c]	3.26 ± 0.073[b]	3.40 ± 0.081[a]	<0.001	0.091	<0.001	2.6–4.5
Glucose, mg/dL	96.2 ± 2.360[a,b]	93.9 ± 2.136[b]	97.8 ± 2.660[a]	0.052	<0.001	0.213	74.0–159.0
Hemolysis, mg/dL	11.5 ± 0.759[a]	15.1 ± 1.699[a]	14.5 ± 1.150[a]	0.128	<0.001	0.489	—
Lipemic, mg/dL	2.73 ± 0.235[b]	3.41 ± 0.296[b]	4.60 ± 0.319[a]	<0.001	0.351	0.004	—
Phosphorus, mg/dL	3.87 ± 0.107[b]	4.01 ± 0.112[b]	4.34 ± 0.087[a]	<0.001	0.473	0.009	3.0–5.6
SDMA, μg/dL	12.3 ± 0.423[a]	11.4 ± 0.377[b]	10.0 ± 0.380[c]	<0.001	0.608	0.599	<14.0
Total protein, g/dL	6.63 ± 0.067[b]	6.74 ± 0.070[b]	6.89 ± 0.077[a]	<0.001	0.325	<0.001	5.8–7.7
Triglycerides, mg/dL	34.5 ± 1.717[b]	43.1 ± 3.380[b]	73.07 ± 9.10[a]	<0.001	0.421	0.001	16.0–223.0
Blood							
Hematocrit, %	31.2 ± 0.606[b]	32.2 ± 0.631[a,b]	32.7 ± 0.700[a]	0.001	0.955	0.122	26.3–45.8
Hemoglobin, g/dL	10.3 ± 0.213[b]	10.6 ± 0.227[a,b]	10.6 ± 0.244[a]	0.075	0.873	0.185	8.4–14.7
IRF, %	5.47 ± 0.817[b]	6.76 ± 0.813[b]	8.91 ± 0.983[a]	<0.001	0.780	0.145	1.1–16.0
MCV, fL	41.3 ± 0.633[b]	41.8 ± 0.596[b]	43.0 ± 0.641[a]	<0.001	0.783	0.060	37.0–52.6
RDW, fL	25.6 ± 0.265[b]	25.6 ± 0.235[b]	26.4 ± 0.321[a]	<0.001	0.954	0.213	23.8–31.8
Urine							
Creatinine, mg/dL	460 ± 16.7[a]	457 ± 16.1[a]	385 ± 14.3[b]	<0.001	0.715	0.001	—
pH	6.45 ± 0.102[a]	6.45 ± 0.075[a]	6.25 ± 0.051[b]	0.018	0.955	0.018	—
Specific gravity	1.04 ± 0.001[b]	1.05 ± 0.001[a,b]	1.05 ± 0.001[a]	0.015	0.818	0.416	—
Feces							
Ammonia, mmol/g	0.020 ± 0.001[c]	0.032 ± 0.001[b]	0.069 ± 0.001[a]	<0.001	—	0.028	—
IgA, mg/g	5.75 ± 0.508[a]	4.90 ± 0.446[a,b]	3.12 ± 0.900[b]	0.010	—	0.968	—
pH	5.31 ± 0.083[c]	5.56 ± 0.060[b]	6.20 ± 0.062[a]	<0.001	—	0.631	—
Fecal score[3]	4.40 ± 0.136[b]	4.66 ± 0.077[a]	4.86 ± 0.05[a]	<0.001	—	0.012	—

[1]Values are means ± SEs for analytes that showed significance ($P < 0.05$) by mixed-model analyses. Mean values were calculated by initially averaging those from both time points (45 and 90 d) for each cat (P28: $n = 54$; P35: $n = 52$; P55: $n = 54$), then averaging by a given treatment. Values in the same row with different letters are significantly different ($P < 0.05$). BUN, blood urea nitrogen; FDR, false discovery rate; IRF, immature reticulocyte fraction; MCV, mean corpuscular volume; RDW, red blood cell distribution width; SDMA, symmetric dimethylarginine.

[2]Reference ranges established from investigators' internal colony.

[3]Fecal score measured on a 1–5 scale with 1 considered as watery and 5 as firm stool (33).

Porphyromonadaceae, were significantly higher with P55 and significantly differed among all foods. Conversely, families such as Veillonellaceae, Bifidobacteriaceae, Prevotellaceae, and Lactobacillaceae were significantly greater when cats consumed P28 or P35 rather than P55.

PCA of the 59 genera showed clear separation of the 95% CIs between the fecal microbiota when cats consumed P28 as opposed to P55 (**Figure 1**A). However, no clear separation was observed between P28 and P35 or P35 and P55. Similarly, hierarchical clustering analysis using the Ward method showed clustering when cats consumed P55 that distinguished this group from P28 and P35; however, when fed the P28 and P35 foods, the samples were indistinguishable as groups (**Supplemental Figure 2**). Bacterial genera such as *Clostridium*, *Fusobacterium*, *Ruminococcus*, *Coprococcus*, *Eubacterium*, *Peptococcus*, *Dorea*, *Blautia*, *Faecalibacterium*, *Adlercreutzia*, and *Slackia* were significantly greater when cats consumed P55 than when they consumed P28 and P35 (Table 3). Several of these (*Coprococcus*, *Peptococcus*, *Blautia*, *Faecalibacterium*, and *Slackia*) also significantly differed between P28 and P35. In contrast, bacterial genera such as *Bifidobacterium*, *Megasphaera*, *Prevotella*, *Lactobacillus*, *Megamonas*, and *Sutterella* were significantly higher when cats consumed P28 than when they consumed P35 and/or P55.

All calculated bacterial α-diversity indexes at the genera level, including the Shannon diversity index, inverse Simpson index, and bacterial richness and evenness, were significantly higher when cats consumed P55 than when they consumed P28 and P35 (Table 3).

Functional capability of the fecal microbiota

PCA of 5554 KEGG functions showed clear separation of the 95% CIs when cats consumed P28 as opposed to P55 (Figure 1B). However, no clear separation was seen when cats consumed P35 as opposed to P28 or P55.

Many KEGG functions associated with amino acid metabolism, mucin foraging pathways, and urea metabolism were significantly higher when cats consumed P55 than when they consumed P28 (**Tables 4** and **5**, **Supplemental Table 5**). In contrast, cats fed P28 had significantly greater carbohydrate-active enzymes and many enzymes involved in SCFA pathways compared with those fed P55 (Table 5, **Supplemental Table 6**).

TABLE 2 Fecal SCFA analysis of cats fed P28, P35, and P55 foods each for 90 d[1]

	Test food			Overall mixed model FDR-adjusted *P* value	
Fatty acids, μg/g	P28	P35	P55	Treatment	Treatment × food fed order
BCFAs					
2-Methylbutyrate	66.1 ± 6.76[c]	99.9 ± 6.89[b]	227 ± 10.3[a]	<0.001	0.166
Isobutyrate	99.9 ± 9.61[c]	139 ± 7.88[b]	297 ± 11.8[a]	<0.001	0.361
Isovalerate	122 ± 10.8[c]	172 ± 10.2[b]	324 ± 13.9[a]	<0.001	0.333
SCFAs					
Acetate	4300 ± 237[a]	3980 ± 155[a]	3960 ± 205[a]	0.264	<0.001
Propionate	909 ± 83.9[c]	1438 ± 125[b]	1900 ± 102[a]	<0.001	0.003
Butyrate	4960 ± 364[a]	3390 ± 336[b]	1530 ± 68.1[c]	<0.001	0.044
Valerate	973 ± 67.5[a]	1034 ± 73.6[a]	635 ± 57.1[b]	<0.001	0.108
Hexanoate	490 ± 57.0[a]	305.7 ± 46.3[b]	52.7 ± 7.18[c]	<0.001	0.073
C2/C3	5.66 ± 0.419[a]	3.47 ± 0.309[b]	2.13 ± 0.063[c]	<0.001	0.040
C2/C4	3.13 ± 1.85[a]	3.32 ± 1.33[a]	2.67 ± 0.157[a]	0.783	0.009

[1] Values are means ± SEs for analytes that showed significance ($P < 0.05$) by mixed-model analyses. Mean values were calculated by initially averaging those from both time points (45 and 90 d) for each cat (P28: $n = 54$; P35: $n = 52$; P55: $n = 54$), then averaging by a given treatment. Values in the same row with different letters are significantly different ($P < 0.05$). BCFA, branched-chain fatty acid; FDR, false discovery rate.

Carbohydrate replacement with protein led to increased metabolism of amino acids by gut microbes

Feces from cats fed P55 showed significantly greater KEGG functions involved in glutamate family biosynthesis than with P28 and P35 (**Supplemental Table 5**, **Supplemental Figure 3**). Both glutamate dehydrogenase (K00261) and glutamine synthetase (K01915) were significantly higher when cats consumed P55 than when they consumed P28 and/or P35.

Feces from when cats consumed P55 had significantly higher KEGG functions for asparagine synthase (glutamine-hydrolyzing) (K01953), aspartate semialdehyde dehydrogenase (K00133), homoserine kinase (K02204), threonine synthase (K01733), and diaminopimelate decarboxylase (K01586) than when they consumed P28 and P35 (**Supplemental Table 5**, **Supplemental Figure 3**). Similarly, KEGG functions involved in serine family biosynthesis such as phosphoglycerate dehydrogenase (K00058), phosphoserine aminotransferase (K00831), serine O-acetyltransferase (K00640), and cysteine synthase A (K01738) were significantly higher when cats consumed P55 than with P28 and P35.

Feces from when cats consumed P55 showed significantly greater KEGG functions involved in pyruvate metabolism, specifically acetolactate synthase_I/II/III subunits (K01652, K01653), dihydroxy-acid dehydratase (K01687), branched-chain amino acid aminotransferase (K00826), and 2-isopropyl malate synthase (K01649), than when they consumed P28 and P35 (**Supplemental Table 5**, **Supplemental Figure 3**).

Aromatic amino acid biosynthesis typically follows the shikimate pathway starting from phosphoenolpyruvate and the pentose phosphate pathway intermediate erythrose 4-phosphate (43). Feces from cats that consumed P55 exhibited significantly higher KEGG functions involved in biosynthesis of L-phenylalanine, L-tryptophan, and L-tyrosine than when they consumed P28 and P35 (**Supplemental Table 5**, **Supplemental Figure 3**).

Feces from when cats consumed P55 had significantly higher KEGG functions involved in catabolism of amino acids than when they consumed P28 and P35 (Table 4). Arginine catabolism can produce agmatine by decarboxylation using arginine decarboxylase (K01583) or polyamines such as putrescine, spermidine, and spermine as part of the polyamine synthesis pathway via agmatinase (K01480), agmatine deiminase (K10536), and spermidine synthase (K00797), all of which were significantly greater when cats consumed P55 as opposed to P28 and P35. Similarly, tyrosine and tryptophan can produce indole, phenol, and pyruvate via tyrosine phenol-lyase (K01668) and tryptophanase (K01667), which were significantly higher when cats consumed P55 than when they consumed P28 and P35. Feces from when cats consumed P55 exhibited significantly greater glycine reductase (K10670), involved in the Stickland reaction, than when cats consumed P28 or P35.

Urea metabolism

KEGG functions involved in the urea cycle were significantly greater when cats consumed P55 than when they consumed P28 and P35 (**Supplemental Table 5**). Specifically, all subunits of urease (K01428, K01429, and K01430), which catalyzes urea into ammonia and carbamate, were significantly higher when cats consumed P55. Other enzymes involved in the rate-limiting steps of the urea cycle, such as carbamoyl-phosphate synthase (K01955 and K01956) and argininosuccinate synthase (K01940), were significantly greater when cats consumed P55 than when they consumed P28.

Carbohydrate-active enzymes and mucin-foraging enzymes

Feces from when cats consumed P28 had significantly higher concentrations of carbohydrate-active enzymes such as glucan 1,3-β-glucosidase (K01210), xylan 1,4-β-xylosidase (K01198), pullulanase (K01200), chitinase (K01183), α-amylase (K01176), endo-1,3(4)-β-glucanase (K01180), and endoglucanase (K01179) than from when they consumed P55 (Table 5, **Supplemental Figure 4**).

Conversely, feces from when cats consumed P55 had significantly higher carbohydrate-active enzymes of the mucin-foraging type (KEGG functions) than from when they consumed P28 and/or P35 (Table 5, **Supplemental Figure 4**). These included α-mannosidase (K01191), α-L-fucosidase (K01206), β-glucuronidase (K01195), β-fructofuranosidase (K01193), and N-acetylneuraminate lyase (K01639).

TABLE 3 Fecal microbiota analysis and diversity measures of cats fed P28, P35, and P55 foods each for 90 d[1]

	Test food			Overall mixed model FDR-adjusted *P* value	
Microbiota	P28	P35	P55	Treatment	Treatment × food fed order
Phyla					
Actinobacteria	6.97 ± 0.167[a]	6.78 ± 0.210[a]	4.75 ± 0.228[b]	<0.001	0.009
Bacteria_unclassified	−0.464 ± 0.234[a]	−0.617 ± 0.261[a]	−1.86 ± 0.160[b]	<0.001	0.007
Bacteroidetes	3.38 ± 0.151[a]	3.39 ± 0.226[a]	2.76 ± 0.172[b]	<0.001	0.417
Firmicutes	7.14 ± 0.144[b]	7.07 ± 0.155[b]	7.78 ± 0.161[a]	<0.001	0.001
Fusobacteria	−7.37 ± 0.355[b]	−7.16 ± 0.414[b]	−5.01 ± 0.428[a]	<0.001	0.017
Proteobacteria	−1.15 ± 0.233[a]	−1.46 ± 0.211[a]	−1.35 ± 0.200[a]	0.435	0.005
TM7	−8.13 ± 0.230[b]	−7.67 ± 0.290[b]	−6.94 ± 0.196[a]	<0.001	0.826
Bacteroidetes/Firmicutes	0.092 ± 0.011[a,b]	0.139 ± 0.034[a]	0.054 ± 0.012[b]	0.006	<0.001
Family					
Alcaligenaceae	−0.161 ± 0.312[a]	−0.932 ± 0.410[a]	−2.63 ± 0.389[b]	<0.001	0.245
Bacteroidaceae	2.25 ± 0.331[b]	2.48 ± 0.289[b]	3.46 ± 0.195[a]	<0.001	0.108
Bifidobacteriaceae	9.90 ± 0.166[a]	9.25 ± 0.236[b]	2.98 ± 0.309[c]	<0.001	0.005
Clostridiaceae	4.55 ± 0.203[c]	5.16 ± 0.226[b]	8.03 ± 0.158[a]	<0.001	0.027
Coriobacteriaceae	9.42 ± 0.183[a]	8.92 ± 0.260[b]	7.63 ± 0.188[c]	<0.001	0.059
Corynebacteriaceae	−4.31 ± 0.251[a]	−5.14 ± 0.249[b]	−4.75 ± 0.269[a,b]	0.026	0.276
Enterococcaceae	−4.51 ± 0.314[b]	−3.49 ± 0.471[a,b]	−2.75 ± 0.674[a]	0.029	0.321
Erysipelotrichaceae	−4.05 ± 0.272[c]	−2.28 ± 0.248[b]	−0.494 ± 0.222[a]	<0.001	0.276
Fusobacteriaceae	−3.62 ± 0.399[b]	−3.80 ± 0.412[b]	−2.01 ± 0.504[a]	<0.001	0.003
Lachnospiraceae	6.88 ± 0.151[c]	7.71 ± 0.212[b]	9.96 ± 0.148[a]	<0.001	<0.001
Lactobacillaceae	2.18 ± 0.945[a]	1.76 ± 0.845[a]	0.158 ± 0.813[b]	0.010	0.481
Mogibacteriaceae	−0.284 ± 0.586[b]	0.059 ± 0.625[b]	2.49 ± 0.401[a]	<0.001	0.264
Peptococcaceae	1.75 ± 0.604[c]	2.94 ± 0.524[b]	4.56 ± 0.546[a]	<0.001	0.060
Porphyromonadaceae	−4.11 ± 0.405[c]	−2.83 ± 0.563[b]	−0.344 ± 0.475[a]	<0.001	0.118
Prevotellaceae	6.96 ± 0.210[a]	6.60 ± 0.305[a]	5.17 ± 0.233[b]	<0.001	0.400
Ruminococcaceae	1.13 ± 0.239[c]	2.38 ± 0.269[b]	5.15 ± 0.182[a]	<0.001	0.090
Streptococcaceae	−2.65 ± 0.378[b]	−2.50 ± 0.486[b]	−1.51 ± 0.579[a]	0.007	0.179
Veillonellaceae	10.2 ± 0.291[a]	9.62 ± 0.306[b]	7.07 ± 0.206[c]	<0.001	0.215
Genera					
Acidaminococcus	3.44 ± 0.901[a]	2.99 ± 0.846[a]	−0.918 ± 0.518[b]	<0.001	<0.001
Actinomyces	−4.32 ± 0.252[a]	−4.58 ± 0.264[a]	−4.86 ± 0.287[a]	0.179	0.725
Adlercreutzia	−0.627 ± 0.314[b]	−0.404 ± 0.330[b]	0.263 ± 0.297[a]	<0.001	0.029
Bacteroides	2.49 ± 0.329[b]	2.50 ± 0.273[b]	3.22 ± 0.217[a]	0.039	0.180
Bifidobacterium	10.1 ± 0.156[a]	9.17 ± 0.246[b]	1.62 ± 0.375[c]	<0.001	0.008
Blautia	5.87 ± 0.208[c]	6.73 ± 0.234[b]	9.07 ± 0.143[a]	<0.001	0.002
Bulleidia	−5.12 ± 0.175[c]	−4.22 ± 0.297[b]	−3.65 ± 0.392[a]	<0.001	0.027
Catenibacterium	−4.77 ± 0.231[b]	−4.66 ± 0.274[b]	−3.97 ± 0.316[a]	0.012	0.304
Clostridium	4.69 ± 0.203[b]	5.14 ± 0.223[b]	7.69 ± 0.153[a]	<0.001	0.044
Coprococcus	−1.43 ± 0.434[c]	0.395 ± 0.435[b]	4.04 ± 0.171[a]	<0.001	0.179
Corynebacterium	−4.10 ± 0.257[a]	−5.09 ± 0.258[b]	−4.98 ± 0.260[b]	0.001	0.169
Dialister	4.02 ± 0.352[a]	3.69 ± 0.460[a]	2.15 ± 0.428[b]	<0.001	0.010
Dorea	1.26 ± 0.391[b]	1.76 ± 0.291[b]	5.48 ± 0.158[a]	<0.001	<0.001
Enterococcus	−4.36 ± 0.317[a]	−3.67 ± 0.502[a]	−3.26 ± 0.724[a]	0.240	0.210
Eubacterium	−4.55 ± 0.195[c]	−3.71 ± 0.306[b]	−1.73 ± 0.248[a]	<0.001	0.095
Faecalibacterium	−3.75 ± 0.273[c]	−2.35 ± 0.458[b]	1.61 ± 0.623[a]	<0.001	0.058
Fusobacterium	−3.58 ± 0.407[a,b]	−4.10 ± 0.380[b]	−2.75 ± 0.520[a]	0.009	0.008
Lactobacillus	2.43 ± 0.954[a]	1.77 ± 0.869[a]	−0.088 ± 0.810[b]	0.001	0.511
Megamonas	3.95 ± 0.285[a]	4.31 ± 0.368[a]	2.82 ± 0.436[b]	<0.001	0.010
Megasphaera	9.98 ± 0.525[a]	8.84 ± 0.565[b]	6.03 ± 0.385[c]	<0.001	0.447
Oscillospira	−1.64 ± 0.385[c]	0.204 ± 0.309[b]	2.41 ± 0.252[a]	<0.001	0.010
Parabacteroides	−3.84 ± 0.393[c]	−2.84 ± 0.542[b]	−0.676 ± 0.490[a]	<0.001	0.055
Peptococcus	1.98 ± 0.598[c]	2.96 ± 0.526[b]	4.32 ± 0.545[a]	<0.001	0.113
Peptostreptococcus	−3.04 ± 0.340[a]	−3.65 ± 0.358[a]	−4.65 ± 0.318[b]	<0.001	0.450
Prevotella	7.20 ± 0.217[a]	6.61 ± 0.310[b]	4.92 ± 0.240[c]	<0.001	0.417
Roseburia	0.222 ± 0.331[a]	−0.235 ± 0.292[a]	−0.447 ± 0.241[a]	0.210	0.333
Ruminococcus	4.42 ± 0.225[b]	4.70 ± 0.257[b]	6.29 ± 0.248[a]	<0.001	0.058

(*Continued*)

TABLE 3 (*Continued*)

Microbiota	Test food			Overall mixed model FDR-adjusted *P* value	
	P28	P35	P55	Treatment	Treatment × food fed order
Slackia	0.421 ± 0.271[c]	1.11 ± 0.151[b]	2.12 ± 0.161[a]	<0.001	0.146
Streptococcus	−2.39 ± 0.388[a]	−2.45 ± 0.482[a]	−1.80 ± 0.590[a]	0.147	0.152
Sutterella	0.071 ± 0.319[a]	−0.928 ± 0.424[a]	−2.89 ± 0.408[b]	<0.001	0.276
Turicibacter	−3.07 ± 0.699[a]	−2.98 ± 0.600[a]	−4.25 ± 0.576[b]	0.032	0.196
Diversity measures					
Shannon index	1.74 ± 0.049[c]	2.01 ± 0.051[b]	2.30 ± 0.045[a]	<0.001	0.055
Inverse Simpson index	3.98 ± 0.215[c]	5.10 ± 0.326[b]	6.04 ± 0.299[a]	<0.001	0.106
Richness	54.6 ± 1.01[b]	57.5 ± 1.81[b]	66.6 ± 1.53[a]	<0.001	0.331
Evenness	0.438 ± 0.012[c]	0.503 ± 0.016[b]	0.548 ± 0.010[a]	<0.001	0.231

[1] Values are means ± SEs of center-log ratio transformations that showed significance ($P < 0.05$) by mixed-model analyses. Mean values were calculated by initially averaging those from both time points (45 and 90 d) for each cat (P28: $n = 54$; P35: $n = 52$; P55: $n = 54$), then averaging by a given treatment. Values in the same row with different letters are significantly different ($P < 0.05$). FDR, false discovery rate.

Microbial functions involved in synthesis of SCFAs and BCFAs

KEGG functions involved in the production of BCFAs such as branched-chain amino acid aminotransferase (K00826) and branched-chain amino acid transport permease protein (K01997 and K01998) were significantly greater in the microbiota when cats consumed P55 than when they consumed P28 and P35 (**Supplemental Table 6**). Similarly, synthesis of acetate through pyruvate was significantly higher in the fecal microbiota when cats consumed P55 as opposed to P28 and P35. In addition, propionate biosynthesis through the threonine and valine catabolic pathway KEGG functions was significantly higher in the fecal microbiota when cats consumed P55 than when they consumed P28 and P35. However, an alternate pathway for propionate production, the acrylate pathway, was greater in feces from when cats consumed P28 than from when they consumed P55. Similarly, cats that consumed P28 showed many significantly higher microbial KEGG functions involved in 4-aminobutanoate degradation to produce butyrate than those that consumed P55.

Associations of bacterial genera with microbial KEGG functions, fecal and blood analytes, and macronutrient intakes

Linear regression analysis performed with the 59 genera and KEGG functions related to pathways of amino acid catabolism and carbohydrate-active enzymes showed that the bacterial genera *Blautia*, *Clostridium*, *Coprococcus*, *Dorea*, *Eubacterium*, *Faecalibacterium*, *Oscillospira*, *Parabacteroides*, *Ruminococcus*, and *Slackia* had positive associations with many KEGG functions involved in amino acid catabolic pathways (**Supplemental Table 7**). Others such as *Megasphaera*, *Prevotella*, *Bifidobacterium*, *Sutterella*, and *Acidaminococcus* showed negative associations with many KEGG functions involved in amino acid catabolic pathways. Bacterial genera that exhibited positive associations with KEGG functions involved in amino acid catabolic pathways also showed positive associations with KEGG functions related to mucin degradation (**Supplemental Table 8**). Similarly, bacterial genera that showed negative associations with KEGG functions involved in amino acid catabolic pathways had positive associations with carbohydrate-active enzymes. The bacterial genera *Bifidobacterium*, *Prevotella*, and *Sutterella* had negative associations with fecal BCFAs and ammonia (**Supplemental Table 9**). Notably, *Bifidobacterium* also showed a negative association with fecal pH. Bacterial genera such as *Blautia*, *Clostridium*, *Coprococcus*, *Dorea*, *Eubacterium*, *Faecalibacterium*, *Oscillospira*, *Ruminococcus*, and *Slackia* showed positive associations with fecal BCFAs, pH, and/or ammonia but a negative association with the SCFA butyric acid.

Discussion

This study documented the effect of varying dietary protein:carbohydrate ratios on the composition of the gut microbiota and its functional capabilities in healthy adult cats, while controlling for fat, fiber, amino acid proportions, and total dietary energy. The AAFCO recommends that cat foods exceed 26% protein (dry matter basis) for adult maintenance (4000 kcal/kg) and 30% protein for growth and reproduction phases (31). In general, commercially available cat foods include 30%–45% protein levels for maintenance diets, with the protein levels sometimes reduced to 28% or increased to 50%–55% for specific conditions such as chronic kidney disease and weight loss/metabolic diseases, respectively (44). Hence, 3 different protein levels were chosen for this study (28%, 35%, and 55%), which reflect the levels in generally available cat foods.

In contrast to prior studies on cats (26–28, 45), foods formulated in the present study used similar ratios of animal and plant protein sources (dried chicken and pea protein). Along with the observation that protein intakes were higher when cats consumed P55 than when they consumed P28, the greater apparent protein digestibility in P55 than in P28 and P35 is expected because of higher protein intake, consistent with previous findings in cats, kittens, and dogs (46–48). Because of the greater protein digestibility of P55, the calculated amount of bypass protein present in the colon was lower than with P28 but higher than with P35, suggesting that the higher protein intake did not raise bypass protein in the colon. In addition, the observed lower apparent protein digestibility in the cats fed P28 is likely due to the significantly higher dietary fiber intakes when cats consumed P28 than when they consumed P55.

Although previous feline microbiota studies used different sequencing technologies (27, 28, 49, 50), the predominant phyla in previous studies, Actinobacteria, Bacteroidetes, Fusobacteria, Firmicutes, and Proteobacteria, were similarly found here. Consistent with prior work, Actinobacteria were lower and Fusobacteria higher with consumption of P55 food (27, 28).

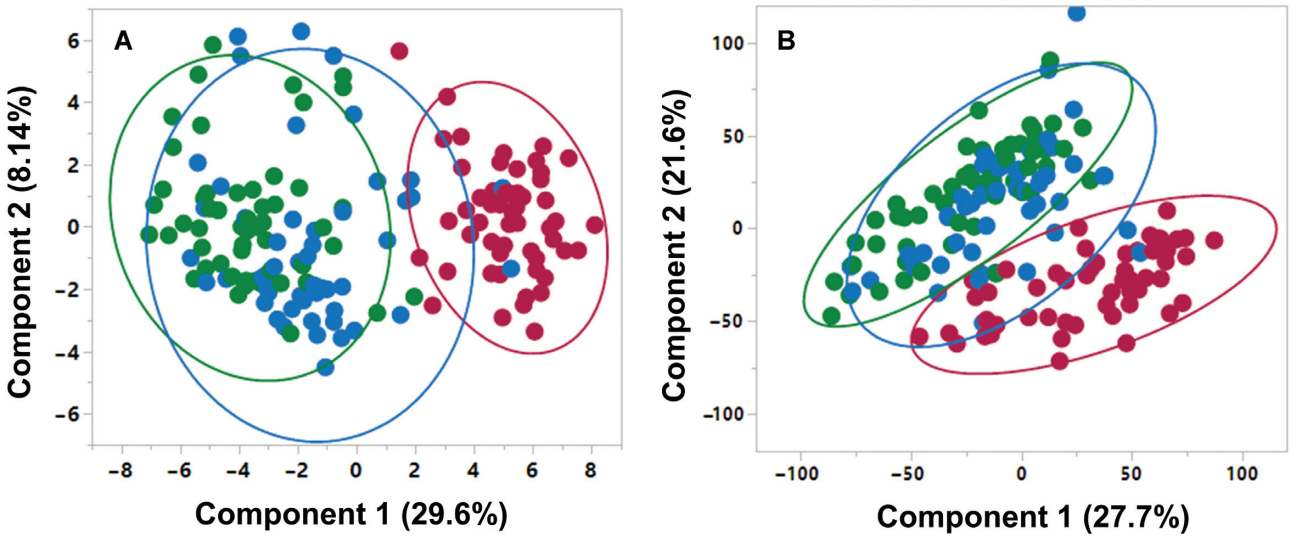

FIGURE 1 Principal component analysis plots of the fecal microbiota (A) and its predictive Kyoto Encyclopedia of Genes and Genomes functions derived by Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (B) in cats fed P28 (green), P35 (blue), and P55 (red) foods each for 90 d. Fecal microbiota and predictive functions were represented at genera level. (A) Positional values for the samples on PC1, which explained 29.6% of the variation, showed significant differences between P28 and P35 ($P < 0.0001$), P28 and P55 ($P < 0.0001$), and P35 and P55 ($P < 0.0001$). The positional values on PC2, which explained 8.14% of the variation, showed significant differences between P28 and P35 ($P = 0.016$) and P35 and P55 ($P = 0.002$) but not between P28 and P55 ($P = 0.83$). (B) PC1, which explained 27.7% of the variation, had significant differences between P28 and P35 ($P < 0.01$), P28 and P55 ($P < 0.0001$), and P35 and P55 ($P < 0.0001$). PC2, which explained 21.6% of the variation, showed significant differences between P28 and P55 ($P < 0.0001$) and P35 and P55 ($P < 0.0001$), but not between P28 and P35 ($P = 0.50$). Data are from 160 samples (P28: $n = 54$; P35: $n = 52$; P55: $n = 54$), which correspond to 2 samples/cat with some missing data. Ovals represent 95% CIs. PC, principal component.

Microbial α diversity, richness, and evenness at the genera level were significantly higher when cats consumed P55 as opposed to P28 and P35, in agreement with a previous report in growing kittens (27). P55 may have raised the availability of specific substrates such as amino acids, dipeptides, and their metabolites that may then increase the diversity and richness of the gut microbiota, whereas P28 provided energy to the microbiota in the form of starch, a less heterogeneous substrate than protein.

In terms of microbiota, there appeared to be a greater difference for P55 compared with P28 and P35 than between the 2 latter foods. That may be due to the skewed distribution of ratios of protein:carbohydrate in the foods (P35 is closer to P28 than to P55). Alternatively, there may be a minimum level of carbohydrates between those found in P35 and P55 capable of sustaining the observed saccharolytic activity of gut microbiota. Feeding P28 or P35 resulted in significantly greater bacterial genera *Bifidobacterium, Lactobacillus, Megasphaera, Prevotella*, and *Acidaminococcus* than feeding P55, consistent with high-protein food also resulting in higher concentrations of these bacteria in growing kittens (27, 28). Similarly, significantly more of the bacterial genera *Bacteroides*, *Blautia, Clostridium, Ruminococcus, Eubacterium*, and *Faecalibacterium* (51–55) were seen when cats consumed P55 than when they consumed P28 or P35 in the present study as well as in growing kittens (27, 28). These genera potentially forage mucins, high-molecular-weight glycoproteins that provide protection to the intestinal epithelium as a mucus barrier (54) and can be used as a microbial substrate (56). In addition to greater potential mucin-foraging bacteria, the gut microbiota when cats consumed P55 showed significantly higher microbial mucin-foraging enzymes than in P28 and P35. The apparent greater ability to degrade mucin may result in more threonine in the colonic environment, which can then be converted into propionate (57) as well as butyrate (58); accordingly, propionate (but not butyrate) was increased in the P55-fed cats. This, along with observed higher threonine catabolic functions, may explain the specificity of greater fecal propionate than butyrate concentrations found with consumption of P55 food. Further, bacterial genera such as *Bacteroides*, *Blautia, Ruminococcus, Eubacterium*, and *Faecalibacterium* that were higher with P55 than with P28 and P35 are generally known for potential saccharolytic activity (21, 41); however, many of these genera are capable of catabolizing both carbohydrate and protein substrates and will utilize those that present least competition with other microbes in a given environment. The "restaurant hypothesis" posits that the GI tract is not a homogeneous culture but rather different microbial communities thrive in this environment through changes in consumption, similar to a restaurant serving different foods (59, 60). Thus, consumption of P55 food, which limited the hindgut availability of carbohydrate and fiber, may have created an environment where microbial foraging on mucin and amino acid metabolism resulted in more proteolytic activity and less saccharolytic activity than with P28 consumption. In line with the restaurant hypothesis, a given microbe could exhibit metabolic activity that best utilizes its available environmental resources. This can affect host health; e.g., normally benign commensal organisms can become pathobionts under specific host conditions or in specific gut locations (61). Overall, these findings further support the idea that microbes in the GI tract are metabolically flexible to exploit availability of substrates, host conditions, and location in the GI environment.

The higher fecal pH, ammonia, and microbial functions associated with amino acid metabolism in feces when cats consumed more dietary protein may be facilitating mucin degradation consequent to the limited availability of carbohydrate and dietary fiber. Further, significantly higher microbial urea cycle KEGG functions when cats consumed P55 can help to explain

TABLE 4 Analysis of fecal microbial amino acid catabolism enzymes from cats fed P28, P35, and P55 foods each for 90 d[1]

KEGG function	Substrates	Products	Test food		
			P28	P35	P55
Amino acid catabolism					
K00491_nitric-oxide synthase, bacterial [EC:1.14.13.39]	L-tyrosine, 2-N-hydroxyarginine	Nitric oxide, L-citrulline	−5.86 ± 0.04^{b}	−5.70 ± 0.06^{b}	−5.39 ± 0.06^{a}
K00505_tyrosinase [EC:1.14.18.1]	L-Tyrosine	3,4-Ihydroxy-L-phenylalanine	−4.62 ± 0.11^{a}	−4.62 ± 0.16^{a}	−5.25 ± 0.06^{b}
K01580_glutamate decarboxylase [EC:4.1.1.15]	L-Glutamate	γ-Aminobutanoate, CO_2	1.16 ± 0.09^{b}	1.35 ± 0.10^{a,b}	1.60 ± 0.05^{a}
K01583_arginine decarboxylase [EC:4.1.1.19]	L-Arginine	Agmatine, CO_2	3.00 ± 0.12^{c}	3.97 ± 0.11^{b}	5.27 ± 0.08^{a}
K01593_aromatic-L-amino-acid decarboxylase [EC:4.1.1.28]	L-Tryptophan, L-histidine, L-tyrosine, L-phenylalanine	Serotonin, histamine, tyramine, tryptamine, phenylethylamine	−6.09 ± 0.07^{b}	−6.03 ± 0.06^{b}	−5.52 ± 0.09^{a}
K01667_tryptophanase [EC:4.1.99.1]	L-Tryptophan	Indole, pyruvate, ammonia	−0.330 ± 0.18^{b}	−0.280 ± 0.11^{b}	1.41 ± 0.10^{a}
K01668_tyrosine phenol-lyase [EC:4.1.99.2]	L-Tyrosine	Phenol, pyruvate, ammonia	−5.26 ± 0.20^{b}	−5.34 ± 0.19^{b}	−3.88 ± 0.25^{a}
K01761_methionine-γ-lyase [EC:4.4.1.11]	L-Methionine	Methanethiol, ammonia, 2- oxobutanoate	4.58 ± 0.12^{a}	4.29 ± 0.12^{a}	3.05 ± 0.07^{b}
Polyamine biosynthesis					
K00797_spermidine synthase [EC:2.5.1.16]	S-adenosylmethionamine	5′-Methylthioadenosine, spermidine	5.71 ± 0.05^{b}	5.82 ± 0.05^{b}	6.26 ± 0.05^{a}
K01480_agmatinase [EC:3.5.3.11]	Agmatine	Putrescine	3.94 ± 0.06^{c}	4.39 ± 0.07^{b}	5.59 ± 0.05^{a}
K01581_ornithine decarboxylase [EC:4.1.1.17]	L-Ornithine	Putrescine, CO_2	4.80 ± 0.08^{a}	4.53 ± 0.10^{a}	2.84 ± 0.11^{b}
K01582_lysine decarboxylase [EC:4.1.1.18]	L-Lysine	Cadaverine, CO	2.18 ± 0.10^{c}	2.76 ± 0.09^{b}	3.95 ± 0.06^{a}
K10536_agmatine deiminase [EC:3.5.3.12]	Agmatine	N-carbamoylputrescine, ammonia	3.43 ± 0.09^{c}	4.06 ± 0.09^{b}	5.34 ± 0.07^{a}
K12251_N-carbamoylputrescine amidase [EC:3.5.1.53]	N-carbamoyl putrescine	Putrescine, CO_2, ammonia	3.77 ± 0.06^{c}	4.30 ± 0.07^{b}	5.40 ± 0.06^{a}
K13747_carboxynorspermidine decarboxylase [EC:4.1.1.-]	Carboxyspermidine	Spermidine, CO_2	4.11 ± 0.07^{c}	4.74 ± 0.07^{b}	6.00 ± 0.05^{a}
Stickland reaction					
K00259_alanine dehydrogenase [EC:1.4.1.1]	Alanine-glycine	Ammonia	2.30 ± 0.12^{a}	2.34 ± 0.10^{a}	2.23 ± 0.06^{a}
K00285_D-amino-acid dehydrogenase [EC:1.4.99.1]	Alanine-glycine	Ammonia	−0.240 ± 0.10^{a}	−0.410 ± 0.14^{a}	−0.630 ± 0.12^{a}
K01478_arginine deiminase [EC:3.5.3.6]	Arginine	Ammonia	4.94 ± 0.06^{a}	4.86 ± 0.07^{a}	4.00 ± 0.08^{b}
K10670_glycine reductase [EC:1.21.4.2]	Alanine-glycine	Ammonia	3.21 ± 0.10^{b}	3.36 ± 0.10^{b}	4.91 ± 0.07^{a}

[1]Values are means ± SEs of center-log ratio transformations that showed significance ($P < 0.05$) by nonparametric analysis followed by the Wilcoxon significance test. Mean values were calculated by initially averaging those from both time points (45 and 90 d) for each cat (P28: $n = 54$; P35: $n = 52$; P55: $n = 54$), then averaging by a given treatment. Values in the same row with different letters are significantly different ($P < 0.05$). KEGG, Kyoto Encyclopedia of Genes and Genomes.

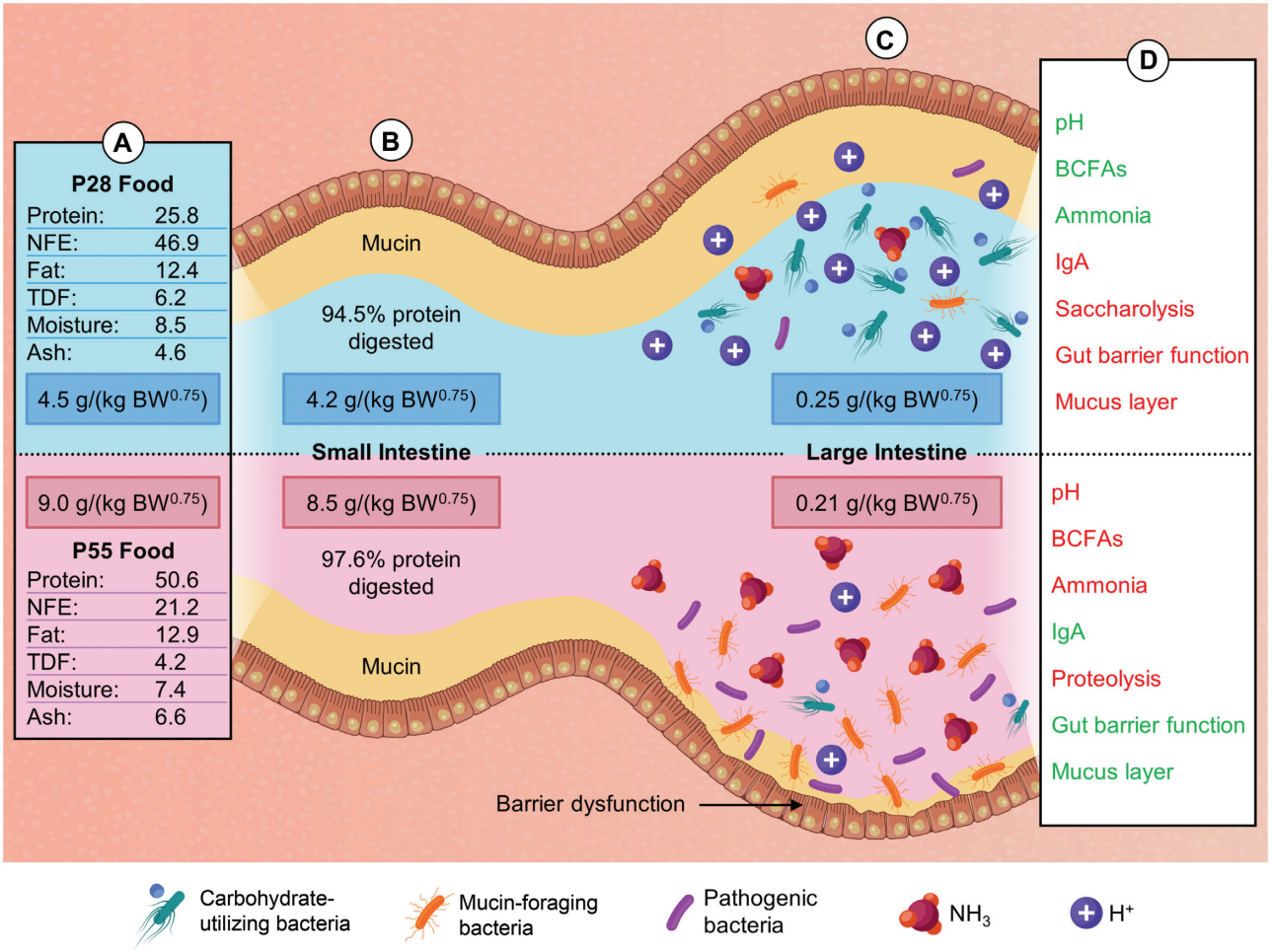

FIGURE 2 Dietary protein and carbohydrate levels affect host gut microbiota and physiology. (A) The P28 and P55 foods fed to healthy adult cats differed in their protein and carbohydrate (NFE) levels. The P28 food provided 4.5 g/(kg $BW^{0.75}$) and P55 food provided 9.0 g/(kg $BW^{0.75}$) protein on an as-fed basis. (B) Based on true protein digestibility, 4.2 g/(kg $BW^{0.75}$) and 8.5 g/(kg $BW^{0.75}$) of protein was assimilated in the small intestine from P28 and P55 foods, respectively. (C) An estimated 0.25 g/(kg $BW^{0.75}$) and 0.21 g/(kg $BW^{0.75}$) of protein bypassed into the large intestine by P28 and P55 foods, respectively, and was available for microbes. (D) For P28 food, the availability of carbohydrate sources led to microbial saccharolytic fermentation; lower BCFAs, pH, and ammonia; increased IgA; and no impact on mucin degradation or gut barrier function. On the contrary, P55 food led to less availability of carbohydrate substrates in the large intestine, leading microbes to forage on mucin, which led to proteolytic fermentation; higher BCFAs, pH, and ammonia; and lower IgA and beneficial commensals. BCFA, branched-chain fatty acid; BW, body weight; NFE, nitrogen-free extract; TDF, total dietary fiber.

the greater fecal pH and ammonia concentrations as a result of urea cycle flux along with amino acid catabolism products such as polyamines. Prior work also showed higher fecal pH due to proteolytic fermentation in dogs fed high-protein food (21, 62, 63).

In contrast, P28 consumption appeared to yield greater saccharolytic activity, evident in significantly lower fecal pH and ammonia due to the availability of carbohydrates and dietary fiber to the colonic microbes. Obviously, all carbohydrates are not completely digestible, but some enter into the colonic environment as bypass and provide substrates to colonic microbes. In this study, the carbohydrates that entered the colon as bypass were probably in the form of resistant starch derived from the carbohydrate sources in the food formulations. Addition of fiber has previously been shown to increase gut microbiota saccharolytic activity in dogs (36). Further, the families Bifidobacteriaceae, Lactobacillaceae, and Prevotellaceae were higher with P28 consumption, whereas Clostridiaceae, Ruminococcaceae, and Bacteroidaceae were higher with P55. These results are comparable with Bermingham et al. (48) showing that Clostridiaceae and Fusobacteriaceae were higher and Prevotellaceae was lower with high meat (or protein). On the contrary, we observed Bacteroidaceae and Ruminococcaceae were higher in P55 (high meat or protein) in this study, whereas they were lower with high meat in Bermingham et al. The differences observed are probably due to the fixed ratio of plant-to-animal protein and species differences because Bermingham et al. completely replaced the plant protein with animal protein without maintaining the ratio and that study was conducted in dogs.

This study also demonstrated that fecal BCFAs, known to be derived from amino acid metabolism, were significantly greater with P55 than with P28, consistent with prior findings in dogs (63). Also, the putrefactive SCFA valerate was higher with greater levels of carbohydrates. The higher concentrations of propionate found with P55 may have been derived from proteolysis of mucin and subsequent putrefaction of the constituent threonine. However, higher propionate may have

TABLE 5 Analysis of fecal microbial carbohydrate-active and mucin-foraging enzymes from cats fed P28, P35, and P55 foods each for 90 d[1]

	Test food		
KEGG function	P28	P35	P55
Starch-, cellulose-, hemicellulose-, trehalose-, fructo-, glucan-, and mannooligosaccharide-utilizing enzymes			
K00700_1,4-α-glucan branching enzyme [EC:2.4.1.18]	6.18 ± 0.04[b]	6.28 ± 0.04[b]	6.74 ± 0.05[a]
K01176_α-amylase [EC:3.2.1.1]	2.91 ± 0.09[a]	2.82 ± 0.11[a]	2.02 ± 0.11[b]
K01178_glucoamylase [EC:3.2.1.3]	−6.13 ± 0.06[b]	−6.02 ± 0.06[b]	−5.71 ± 0.07[a]
K01179_endoglucanase [EC:3.2.1.4]	5.10 ± 0.08[a]	5.12 ± 0.07[a]	4.83 ± 0.05[b]
K01180_endo-1,3(4)-β-glucanase [EC:3.2.1.6]	−4.61 ± 0.12[a]	−4.57 ± 0.19[a]	−5.25 ± 0.06[b]
K01181_endo-1,4-β-xylanase [EC:3.2.1.8]	1.52 ± 0.14[b]	1.55 ± 0.13[b]	2.72 ± 0.09[a]
K01182_oligo-1,6-glucosidase [EC:3.2.1.10]	5.90 ± 0.05[c]	6.17 ± 0.05[b]	6.90 ± 0.06[a]
K01183_chitinase [EC:3.2.1.14]	4.77 ± 0.06[a]	4.61 ± 0.07[a]	2.51 ± 0.07[b]
K01184_polygalacturonase [EC:3.2.1.15]	−4.74 ± 0.06[a]	−4.96 ± 0.05[b]	−5.14 ± 0.06[b]
K01185_lysozyme [EC:3.2.1.17]	3.59 ± 0.14[a]	3.52 ± 0.13[a]	2.62 ± 0.14[b]
K01194_α, α-trehalase [EC:3.2.1.28]	−2.15 ± 0.27[a]	−2.48 ± 0.24[a]	−2.31 ± 0.21[a]
K01198_xylan 1,4-β-xylosidase [EC:3.2.1.37]	5.68 ± 0.09[a]	5.66 ± 0.08[a]	4.02 ± 0.05[b]
K01199_glucan endo-1,3-β-D-glucosidase [EC:3.2.1.39]	−4.70 ± 0.06[a]	−4.77 ± 0.07[a]	−5.20 ± 0.07[b]
K01200_pullulanase [EC:3.2.1.41]	5.42 ± 0.06[a]	5.36 ± 0.05[a]	4.61 ± 0.06[b]
K01201_glucosylceramidase [EC:3.2.1.45]	4.77 ± 0.06[a]	4.65 ± 0.06[a]	2.60 ± 0.06[b]
K01207_β-N-acetylhexosaminidase [EC:3.2.1.52]	5.33 ± 0.06[a]	5.19 ± 0.06[a]	4.69 ± 0.04[b]
K01208_cyclomaltodextrinase [EC:3.2.1.54]	−1.16 ± 0.48[a]	−1.26 ± 0.45[a]	−1.98 ± 0.38[a]
K01209_α-N-arabinofuranosidase [EC:3.2.1.55]	5.43 ± 0.06[b]	5.57 ± 0.05[a,b]	5.66 ± 0.06[a]
K01210_glucan 1,3-β-glucosidase [EC:3.2.1.58]	5.77 ± 0.06[a]	5.65 ± 0.07[a]	3.86 ± 0.08[b]
K01212_levanase [EC:3.2.1.65]	3.65 ± 0.07[c]	4.34 ± 0.08[b]	5.31 ± 0.07[a]
K01215_glucan 1,6-α-glucosidase [EC:3.2.1.70]	−3.30 ± 0.33[b]	−2.79 ± 0.29[a,b]	−1.92 ± 0.30[a]
K01216_licheninase [EC:3.2.1.73]	−4.01 ± 0.23[a]	−4.54 ± 0.12[a,b]	−4.89 ± 0.13[b]
K01219_agarase [EC:3.2.1.81]	−6.14 ± 0.04[b]	−6.00 ± 0.06[b]	−4.78 ± 0.28[a]
K01220_6-phospho-β-galactosidase [EC:3.2.1.85]	5.14 ± 0.07[a]	5.23 ± 0.08[a]	4.71 ± 0.09[b]
K01222_6-phospho-β-glucosidase [EC:3.2.1.86]	2.07 ± 0.12[b]	2.18 ± 0.10[a,b]	2.52 ± 0.10[a]
K01223_6-phospho-β-glucosidase [EC:3.2.1.86]	6.07 ± 0.04[c]	6.22 ± 0.04[b]	6.61 ± 0.04[a]
K01225_cellulose 1,4-β-cellobiosidase [EC:3.2.1.91]	−1.34 ± 0.13[c]	−0.75 ± 0.11[b]	1.25 ± 0.11[a]
K01226_trehalose-6-phosphate hydrolase [EC:3.2.1.93]	1.94 ± 0.16[a]	1.87 ± 0.15[a]	2.19 ± 0.09[a]
K01227_mannosyl-glycoprotein endo-β-N-acetylglucosaminidase [EC:3.2.1.96]	3.46 ± 0.10[a]	2.85 ± 0.21[a]	−0.590 ± 0.23[b]
K01234_neopullulanase [EC:3.2.1.135]	2.34 ± 0.14[b]	2.37 ± 0.13[b]	3.68 ± 0.12[a]
K01236_maltooligosyltrehalose trehalohydrolase [EC:3.2.1.141]	−1.66 ± 0.13[a]	−1.70 ± 0.16[a]	−2.95 ± 0.09[b]
Mucin-foraging enzymes			
K00737_β-1,4-mannosyl-glycoprotein β-1,4-N-acetylglucosaminyltransferase [EC:2.4.1.144]	−0.380 ± 0.09[c]	0.010 ± 0.13[b]	1.19 ± 0.08[a]
K01187_α-glucosidase [EC:3.2.1.20]	5.97 ± 0.04[b]	6.07 ± 0.03[b]	6.66 ± 0.05[a]
K01188_β-glucosidase [EC:3.2.1.21]	5.34 ± 0.06[c]	5.55 ± 0.05[b]	5.99 ± 0.05[a]
K01190_β-galactosidase [EC:3.2.1.23]	6.20 ± 0.05[c]	6.40 ± 0.05[b]	7.20 ± 0.05[a]
K01191_α-mannosidase [EC:3.2.1.24]	4.72 ± 0.07[c]	4.98 ± 0.06[b]	5.98 ± 0.05[a]
K01192_β-mannosidase [EC:3.2.1.25]	3.22 ± 0.10[c]	4.05 ± 0.10[b]	5.65 ± 0.07[a]
K01193_β-fructofuranosidase [EC:3.2.1.26]	6.28 ± 0.05[b]	6.45 ± 0.05[b]	7.08 ± 0.05[a]
K01195_β-glucuronidase [EC:3.2.1.31]	2.95 ± 0.10[b]	3.56 ± 0.08[a]	3.75 ± 0.06[a]
K01197_hyaluronoglucosaminidase [EC:3.2.1.35]	0.010 ± 0.19[b]	0.340 ± 0.19[b]	2.01 ± 0.17[a]
K01205_α-N-acetylglucosaminidase [EC:3.2.1.50]	−1.19 ± 0.20[b]	−1.03 ± 0.18[b]	−0.060 ± 0.12[a]
K01206_α-L-fucosidase [EC:3.2.1.51]	4.40 ± 0.07[c]	4.99 ± 0.08[b]	6.27 ± 0.06[a]
K01218_mannan endo-1,4-β-mannosidase [EC:3.2.1.78]	0.060 ± 0.11[b]	0.400 ± 0.13[b]	1.67 ± 0.07[a]
K01235_α-glucuronidase [EC:3.2.1.139]	−3.81 ± 0.11[b]	−3.47 ± 0.18[b]	−2.61 ± 0.21[a]
K01639_N-acetylneuraminate lyase [EC:4.1.3.3]	4.18 ± 0.07[c]	4.74 ± 0.07[b]	5.94 ± 0.05[a]

[1] Values are means ± SEs of center-log ratio transformations that showed significance ($P < 0.05$) by nonparametric analysis followed by the Wilcoxon significance test. Mean values were calculated by initially averaging those from both time points (45 and 90 d) for each cat (P28: $n = 54$; P35: $n = 52$; P55: $n = 54$), then averaging by a given treatment. Values in the same row with different letters are significantly different ($P < 0.05$). KEGG, Kyoto Encyclopedia of Genes and Genomes.

also had contributions from greater colonic microbe conversion of acetate into propionate.

Several studies have demonstrated that SCFAs that are derived from saccharolytic fermentation (e.g., butyrate) rather than putrefaction (e.g., valerate) confer benefits to the host, in particular due to inhibitory effects on pathogenic bacteria of lowering luminal pH and anti-inflammatory properties (64). BCFAs also have some beneficial properties such as suppression of proinflammatory markers in intestinal cells (65) and reduction of necrotizing colitis in neonatal rats (66). Both SCFAs and BCFAs serve as sources of energy for colonocytes (67). Further, these BCFAs are generated by microbes via proteolysis of undigested dietary protein and deamination of branched-chain amino acids, suggesting that consumption of

P55 would lead to greater concentrations of amino acids or dipeptides entering the colonic environment. Based on changes in imputed microbial function, greater microbial biosynthesis of branched-chain amino acids seems to have been occurring alongside the observed higher fecal BCFAs when cats consumed P55.

The genera *Bifidobacterium, Megasphaera, Lactobacillus*, and *Prevotella*, all higher with P28, tend to produce more saccharolytic-type SCFAs (e.g., butyrate). *Bacteroides* and *Ruminococcus*, greater with P55, may have been shunted toward foraging mucin; these genera harbor genetic elements competent to degrade mucin (68). Furthermore, greater *Prevotella* with P28 and greater *Bacteroides* with P55 are consistent with data in which *Prevotella* was more abundant in people who ate more carbohydrates and *Bacteroides* was more abundant in those who consumed high levels of protein and animal fat (69).

Overall, feces from when cats consumed P28 favored gut microbial saccharolytic activity and P55 favored more mucin-foraging and proteolytic activity. Greater proteolytic activity produces metabolites that are potential uremic toxins such as polyamines, ammonia, and indoles (70) that may affect the progression of existing renal disease (21). Further, this study also observed significantly higher BUN, BUN:creatinine ratio, phosphorus, total protein, and glucose in blood analyses as well as higher urine creatinine and specific gravity when cats consumed P55 as opposed to P28. However, consistent with a prior study (22), these higher blood/serum and urinary analytes are within the range of reference intervals, so they may not affect a healthy host.

To our knowledge, this is the first study in cats to systematically test varying dietary protein levels with compensating carbohydrate levels, without affecting the proportions of fat, animal:plant protein ratio, and amino acids. Further, total dietary fiber was similar across diets. Overall, this study provides a model (**Figure 2**) in which P55 had greater protein digestibility in the small intestine and less bypass protein in the colon than P28. However, presumably due to lower availability of carbohydrate substrates and dietary fiber in the colonic environment of P55-fed cats, gut microbiota foraging of mucin glycans and amino acid biosynthesis was higher as previously observed (71). In parallel, amino acid catabolism was also greater, and the production of various metabolic byproducts resulted in higher fecal pH, ammonia, and BCFA production. Although not tested here, it is proposed that there may have been consequent depletion of the mucin layer.

There are a few limitations to this study. Although this study utilized only healthy adult cats, the impact of increasing dietary protein intake on protein digestibility in senior cats requires further investigation because the aging process reduces the absorption of proteins. Further, the higher concentrations of certain blood/serum and urinary analytes when healthy cats consumed P55 than when they consumed P28 were within normal reference ranges, but those higher concentrations may affect the health of cats with conditions such as chronic kidney disease or cardiovascular disease. Hence, further studies are needed to understand how foods with differing protein levels with or without dietary fiber may affect these health conditions. In addition, the functional attributes of microbiota analyzed through PiCRUSt have constraints in terms of predicting functional attributes by a single marker gene, in that the sequences/annotated genomes of phylogenetically close relatives may not be present in the reference database. However, the microbiota functional attributes presented in this study were partially validated by analyzing fecal pH, SCFAs, and ammonia. Nonetheless, additional studies in this area are warranted, specifically in metabolomics, to understand how high intake of protein alters postbiotics and their role in the overall well-being of cats, aligned with microbiota-predicted functional attributes.

Acknowledgments

We thank Jennifer L Giel, who assisted with the writing and development of the manuscript.

The authors' responsibilities were as follows—DVB designed the research, conducted the research, and had primary responsibility for the final content; DEJ provided essential reagents or materials; and all authors analyzed the data, wrote the paper, and read, edited, and approved the final manuscript.

References

1. Wernimont SM, Radosevich J, Jackson MI, Ephraim E, Badri DV, MacLeay JM, Jewell DE, Suchodolski JS. The effects of nutrition on the gastrointestinal microbiome of cats and dogs: impact on health and disease. Front Microbiol 2020;11:1266.
2. Mondo E, Marliani G, Accorsi PA, Cocchi M, Di Leone A. Role of gut microbiota in dog and cat's health and diseases. Open Vet J 2019;9(3):253–8.
3. Barko PC, McMichael MA, Swanson KS, Williams DA. The gastrointestinal microbiome: a review. J Vet Intern Med 2018;32(1):9–25.
4. Blake AB, Suchodolski JS. Importance of gut microbiota for the health and disease of dogs and cats. Anim Front 2016;6(3):37–42.
5. De Angelis M, Ferrocino I, Calabrese FM, De Filippis F, Cavallo N, Siragusa S, Rampelli S, Di Cagno R, Rantsiou K, Vannini L, et al. Diet influences the functions of the human intestinal microbiome. Sci Rep 2020;10(1):4247.
6. Shortt C, Hasselwander O, Meynier A, Nauta A, Fernandez EN, Putz P, Rowland I, Swann J, Turk J, Vermeiren J, et al. Systematic review of the effects of the intestinal microbiota on selected nutrients and non-nutrients. Eur J Nutr 2018;57(1):25–49.
7. Singh RK, Chang H-W, Yan D, Lee KM, Ucmak D, Wong K, Abrouk M, Farahnik B, Nakamura M, Zhu TH, et al. Influence of diet on the gut microbiome and implications for human health. J Transl Med 2017;15(1):73.
8. Rothschild D, Weissbrod O, Barkan E, Kurilshikov A, Korem T, Zeevi D, Costea PI, Godneva A, Kalka IN, Bar N, et al. Environment dominates over host genetics in shaping human gut microbiota. Nature 2018;555(7695):210–15.
9. Tindall AM, Petersen KS, Kris-Etherton PM. Dietary patterns affect the gut microbiome—the link to risk of cardiometabolic diseases. J Nutr 2018;148(9):1402–7.
10. Willson K, Situ C. Systematic review on effects of diet on gut microbiota in relation to metabolic syndromes. J Clin Nutr Metab 2017;1:2.
11. Kim B, Choi H-N, Yim J-E. Effect of diet on the gut microbiota associated with obesity. J Obes Metab Syndr 2019;28(4):216–24.
12. Gurung M, Li Z, You H, Rodrigues R, Jump DB, Morgun A, Shulzhenko N. Role of gut microbiota in type 2 diabetes pathophysiology. EBioMedicine 2020;51:102590.
13. Seesaha PK, Chen X, Wu X, Xu H, Li C, Jheengut Y, Zhao F, Liu L, Zhang D. The interplay between dietary factors, gut microbiome and colorectal cancer: a new era of colorectal cancer prevention. Future Oncol 2020;16(7):293–306.
14. Martinez KB, Leone V, Chang EB. Microbial metabolites in health and disease: navigating the unknown in search of function. J Biol Chem 2017;292(21):8553–9.
15. Heintz-Buschart A, Wilmes P. Human gut microbiome: function matters. Trends Microbiol 2018;26(7):563–74.
16. Verbrugghe A, Hesta M. Cats and carbohydrates: the carnivore fantasy? Vet Sci 2017;4(4):55.
17. Zoran DL. The carnivore connection to nutrition in cats. J Am Vet Med Assoc 2002;221(11):1559–67.
18. Hall JA, Vondran JC, Vanchina MA, Jewell DE. When fed foods with similar palatability, healthy adult dogs and cats choose different macronutrient compositions. J Exp Biol 2018;221(Pt 14): jeb173450.

19. Hewson-Hughes AK, Hewson-Hughes VL, Miller AT, Hall SR, Simpson SJ, Raubenheimer D. Geometric analysis of macronutrient selection in the adult domestic cat, *Felis catus*. J Exp Biol 2011;214(6):1039–51.
20. Plantinga EA, Bosch G, Hendriks WH. Estimation of the dietary nutrient profile of free-roaming feral cats: possible implications for nutrition of domestic cats. Br J Nutr 2011;106(Suppl 1):S35–48.
21. Ephraim E, Cochrane C-Y, Jewell DE. Varying protein levels influence metabolomics and the gut microbiome in healthy adult dogs. Toxins (Basel) 2020;12(8):517.
22. Backlund B, Zoran DL, Nabity MB, Norby B, Bauer JE. Effects of dietary protein content on renal parameters in normal cats. J Feline Med Surg 2011;13(10):698–704.
23. Zhao J, Zhang X, Liu H, Brown MA, Qiao S. Dietary protein and gut microbiota composition and function. Curr Protein Pept Sci 2019;20(2):145–54.
24. Ma N, Tian Y, Wu Y, Ma X. Contributions of the interaction between dietary protein and gut microbiota to intestinal health. Curr Protein Pept Sci 2017;18(8):795–808.
25. O'Keefe SJD, Li JV, Lahti L, Ou J, Carbonero F, Mohammed K, Posma JM, Kinross J, Wahl E, Ruder E, et al. Fat, fibre and cancer risk in African Americans and rural Africans. Nat Commun 2015;6(1):6342.
26. Lubbs DC, Vester BM, Fastinger ND, Swanson KS. Dietary protein concentration affects intestinal microbiota of adult cats: a study using DGGE and qPCR to evaluate differences in microbial populations in the feline gastrointestinal tract. J Anim Physiol Anim Nutr (Berl) 2009;93(1):113–21.
27. Deusch O, O'Flynn C, Colyer A, Morris P, Allaway D, Jones PG, Swanson KS. Deep Illumina-based shotgun sequencing reveals dietary effects on the structure and function of the fecal microbiome of growing kittens. PLoS One 2014;9(7):e101021.
28. Hooda S, Vester Boler BM, Kerr KR, Dowd SE, Swanson KS. The gut microbiome of kittens is affected by dietary protein:carbohydrate ratio and associated with blood metabolite and hormone concentrations. Br J Nutr 2013;109(9):1637–46.
29. National Research Council (US) Committee for the Update of the Guide for the Care and Use of Laboratory Animals. Guide for the care and use of laboratory animals. 8th ed. Washington (DC): National Academies Press (US), National Academy of Sciences; 2011.
30. Wang B-S, Wang X-J, Gong L-K. The construction of a Williams design and randomization in cross-over clinical trials using SAS. J Stat Soft 2009;29(Code Snippet 1):1–10.
31. Association of American Feed Control Officials (AAFCO). AAFCO methods for substantiating nutritional adequacy of dog and cat foods[Internet]. Champaign, IL: AAFCO; 2014[cited 7 October, 2019]. Available from: https://www.aafco.org/Portals/0/SiteContent/Regulatory/Committees/Pet-Food/Reports/Pet_Food_Report_2013_Midyear-Proposed_Revisions_to_AAFCO_Nutrient_Profiles.pdf.
32. AOAC International. Official methods of analysis. 21st ed. Rockville, MD: AOAC international; 2019.
33. Hall JA, Melendez LD, Jewell DE. Using gross energy improves metabolizable energy predictive equations for pet foods whereas undigested protein and fiber content predict stool quality. PLoS One 2013;8(1):e54405.
34. Chaney AL, Marbach EP. Modified reagents for determination of urea and ammonia. Clin Chem 1962;8(2):130–2.
35. Kendall PT, Blaza SE, Holme DW. Assessment of endogenous nitrogen output in adult dogs of contrasting size using a protein-free diet. J Nutr 1982;112(7):1281–6.
36. Jackson MI, Jewell DE. Balance of saccharolysis and proteolysis underpins improvements in stool quality induced by adding a fiber bundle containing bound polyphenols to either hydrolyzed meat or grain-rich foods. Gut Microbes 2019;10(3):298–320.
37. Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, Lesniewski RA, Oakley BB, Parks DH, Robinson CJ, et al. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl Environ Microbiol 2009;75(23):7537–41.
38. Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R. UCHIME improves sensitivity and speed of chimera detection. Bioinformatics 2011;27(16):2194–200.
39. Wang Q, Garrity GM, Tiedje JM, Cole JR. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl Environ Microbiol 2007;73(16):5261–7.
40. Langille MG, Zaneveld J, Caporaso JG, McDonald D, Knights D, Reyes JA, Clemente JC, Burkepile DE, Vega Thurber RL, Knight R, et al. Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. Nat Biotechnol 2013;31(9):814–21.
41. Fernandes AD, Reid JN, Macklaim JM, McMurrough TA, Edgell DR, Gloor GB. Unifying the analysis of high-throughput sequencing datasets: characterizing RNA-seq, 16S rRNA gene sequencing and selective growth experiments by compositional data analysis. Microbiome 2014;2(1):15.
42. Cohen J. Statistical power analysis for the behavioral sciences. New York, NY: Routledge Academic; 1988.
43. Portune KJ, Beaumont M, Davila A-M, Tomé D, Blachier F, Sanz Y. Gut microbiota role in dietary protein metabolism and health-related outcomes: the two sides of the coin. Trends Food Sci Technol 2016;57:213–32.
44. Gross KL, Becvarova I, Armstrong PJ, Debraekeleer J. Feeding young adult cats: before middle age. In: Hand MS, Thatcher CD, Remillard RL, Roudebush P, Novotny B , editors. Small animal clinical nutrition. 5th ed. Topeka, KS: Mark Morris Institute; 2010. p. 389–400.
45. Vester BM, Belsito KR, Swanson KS. Serum metabolites, ghrelin and leptin are modified by age and/or diet in weanling kittens fed either a high- or moderate-protein diet. Anim Sci J 2012;83(5):426–33.
46. Golder C, Weemhoff JL, Jewell DE. Cats have increased protein digestibility as compared to dogs and improve their ability to absorb protein as dietary protein intake shifts from animal to plant sources. Animals (Basel) 2020;10(3):541.
47. Hamper BA, Kirk CA, Bartges JW. Apparent nutrient digestibility of two raw diets in domestic kittens. J Feline Med Surg 2016;18(12):991–6.
48. Bermingham EN, Maclean P, Thomas DG, Cave NJ, Young W. Key bacterial families (Clostridiaceae, Erysipelotrichaceae and Bacteroidaceae) are related to the digestion of protein and energy in dogs. PeerJ 2017;5:e3019.
49. Ritchie LE, Steiner JM, Suchodolski JS. Assessment of microbial diversity along the feline intestinal tract using 16S rRNA gene analysis. FEMS Microbiol Ecol 2008;66(3):590–8.
50. Ritchie LE, Burke KF, Garcia-Mazcorro JF, Steiner JM, Suchodolski JS. Characterization of fecal microbiota in cats using universal 16S rRNA gene and group-specific primers for *Lactobacillus* and *Bifidobacterium* spp. Vet Microbiol 2010;144(1–2):140–6.
51. Bunesova V, Lacroix C, Schwab C. Mucin cross-feeding of infant Bifidobacteria and *Eubacterium hallii*. Microb Ecol 2018;75(1):228–38.
52. Crost EH, Tailford LE, Le Gall G, Fons M, Henrissat B, Juge N. Utilisation of mucin glycans by the human gut symbiont *Ruminococcus gnavus* is strain-dependent. PLoS One 2013;8(10):e76341.
53. Crost EH, Tailford LE, Monestier M, Swarbreck D, Henrissat B, Crossman LC, Juge N. The mucin-degradation strategy of *Ruminococcus gnavus:* the importance of intramolecular *trans*-sialidases. Gut Microbes 2016;7(4):302–12.
54. Tailford LE, Crost EH, Kavanaugh D, Juge N. Mucin glycan foraging in the human gut microbiome. Front Genet 2015;6:81.
55. Van Herreweghen F, De Paepe K, Roume H, Kerckhof FM, Van de Wiele T. Mucin degradation niche as a driver of microbiome composition and *Akkermansia muciniphila* abundance in a dynamic gut model is donor independent. FEMS Microbiol Ecol 2018;94(12):fiy186.
56. Pacheco AR, Curtis MM, Ritchie JM, Munera D, Waldor MK, Moreira CG, Sperandio V. Fucose sensing regulates bacterial intestinal colonization. Nature 2012;492(7427):113–17.
57. Gonzalez-Garcia RA, McCubbin T, Navone L, Stowers C, Nielsen LK, Marcellin E. Microbial propionic acid production. Fermentation 2017;3(2):1–20.
58. Levine UY, Looft T, Allen HK, Stanton TB. Butyrate-producing bacteria, including mucin degraders, from the swine intestinal tract. Appl Environ Microbiol 2013;79(12):3879–81.
59. Conway T, Cohen PS. Commensal and pathogenic *Escherichia coli* metabolism in the gut. Microbiol Spectr 2015;3(3):MBP–0006-2014.
60. Hutchinson GE. Concluding remarks. Cold Spring Harb Symp Quant Biol 1957;22:415–27.
61. Mazmanian SK, Round JL, Kasper DL. A microbial symbiosis factor prevents intestinal inflammatory disease. Nature 2008;453(7195):620–5.
62. Herstad KMV, Gajardo K, Bakke AM, Moe L, Ludvigsen J, Rudi K, Rud I, Sekelja M, Skancke E. A diet change from dry food to beef

induces reversible changes on the faecal microbiota in healthy, adult client-owned dogs. BMC Vet Res 2017;13(1):147.

63. Hang I, Heilmann RM, Grutzner N, Suchodolski JS, Steiner JM, Atroshi F, Sankari S, Kettunen A, de Vos WM, Zentek J, et al. Impact of diets with a high content of greaves-meal protein or carbohydrates on faecal characteristics, volatile fatty acids and faecal calprotectin concentrations in healthy dogs. BMC Vet Res 2013;9(1):201.
64. Morrison DJ, Preston T. Formation of short chain fatty acids by the gut microbiota and their impact on human metabolism. Gut Microbes 2016;7(3):189–200.
65. Yan Y, Wang Z, Greenwald J, Kothapalli KS, Park HG, Liu R, Mendralla E, Lawrence P, Wang X, Brenna JT. BCFA suppresses LPS induced IL-8 mRNA expression in human intestinal epithelial cells. Prostaglandins Leukot Essent Fatty Acids 2017;116:27–31.
66. Ran-Ressler RR, Khailova L, Arganbright KM, Adkins-Rieck CK, Jouni ZE, Koren O, Ley RE, Brenna JT, Dvorak B. Branched chain fatty acids reduce the incidence of necrotizing enterocolitis and alter gastrointestinal microbial ecology in a neonatal rat model. PLoS One 2011;6(12):e29032.
67. Jaskiewicz J, Zhao Y, Hawes JW, Shimomura Y, Crabb DW, Harris RA. Catabolism of isobutyrate by colonocytes. Arch Biochem Biophys 1996;327(2):265–70.
68. Oliphant K, Allen-Vercoe E. Macronutrient metabolism by the human gut microbiome: major fermentation by-products and their impact on host health. Microbiome 2019;7(1):91.
69. Wu GD, Chen J, Hoffmann C, Bittinger K, Chen Y-Y, Keilbaugh SA, Bewtra M, Knights D, Walters WA, Knight R, et al. Linking long-term dietary patterns with gut microbial enterotypes. Science 2011;334(6052):105–8.
70. Aronov PA, Luo FJ, Plummer NS, Quan Z, Holmes S, Hostetter TH, Meyer TW. Colonic contribution to uremic solutes. J Am Soc Nephrol 2011;22(9):1769–76.
71. Holmes AJ, Chew YV, Colakoglu F, Cliff JB, Klaassens E, Read MN, Solon-Biet SM, McMahon AC, Cogger VC, Ruohonen K, et al. Diet-microbiome interactions in health are controlled by intestinal nitrogen source constraints. Cell Metab 2017;25(1):140–51.