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Consumption of Cooked Navy Bean Powders Modulate the Canine Fecal and Urine Metabolome

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Abstract: Common beans (*Phaseolus vulgaris,* L.), are a safe and digestible source of macro- and micro-nutrients for dogs and supply phytochemicals that are associated with improving human and animal health. In this study, we utilized a targeted and non-targeted metabolomic approach to evaluate dietary cooked navy bean powder (25% weight/weight) as part of the canine food metabolome. GC- and LC-MS were used to evaluate the effects of a navy bean diet on serum biochemistry and chemical composition of feces and urine in clinically healthy adult dogs (n=10), compared to a control diet (n=11). The navy bean diet was higher in 2-piperdinecarboxylic acid, s-methyl cysteine, β-sitosterol, γ-tocopherol, sucrose, and fructose. Companion dogs consuming the 25% navy bean diet for one month had lower serum cholesterol and showed changes in fecal and urine metabolites that were consistent with modulation of lipid and carbohydrate metabolism. Metabolite biomarkers of bean intake were identified in canine diets and urine. These data support the continued investigation of the potential for bean-based diets to improve companion dog health and the utility of nutritional metabolomics in companion animal studies.

Keywords: Beans (*Phaseolus vulgaris),* carbohydrate, canine, cholesterol, lipid, metabolomics.

INTRODUCTION

Dry common beans (*Phaseolus vulgaris,* L.) are a staple food ingredient that is rich in macro- and micro-nutrients, phytochemicals, and readily incorporated into commercial dog diet formulations [1]. To this end, we recently showed that cooked dry bean consumption at 25% weight/weight (w/w) is safe and digestible in clinically healthy, normal weight dogs [2]. Furthermore, bean-based diets modulate serum lipid profiles in overweight dogs undergoing caloric restriction [3].

In addition to providing a unique source of nutrients that may improve nutritional status [4], dry beans also contain a rich array of phytochemicals with health promoting properties, such as tocopherols, flavonoids, phytosterols, and enzyme inhibitors [5-7]. Consumption of dry beans has been shown in humans and laboratory animal models to decrease risk factors for developing metabolic syndrome, cardiovascular disease, cancer, and diabetes [6, 8, 9]. Dry bean intake has also been associated with improving longevity, promoting gastrointestinal health, facilitating healthy weight maintenance, and supporting hepatic function [10-14]. One mechanism by which cooked bean intake may promote health is by altering fat metabolism: for example, serum lipid modulation is one of the most consistently reported effects of bean intake in humans and animals. Multiple studies demonstrate that increased bean consumption is associated with reduced serum cholesterol, low density lipoprotein cholesterol (LDL-C), and triglycerides with increased high-density lipoprotein cholesterol (HDL-C) [8, 15-17]. The combination of dry bean macronutrients, micronutrients, and phytochemicals work together to promote health and reduce disease risk [1] and may have potential to improve the health of companion dogs as well.

Metabolomics is becoming more frequently utilized in nutrition and veterinary sciences to evaluate phytochemical diversity in foods and to elucidate the functional responses of mammals to dietary interventions [18-20]. Metabolomics can also provide candidate dietary biomarkers for specific food and diet patterns in serum, urine, or feces, as well as measure the effects of diet on disease risk factors [21, 22].

In this study, we evaluated the effect of adding 25% w/w cooked navy bean (NB) powder on 1) the canine food metabolome and 2) the effects of this diet on serum biochemis-

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try and the chemical composition of feces and urine in clinically healthy adult dogs, compared to a control (CON) diet. The dogs consumed NB or CON diets for one month, and metabolites were characterized in the diet, urine, and feces using a combination of gas and liquid chromatography coupled with mass spectrometry (GC- and LC- MS). Serum analytes were determined using standard, clinical biochemistry panels [2]. We hypothesized that the NB diet contains distinct phytochemicals compared to the CON diet and that NB consumption modulates the chemical composition of the canine fecal and urine metabolome.

MATERIAL AND METHODS

Canine Participants, Sample Collection, and Ethics Statement

Ethics Statement

Clinical trial operations, animal care procedures, and biological sample collections were approved by the Colorado State University Institutional Animal Care and Use Committee (IACUC Protocol 10-1932A).

Participants

Twenty-one clinically healthy, normal weight, male and female dogs between the ages of 2 and 7 years, and a variety of breeds were enrolled in a controlled, double-blinded, restrictively randomized, 4 week dietary intervention at the Flint Animal Cancer Center, Veterinary Teaching Hospital at Colorado State University as previously reported [2]. A body condition score (BCS) was assigned to each dog by the study clinician using a 9-point scale [23]. Dogs were evenly distributed and no differences were detected between diet groups with respect to BCS, age, weight, and sex (Table **1**).

Sample Collection and Processing

Fecal, plasma, and urine samples were collected for clinical and analytical analysis at baseline, 2, and 4 week time points. Owners were instructed to collect fecal samples within 5 hours of being voided. Samples were either immediately frozen at -20 °C in provided containers and transferred to -80 °C, or brought to the study site and immediately stored at -80 °C until analysis. Serum samples for biochemical analysis were collected via venipuncture into a tube without anticoagulant, allowed to coagulate for 15 min and centrifuged for 15 min at 1,500 rcf. Blood was collected at approximately the same time of day throughout the study to minimize postprandial variation. Urine was collected by owners or a study technician using a free-catch method, however in some cases where a urine sample was not obtained via free-catch, ultrasound guided cystocentesis was utilized at the clinician's discretion. Serum cholesterol levels were obtained using standard clinical analytical methods as previously described [2].

Canine Diet Formulations and Nutrient Analysis

A standard ingredient, CON diet was formulated to meet the Association of American Feed Control Officials (AAFCO) and National Research Council (NRC) adult dog maintenance requirements [24, 25]. A second iso-nutrient and isocaloric diet containing 25 % w/w cooked NB powder was formulated to investigate the utility of common bean ingredients in dog food. A detailed ingredient and nutrient profile for each diet was previously published [2]. Table **2** provides a summary of the major ingredients in each diet. Energy requirements and daily food intake for weight maintenance were calculated for each individual dog as previously described [2]. Briefly, the amount of food was determined by the energy density of each diet and the daily metabolizable energy (ME) requirement of each dog. Daily ME requirement was determined by the formula ME (kcal/day) =110 x (body weight, kg)$^{0.75}$ [25].

Targeted Analysis of Dietary Nutrient Profiles

The macronutrient composition, on a dry matter basis, for the CON and NB diets respectively, is as follows: crude protein was 31.15 % and 29.91 %; crude fat was 14.00 % and 13.58 %; organic matter was 91.37 % and 91.83 %; crude fiber was 2.95 % and 3.18 %; and gross energy was 4,967 kcal/kg and 4,957 kcal/kg. All macronutrients were equally digestible between the CON and 25 % NB diets [2]. To determine the lipid profiles of the diets, total fat was extracted

Table 1. Baseline characteristics of canine study participants.

Characteristic	Control (n=10)	Navy Bean (n=11)	P-value[1]
Age[1], years	3.0 (2.0-4.0)	4.0 (2.8 - 5.3)	0.206
Body Weight, kg	28.5 (19.0 – 33.0)	21.8 (19.9 - 25.88)	0.260
Sex[3]			0.670
Female	5	6	
Male	6	4	
BCS[4]			1.00
BCS 4 & 5	9	8	
BCS 6 & 7	2	2	

[1]Age and weight are reported as median and interquartile range and differences between groups were evaluated with a Mann-Whitney t-test. Sex and Body Condition Score (BCS) are reported as number of dogs and differences were evaluated with Fisher's exact test. [2]Age is presented as reported by owner. [3]All dogs were neutered with the exception of one male in the control group. [4] BCS was assigned on a 9 point scale [23].

and separated using thin layer chromatography (TLC) and analyzed via GC under previously reported conditions [26]. Amino acid analysis was performed by Midwest Laboratories, Inc. (Omaha, NE) using standard methods (AOAC 994.12 (III)). Cystine, methionine, and tryptophan were analyzed using specialized protocols (AOAC 994.12 (Alt I) and 998.15). Total dietary fiber (TDF) and the soluble and insoluble fiber fractions were determined as previously described [27].

Non-targeted Metabolomic Analysis

Canine Diet Preparation for GC-MS Analysis

The diets were ground to a powder using a mortar and pestle, and metabolites were extracted by adding 1 ml of ice-cold methanol:water (80:20, v:v) to 100 mg of diet and incubated for 1 hr at -80 °C. Samples were then centrifuged at 1,500 rcf for 5 min and the extract was transferred into 1.5 ml microcentrifuge tubes as previously described [28]. Extracts were dried in a speedvac and methoximated by resuspending in pyridine (50 µL) with 25 mg/mL of methoxyamine hydrochloride and incubated at 60 ºC for 45 min twice, with 10 min sonication in between. After methoximation, samples were incubated with 50 µL of N-methyl-N-trimethylsilyltrifluoroacetamide with 1 % trimethylchlorosilane (MSTFA + 1 % TMCS, Thermo Scientific) at 60 ºC for 30 min, followed by centrifugation at 3,000 rcf for 5 min. Samples were then cooled to room temperature (~22 °C), and the supernatant (80 µL) was transferred to a 150 µL glass insert in a GC-MS autosampler vial.

Canine Fecal and Urine Sample Preparation, Metabolite Separation and Detection by GC-MS and LC-MS

Fecal metabolites were extracted using identical methods as the diets. Extracts were prepared with 100 mg of fecal material and methanol:water (80:20, v:v) as described above for the diets. One hundred ul of each urine sample was centrifuged at 13,000 rcf at 4 °C. The supernatant was transferred to an autosampler vial and directly injected. Extracted metabolites from diet and fecal samples were analyzed by GC-MS and urine samples were analyzed by LC-MS under similar conditions as previously described with minor differences [29-30]. Specific run conditions are given in Specific run conditions are given in supplemental appendix A.

Mass Spectrometry Data Processing and Metabolite Annotation

For each sample, a matrix of molecular features as defined by retention time and mass (m/z) was generated using XCMS [31]. Each sample was injected twice and the relative abundance of each molecular feature was measured by determining the mean chromatographic peak area after normalization to total ion current. Mass spectra were generated using an algorithm that clusters molecular features into spectra ('clusters' or 'compounds', C) based on co-variation and co-elution in the data set [32]. The abundance of each cluster was determined as the weighted sum of the peak areas for all molecular features within a cluster. Mass spectral clusters were searched against in-house and external databases including NIST v12 (www.nist.gov), Massbank, Metlin, and Golm (http://gmd.mpimp-golm.mpg.de) metabolite databases for annotation. The relative abundance of each fecal and urine metabolite was scaled to a median value of one. Each annotated metabolite is presented in Supplementary Table **1** with its corresponding "cluster" number (C), identification confidence level [33], PubChem Compound Identifier (CID), and Simplified Molecular Input Line Entry Specification (SMILES). Spectral libraries for diet, fecal, and urine metabolites are also available as supplementary materials.

Statistical Methods

Continuous baseline characteristics of the dogs were evaluated by a Mann-Whitney t-test (age and weight) and categorical data were evaluated with a Fisher's exact test (sex and BCS).

Diet, fecal and urine metabolites were evaluated with a principal components analysis (PCA, SIMCA P+ v12, Umetrics, Umea, Sweden) to determine global pattern changes or differences within each matrix. PCA was conducted with metabolite abundances that were mean-centered and Pareto-scaled, and 95 % confidence intervals for the PCA model were utilized to identify outliers. Significance of each component was determined using analysis of variance of PC scores for each component with a threshold of $p < 0.05$.

The relative abundance of each fecal and urine metabolite was scaled to a median value of one and is presented as the median and interquartile range (IQR).To determine differences in the relative abundance of each fecal and urine cluster/metabolite between diet groups and time, a mixed model linear regression analysis was applied using the lme4 package [34] in R [35]. The model included fixed effects for diet x time and the random effect for animal ID to control for repeated measures. A false discovery rate (FDR) correction was used to control for multiple comparisons [36]. An adjusted p-value < 0.1 was accepted as significant. When the time factor was significant, a post-hoc Wilcoxon matched-pairs signed rank test was used to determine the group in which a significant change occurred (GraphPad Prism version 5.03 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com). When metabolites were significant by diet, a Mann Whitney t-test was used to determine the timepoint at which the differences were significant. Diet metabolite differences are shown as a fold difference between the NB and CON diet (NB:CON). Metabolites that varied by more than 10 % relative abundance between diets were reported. For serum cholesterol levels, a repeated measures, 2-way ANOVA for diet, time, and diet x time interaction was used, a p-value < 0.05 was accepted as statistically significant.

RESULTS

Targeted Nutrient Profiles were Similar between Navy Bean and Control Diets

The major ingredients included in the CON and NB diets were wheat, corn, rice, and meat and bone meal, and poultry fat (Table **2**). A detailed ingredient list has been previously reported [2]. Total fat content (as-fed) was 13.0 % in the CON diet and 12.8 % NB diet. Sixteen individual fatty acids were detected in the diets, and in order of total fat composition in the NB and CON diets, respectively were: oleic acid,

Table 2. Major diet ingredients fed to twenty-one clinically healthy, adult dogs.

Ingredient, % weight/weight	Control Diet	Navy Bean Diet
cooked navy bean powder	-	25.00
wheat	29.00	9.94
corn	25.49	20.60
meat, bone, and poultry by-product meal	21.33	20.36
rice	12.50	12.50
poultry fat	7.77	7.77
supplements, fiber, and palatants	3.95	3.91

Ingredients are reported on an-fed basis. A detailed ingredient list has previously been published [2]

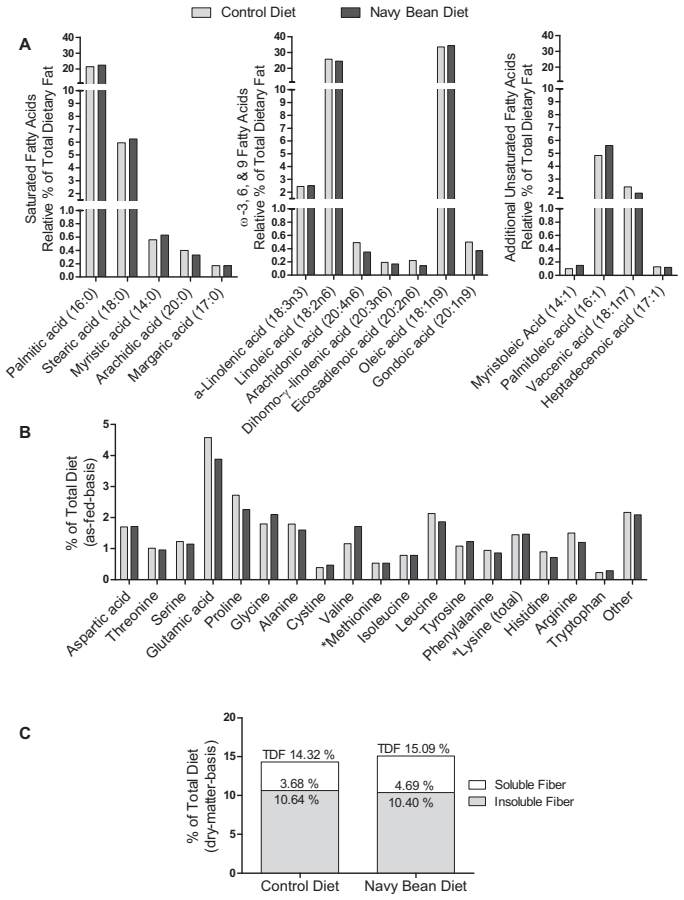

Fig. (1). Macronutrient diet components in 25 % navy bean powder and control diets. **A**) Detected saturated, ω -3, 6, 9, 5, & 7 fatty acids in navy bean and control diets as a relative percent of total dietary fat. **B**) Dietary amino acid profiles, as a percentage of total diet. Amino acids supplemented in the diets are indicated with an asterisk (*). **C**) Total dietary fiber (TDF) with soluble and insoluble fiber.

34.39 % and 33.5 %; linoleic acid, 24.49 % and 25.71 %; palmitic acid, 22.44 % and 21.40 %; stearic acid, 6.2 5% and 5.95 %; palmitoleic acid, 5.6 % and 4.83 %, α-linolenic acid, 2.5 % and 2.44 %; vaccenic acid, 1.9 % and 2.38 %. Myristic acid, gondoic acid, arachidonic acid, Arachidic acid, margaric acid, dihomo-γ-linolenic acid, myristoleic acid, eicosadienoic acid and heptadecenoic acid each comprised less than 1 % of total fat (Fig. **1A**). Saturated fat content was 29.82 % and 28.48 % of the total fat content, and unsaturated fat was 70.18 % and 70.49 % of the total fat for the NB and CON diets, respectively. For the NB diet, ω-3, ω-6, and ω-9 fatty acids comprised 2.50 %, 25.15 %, and 34.76 % of total fat, respectively. For the CON diet, ω-3, ω-6, and ω-9 fatty acids comprised 2.44 %, 26.61 %, and 34.00 % of total fat, respectively.

Crude protein levels were similar between the diets [2], and total amino acid profiles were likewise similar (Fig. **1B**). In order of percent of total diet in the NB and CON diets, respectively, amino acid content was: glutamic acid, 3.88 % and 4.58 %; proline, 2.26 % and 2.72 %; glycine, 2.10 % and 1.80 %; Leucine, 1.87 % and 2.13 %; aspartic acid, 1.72 % and 7.70 %; valine, 1.72 % and 1.16 %; alanine, 1.16 % and 1.79 %; lysine, 1.47 % and 1.45 %, tyrosine, 1.23 % and 1.08 %; arginine 1.20 % and 1.50 %, serine, 1.15 % and 1.23 %; threonine, 0.96 % and 1.01 %; phenylalanine, 0.86 % and 0.95 %; isoleucine, 0.78 % and 0.78 %; histidine, 0.72 % and 0.90 %; methionine, 0.53 % and 0.53 %; cysteine, 0.47 % and 0.39 %; and tryptophan, 0.29 % and 0.23 %. Other amino acids not individually identified (e.g. choline and taurine) comprised 2.09 % and 2.17 % for the NB and CON diets, respectively. Lysine and methionine were supplemented in both diets [2].

Crude fiber was similar between diets [2]. TDF was 15.09 % and 14.32 % for the NB and CON diets, respectively. Total soluble fiber was 4.69 % and 3.68 %, and total insoluble fiber was 10.40 % and 10.64 % for the NB and CON diets, respectively (Fig. **1C**).

Non-Targeted Revealed Phytochemical Profile Differences between Control and Navy Bean Diets

GC-MS analysis of the diets resulted in detection of 7,742 isolated features described by time and mass/charge ratio (*m/z*). These ions were collapsed into 833 clusters and showed significant variation between diets (Fig. **2a**). Of these 833 clusters, 18 metabolites were identified that had relative abundance differences of at least 10 % between diets. Metabolites that were greater in the NB diet compared to CON diet were 2-piperdinecarboxylic acid (41.49 fold increase); s-methyl cysteine (5.15 fold increase); 5-hydroxynorvaline (4.90 fold increase); sucrose (1.63 fold increase); citric acid (1.44 fold increase); γ-tocopherol (1.42 fold increase); β-sitosterol (1.36 fold increase); taurine (1.28 fold increase); α-tocopherol acetate (1.17 fold increase); fructose (1.15 fold increase); and Glycerol-3-Phosphate (G3P, 1.11 fold increase). Decreased metabolites included palmitelaidic acid (-1.12 fold decrease); L-threitol (-1.17 fold decrease); hydroquinone (-1.33 fold decrease); glucose (-1.46 fold decrease); D-pinitol (-1.57 fold decrease), an unidentified monosaccharide (**-**1.75 decrease); and trehalose (-1.84 fold decrease) (Fig. **2b**).

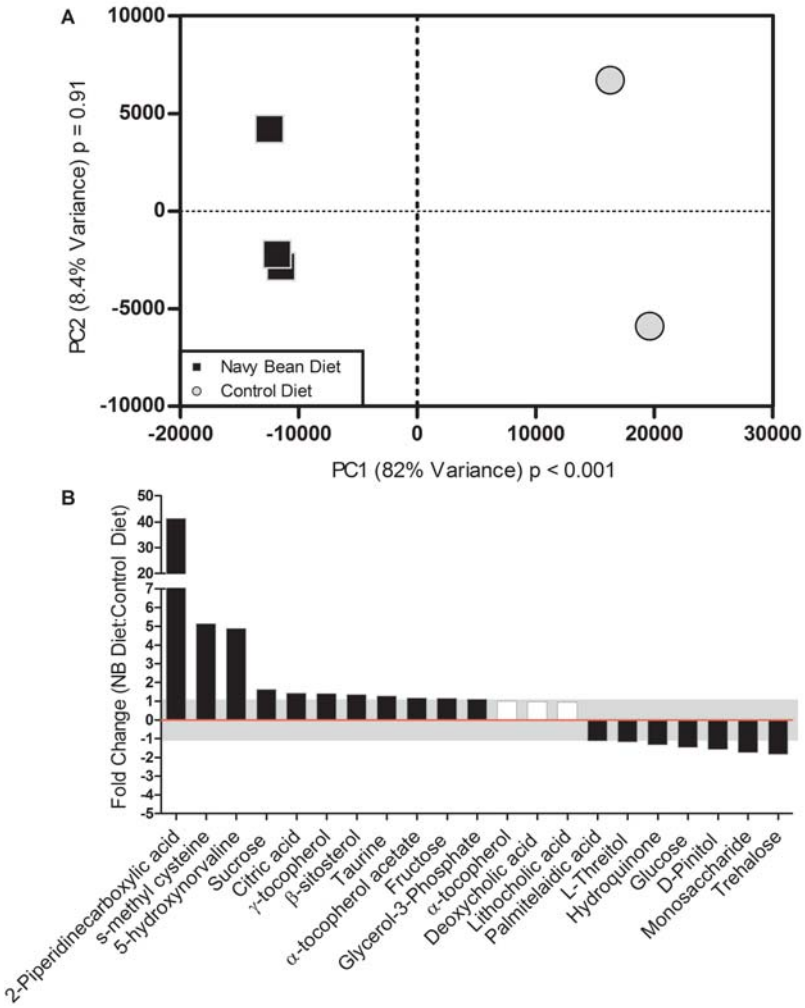

Fig. (2). Metabolite profiles of a nutritionally complete, standard ingredient diet compared to an isocaloric, nutrient matched diet containing 25 % w/w cooked navy bean powder. **A**) Metabolite variance between 25 % navy bean powder and control diets. The Navy Bean diet is represented by squares and the control diet is represented by circles. **B**) Ratios of the relative abundance of identified metabolites in the navy bean diet compared to control.

Lower Serum Cholesterol Levels in Dogs Consuming Navy Beans

After 4 weeks of dietary intervention, dogs in the NB diet group had lower cholesterol levels than dogs in the CON diet group (Fig. **3**). At baseline, for NB and CON diet groups, respectively, median serum cholesterol was 226 mg/dL (IQR: 177 to 243 mg/dL) and 244 mg/dL (IQR: 175 to 296 mg/dL), at two weeks serum cholesterol was 209 mg/dL (IQR: 202 to 233 mg/dL) and 245 mg/dL (IQR: 220 to 313 mg/dL), and at 4 weeks 209 mg/dL (IQR: 195 to 225 mg/dL) and 263 mg/dL (IQR: 233 to 289 mg/dL). Changes in serum cholesterol within group over time were not significant, and remained within normal ranges throughout the study.

Dietary Modulation of the Canine Fecal Metabolome

12,121 peaks were detected by GC-MS in the canine fecal samples and were reduced to 911 clusters. Principal components analysis revealed one dog in the NB group that was an outlier (95 % confidence across PC1 and PC2) which was subsequently excluded from statistical analysis. There were no significant differences in the principal component scores between or within groups at baseline or day 28 (data not shown), however, in dogs consuming the NB diet, we observed a consistent pattern shift in components 1 and 2 that was not observed in the CON group. To demonstrate this shift, fecal metabolite variance between groups and across time is shown as a PCA (Fig. **4A**). Fecal metabolite change was highly variable over time within the CON diet group: 7/11 dogs showed a positive directional change in PC1 and 4/11 had a negative directional change. For PC2, 7/11 had a negative directional change and 4/11 had a positive directional change (Fig. **4B**). The sum effect of these directional changes resulted in approximately random distribution in the PC1 and PC2 score plots. In the NB group within PC1, 4/9 dogs had a positive directional change and

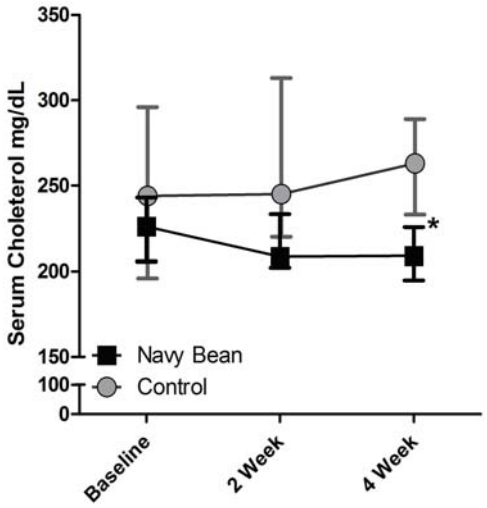

Fig. (3). Serum cholesterol levels of 21 clinically healthy adult dogs during a 4 week dietary intervention trial establishing the safety and digestibility of 25 % w/w navy bean powder consumption compared to an isonutrient control diet. Dogs consuming the navy bean diet (n=10) had significantly lower serum cholesterol levels at 4 weeks compared to dogs consuming the control diet (n=11). * = $p < 0.05$. Data are shown as median and interquartile range.

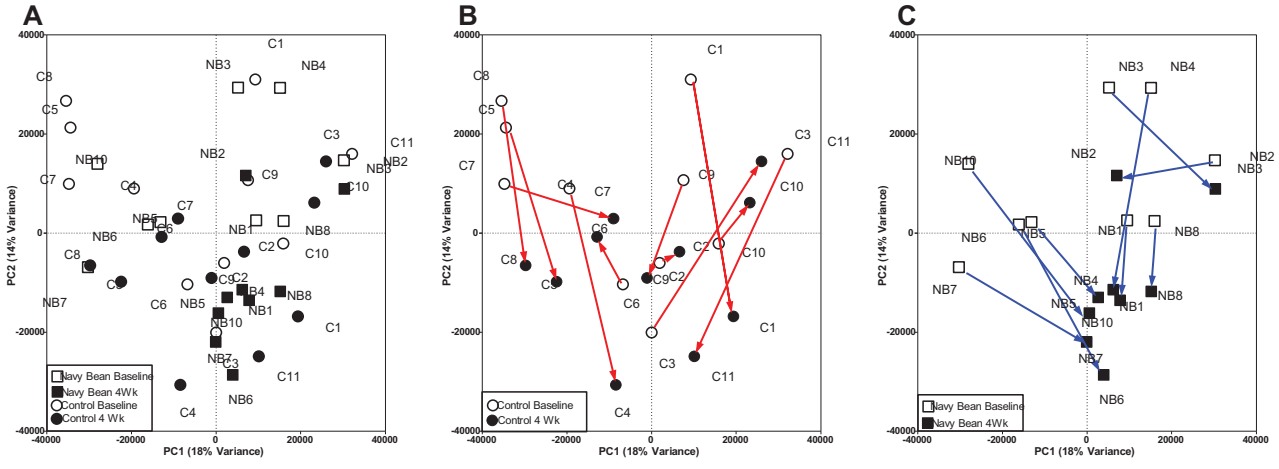

Fig. (4). Fecal metabolite variance between both diet groups, control and navy bean, at baseline and after 4 weeks of consuming either a 25 % w/w navy bean powder or control diet (**A**). Dogs consuming the Navy Bean diet (n=9) are represented by squares and dogs consuming the control diet (n=11) are represented by circles. Dog NB9 was determined to be an outlier at 4 weeks and was not included in the analysis. Variance between the dogs is represented by the distance between each symbol. Variance was not significant between diet groups. The direction of change in variance within the whole model in the control group (**B**) and navy bean group (**C**) is emphasized with arrows.

5/9 dogs had a negative directional change and all dogs had a negative directional change in PC 2. In contrast with the CON group, the sum effect of these directional changes resulted in dogs consuming the NB diet to have less variation from each other compared to baseline (Fig. **4C**).

Fifteen fecal metabolites were identified that showed significant changes over time within each group, or were different between groups at 4 weeks (Table **3**). The relative abundance of these metabolites was not statistically significant between the NB and CON groups at baseline (data not shown). Six metabolites were identified that changed in both the CON and NB diet groups after 4 weeks of consuming the micro- and macro-nutrient matched diets. Data are presented as relative abundance and median fold change from baseline, range of the data within group is given as IQR. The fecal metabolites that decreased in the CON and NB diet groups, respectively, were α-tocopherol: 0.30 (0.20-0.41) and 0.37; lithocholic acid: 0.94 (0.38-1.18) and 0.57 (0.48-1.08); myristic acid: 0.76 (0.71-0.92) and 0.81 (0.71-0.86); margaric acid: 0.73 (0.48-0.88) and 0.69 (0.41-0.96); and a poly-

Table 3. Diet Responsive Canine Fecal Metabolites.

	Metabolite (Library Cluster)	Control Diet Group			Navy Bean Diet Group			*P values*		
		Abundance		*Fold Change*	*Abundance*		*Fold Change*			
		Day 0	Day 28	Day 28:Day 0	Day 0	Day 28	Day 28:Day 0	Diet	Time	Interaction
NB and CON	α-Tocopherol (C40)	3.73 (1.43-5.72)	0.84 (0.75-0.98)	0.30 ↓ (0.20-0.41)	1.72 (0.92-5.54)	0.76 (0.65-0.99)	0.37 ↓ (0.20-0.94)	0.751	0.010	0.850
	Lithocholic acid (C59)	1.10 (0.77-1.95)	0.87 (0.75-1.05)	0.94 ↓ (0.38-1.18)	1.60 (0.81-1.81)	0.92 (0.79-1.02)	0.57 ↓ (0.48-1.08)	0.842	0.076	0.972
	Myristic Acid (C246)	1.04 (0.95-1.37)	0.89 (0.76-1.01)	0.76↓ (0.71-0.92)	1.10 (1.02-1.25)	0.89 (0.79-0.89)	0.81 ↓ (0.71-0.86)	0.950	0.043	0.972
	Margaric acid, putative (C688)	1.23 (0.95-2.31)	0.93 (0.77-1.10)	0.73 ↓ (0.48-0.88)	1.40 (0.79-2.60)	0.84 (0.77-0.96)	0.69 ↓ (0.41-0.96)	0.880	0.027	0.916
	Polycyclic Hydrocarbon (190)	2.76 (0.39-5.06)	1.20 (0.90-1.38)	0.48 ↓ (0.25-2.02)	1.71 (0.34-7.45)	0.71 (0.63-1.01)	0.44 ↓ (0.11-2.37)	0.730	0.085	0.745
	Arachidic Alcohol (C691)	0.38 (0.19-0.79)	1.23 (0.67-2.57)	2.83 ↑ (1.32-5.99)	0.88 (0.43-1.49)	1.82 (1.47-2.14)	1.97 ↑ (1.08-4.29)	0.502	0.011	0.827
CON	Hydroquinone (C428)	0.78 (0.57-0.92)	1.69 (1.38-2.15)	2.44 ↑ (1.84-3.13)	0.81 (0.67-1.14)	1.06 (1.00-1.22)	1.24 (0.89-1.80)	0.694	0.011	0.101
	Cycloartenol (C292)	1.53 (0.97-2.07)	0.76 (0.76-1.00)	0.65 ↓ (0.37-0.79)	1.07 (0.63-1.44)	1.00 (0.87-1.15)	0.90 (0.80-1.41)	0.085	0.080	0.114
NB	Deoxycholic acid (C4)	1.64 (0.77-3.08)	0.88 (0.60-1.15)	0.67 (0.27-1.09)	1.71 (1.00-2.49)	0.93 (0.63-1.27)	0.52 ↓ (0.42-0.93)	0.978	0.058	0.972
	α-Tocopherol acetate (C232)	5.03 (2.07-7.70)	0.70 (0.51-0.96)	0.19 (0.11-0.33)	1.11 (0.97-3.30)	0.56 0.40-0.68)	0.34 ↓ (0.17-0.56)	0.164	0.065	0.438
	Glucose (C1)	0.71 (0.66-1.46)	1.09 (0.92-1.28)	1.43 (0.94-2.13)	0.91 (0.57-1.11)	1.55 (0.86-1.73)	1.64 ↑ (1.22-2.18)	0.958	0.054	0.552
	Steroid (C30)	0.99 (0.87-1.05)	0.86 (0.77-1.09)	0.92 (0.74-1.33)	0.84 (0.67-1.01)	7.22 (5.85-10.07)	9.04 ↑ (6.78-10.86)	0.956	<0.001	0.003
	Steroid (C53)	0.96 (0.24-1.79)	0.41 (0.37-0.90)	0.52 (0.35-3.13)	0.47 (0.21-1.72)	2.95 (1.98-4.38)	4.13 ↑ (1.84-20.5)	0.999	0.065	0.043
	Steroid (C179)	0.54 (0.09-2.56)	0.32 (0.24-0.92)	0.57 (0.09-0.88)	1.20 (0.11-2.31)	12.33 (7.96-24.98)	11.43 ↑ (4.89-52.47)	0.916	0.056	0.102

The normalized, scaled, relative abundance is reported as median (IQR). A mixed model linear regression with a false discovery rate correction was used to determine metabolites that changed over time or between diet groups ($p < 0.10$). Significant differences over time were confirmed by Wilcoxon matched-pairs signed rank test and are indicated by arrows showing the direction of change (↓ or ↑), $p < 0.05$. A Mann Whitney t-test was used to determine if the metabolite was different at baseline. No significant differences between groups were detected at baseline for any of the reported metabolites. Metabolites are sorted by the diet group in which a significant change occurred. NB: navy bean diet group; CON: control diet group.

cyclic hydrocarbon: 0.48 (0.25-2.02) and 0.44 (0.11-2.37). One metabolite increased in the CON and NB diet groups, respectively: arachidic alcohol 2.38 (1.32-5.99) and 1.97 (1.08-4.29). Within the CON diet group, two metabolites changed significantly over time ($p < 0.10$). Excretion of hydroquinone increased by 2.44 fold (1.84-3.13) and cycloartenol, a metabolic precursor of phytosterols, decreased by 0.65 fold (0.37-0.79). While the change over time within the CON group was significant, differences between the CON and NB groups were not significant at either time point for these metabolites. Within the NB diet group, fecal excretion of glucose significantly increased by 1.64 fold (1.22-2.18), deoxycholic acid decreased by 0.52 (0.42-0.93), and α-tocopherol acetate decreased by 0.34 (0.17-0.56). Excretion of three steroid like compounds increased in the NB group: steroid metabolite C30 increased by 9.04 fold (6.78-10.86); steroid metabolite 53 increased by 4.13 (1.84-20.50); and steroid metabolite 179 increased by 11.43 fold (4.89-52.47). These results are shown as the average scaled values at baseline and 4 weeks, and an average fold change within each group in Table **3**.

Phytochemical Detection in Canine Urine by Non-targeted Metabolomics

Urine samples were collected from the 21 dogs in at least one time point; however, sufficient urine samples at both time timepoints were not obtained in three dogs: two CON dogs and one NB dog. Repeated measures statistical analysis was therefore carried out in 9 NB dogs and 9 CON dogs. In the canine urine samples 5,585 ions were detected by LC-MS, these were collapsed into 1,110 clusters. The change in the global profile of urine metabolites was not different between groups, as was seen in the fecal metabolome (Fig. **5**). Fifteen metabolites were identified that were significantly different by diet or time (Table **4**). Data are reported as median and IQR. In both groups, urinary excretion of estradiol, suberic acid, and two peptides increased, while excretion of a third peptide and kynurenine decreased. In the CON group, urinary excretion of a peptide (C63) increased by 514 fold (41.08-1,961.00), excretion of a saccharide (C625) increased by 17.92 fold (6.64-113.33), peptide C180 increased by 10.11 fold (4.01-15.03), and excretion of peptide C17 increased by 3.49 fold (2.23-4.35). In the NB group, a compound with an exact spectral match for trigonelline was detected, but un- retained within the chromatography, and was therefore putatively annotated as trigonelline. In the NB group, urinary trigonelline (putative) excretion increased by 9.73 fold (5.12-13.50), a dipeptide (C59) increased by 2.88 fold (1.69-3.84), peptide C909 increased by 2.23 fold (1.43-2.56), and the flavonoid homoeridoictyol chalcone, an intermediate identified in common bean flavonoid biosynthesis, (as well as many other plants), increased by 2.40 fold (1.11-6.43).

DISCUSSION

This study describes the food metabolome distinctions between an extruded canine diet formulated with 25 % w/w cooked NB powder compared to a nutrient matched CON diet. This study also demonstrates that dogs consuming the NB diet had fecal and urine metabolome changes that indicate modulation of lipid and carbohydrate metabolism.

The targeted analysis of the nutrient profiles demonstrated that lipid, protein, and fiber were similar between the CON and NB diets, while the NB diet had slightly higher levels of soluble fiber (Fig. **1**). The non-targeted GC-MS metabolomics provided an advanced layer of food composition analysis, whereby significant differences in the bean-based food metabolome were detected. (Fig. **2A**). Increases in specific food metabolites such as 2-piperdinecarboxylic acid and s-methyl cysteine (Fig. **2B**) have been reported in multiple common bean cultivars [37] and validated in human and mouse studies as a predictive biomarker of dry bean intake in plasma and fecal samples [38-39]. 2-Piperidinecarboxylic acid was detected in the fecal samples, however its relative abundance did not change over time in either the NB or CON group, and was not significantly different between groups (data not shown). γ-tocopherol and γ-tocopherol acetate were both higher in the NB diet compared

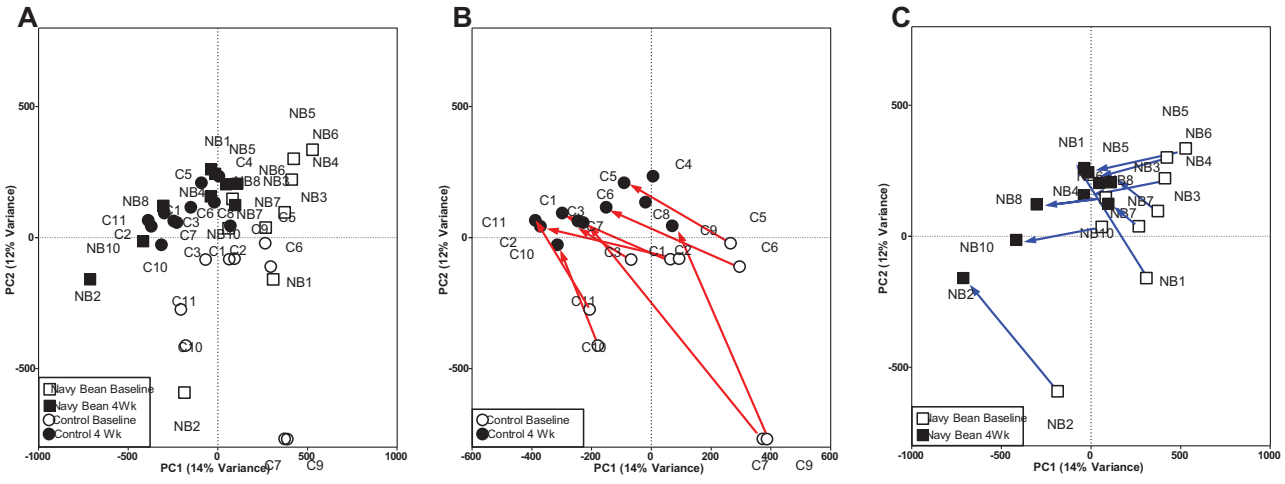

Fig. (5). Urine metabolite variance between both diet groups, control and navy bean at baseline and after 4 weeks of consuming either a 25 % w/w navy bean powder or control diet (**A**). Dogs consuming the navy bean diet (n=9) are represented by squares and dogs consuming the control diet (n=9) are represented by circles. Variance between the dogs is represented by the distance between each symbol. Variance was not significant between diet groups. The direction of change in variance within the whole model in the control group (**B**) and navy bean group (**C**) is emphasized with arrows.

Table 4. Diet Responsive Canine Urine Metabolites.

	Metabolite (Library Cluster)	Control Diet Group			Navy Bean Diet Group			*P values*		
		Abundance		*Fold Change*	*Abundance*		*Fold Change*			
		Day 0	Day 28	Day 28:Day 0	Day 0	Day 28	Day 28:Day 0	Diet	Time	Interaction
NB and CON	Estradiol (C6)	0.41 (0.25-0.80)	3.24 (2.26-5.06)	11.91 ↑ (4.39-17.52)	0.39 (0.24-0.74)	1.39 (0.71-1.50)	3.79 ↑ (1.82-4.42)	0.022	<0.001	0.008
	Peptide (C24)	0.43 (0.37-1.25)	2.40 (1.58-3.07)	5.84 ↑ (2.02-8.14)	0.41 (0.23-0.56)	1.11 (0.82-1.66)	3.33 ↑ (1.30-5.61)	0.054	0.002	0.424
	Peptide (C36)	3.87 (1.22-8.00)	0.62 (0.45-0.74)	0.19 ↓ 0.09-0.46)	3.26 (1.04-4.49)	0.71 (0.50-1.47)	0.40 ↓ (0.19-0.56)	0.665	0.011	0.516
	Peptide (C69)	0.35 (0.23-0.92)	1.38 (1.09-2.15)	3.37 ↑ (1.33-9.83)	0.29 (0.16-0.39)	1.35 (0.87-2.38)	5.88 ↑ (2.58-9.18)	0.925	<0.001	0.750
	Suberic acid (C18)	0.71 (0.54-1.21)	1.53 (1.26-1.81)	2.29 ↑ (1.10-2.77)	0.68 (0.45-0.74)	1.25 (0.98-1.57)	2.18 ↑ (1.40-2.63)	0.334	0.002	0.864
	Kynurenine (C5)	1.22 (0.88-1.76)	0.49 (0.40-0.74)	0.48 ↓ (0.35-0.73)	2.30 (0.75-2.77)	1.08 (0.45-1.80)	0.59 ↓ (0.42-0.76)	0.619	0.007	0.884
CON	Peptide (C63)	0.05 (0.01-0.31)	23.78 (15.90-31.40)	514.10 ↑ (41.08-1961.0)	0.40 (0.24-1.77)	1.17 (0.69-3.28)	2.52 (0.50-4.15)	0.001	<0.001	<0.001
	Peptide (C180)	0.51 (0.27-1.22)	5.16 (2.31-6.23)	10.11 ↑ (4.01-15.03)	0.44 (0.32-0.98)	1.07 (0.39-1.51)	0.15 (0.58-2.63)	0.011	0.002	0.003
	Saccharide (C625)	0.00 (0.00-0.39)	11.34 (4.50-15.29)	17.92 ↑ (6.64-18.33)	1.10 (0.10-2.30)	0.94 (0.00-4.02)	1.05 (0.00-2.56)	0.032	0.002	0.004
	Peptide (C17)	0.64 (0.31-0.75)	1.55 (1.27-2.09)	3.49 ↑ (2.23-4.35)	0.85 (0.56-1.22)	1.37 (1.08-1.64)	1.72 (0.90-2.81)	0.927	<0.001	0.178
NB	Trigonelline, putative (C196)	0.73 (0.54-1.79)	0.53 (0.49-0.92)	0.72 (0.39-1.24)	0.87 (0.49-1.37)	7.19 (4.96-9.61)	9.73 ↑ (5.12-13.50)	<0.001	<0.001	<0.001
	Peptide (C909)	0.92 (0.87-1.16)	1.00 (0.62-1.23)	0.86 (0.74-1.18)	0.75 (0.52-0.96)	1.46 (1.18-1.72)	2.23 ↑ (1.43-2.56)	0.670	0.004	0.002
	homoeriodictyol chalcone (C156)	1.14 (0.37-1.87)	0.51 (0.38-0.63)	0.44 (0.26-0.93)	1.23 (0.17-2.62)	3.70 (1.83-4.75)	2.40 ↑ (1.11-6.43)	0.083	0.350	0.004
	Dipeptide (C59)	1.01 (0.52-1.68)	0.77 (0.54-1.08)	0.82 (0.50-1.53)	0.87 (0.53-1.17)	2.14 (1.79-2.61)	2.88 ↑ (1.69-3.84)	0.087	0.262	0.006
	Kynurenic acid (C32)	0.99 (0.95-1.02)	0.94 (0.85-1.01)	0.94 (0.86-1.02)	1.12 (1.00-1.19)	1.01 (0.80-1.06)	0.89 ↓ (0.78-0.96)	0.897	0.041	0.598

The normalized, scaled, relative abundance is reported as median (IQR). A mixed model linear regression with a false discovery rate correction was used to determine metabolites that changed over time or between diet groups ($p < 0.10$). Significant differences over time were confirmed by Wilcoxon matched-pairs signed rank test and are indicated by arrows showing the direction of change (↓ or ↑), $p < 0.05$. A Mann Whitney t-test was used to determine if the metabolite was different at baseline. No significant differences between groups were detected at baseline for any of the reported metabolites. Metabolites are sorted by the diet group in which a significant change occurred. NB: navy bean diet group; CON: control diet group.

to CON, while α-tocopherol content was similar across diets (Fig. **2B**). This finding is consistent with previous reports for legume seeds showing that the most abundant vitamin E isoform is γ-tocopherol [40]. β-sitosterol is the most abundant phytosterol in beans [41] and was elevated in the NB compared to CON diet. One of the mechanisms by which beans may modulate serum lipid profiles is via the activity of phytosterols which decrease cholesterol solubility in the intestinal lumen, thereby preventing its absorption [5].

This food metabolome analysis also revealed differences in the carbohydrate profiles of the CON and NB diets. Sucrose, and surprisingly, fructose (given the reduced amount of corn) were higher in the NB diet compared to CON, while

glucose and trehalose were lower. Although dietary glucose was lower in the NB diet, dogs consuming the NB diet had increased glucose excretion compared to CON, supporting that the NB diet also resulted in modulation of carbohydrate metabolism. Previous studies evaluating the carbohydrate profiles of whole, boiled beans have found that while relatively low, glucose is slightly higher than fructose, and that the most common carbohydrates present are the complex carbohydrates stachyose, kestose, and raffinose [42]. These α-galactosides are highly fermentable and thought to be the primary inducers of bean-associated flatulence, but may also be responsible for multiple health benefits; including inhibiting growth of pathogenic bacteria, preventing colon cancer, increasing mineral absorption, improving lipid metabolism, and increasing SCFA acid production in the large intestine [43]. Oligosaccharide content of the diets was not determined in this study, however extrusion of bean powders has been shown to reduce oligosaccharide content [44]. One explanation for the higher fructose content in the NB diet is that a fraction of the complex carbohydrates or oligosaccharides, all of which contain fructose, were reduced to simple carbohydrates first by the flour processing method and subsequent extrusion process to make the kibble. This hypothesis is supported by the observation that none of the dog owners reported a change in perceived flatulence in their dogs regardless of the diet consumed [2].

Dogs consuming a 25 % w/w NB bean diet for 4 weeks had significantly lower serum cholesterol levels compared to dogs consuming an iso-caloric and nutrient matched, standard ingredient diet. While blood samples were all collected at approximately the same time of day throughout the study, the dogs were not strictly fasted, which could affect cholesterol levels. However, variation within dog was not significant, supporting that the reported cholesterol levels were reliable. One of the most frequently reported effects of bean intake, in human and laboratory animal studies, is beneficially altered serum lipid profiles [8,12]. This is also consistent with the reported changes in triglycerides and lipid carrier proteins (LDL and HDL) reported from overweight and obese dogs consuming a bean based diet during weight loss [3]. The significant modulation of serum cholesterol by cooked, dry beans in dogs, without concurrent weight loss, is novel support for bean modulation of metabolism in a healthy weight host. The mechanism by which dry bean consumption reduced serum cholesterol in dogs is unknown, but may be attributed to dry bean components that inhibit cholesterol absorption in the small intestine [5, 45-46], prevent reuptake of bile acids via enterohepatic recirculation [47, 48], or increase biliary bile acid and cholesterol secretion [49-51]. Contrary to expectations, given the increased cholesterol and bile acid content in bile associated with bean intake, fecal cholesterol excretion does not increase [48] and fecal bile acid excretion, especially secondary bile acids, actually decrease [47, 49, 52]. Our results confirm these observations in dogs, as no changes in fecal cholesterol excretion were observed (data not shown), and fecal excretion of deoxycholic acid decreased in the NB group (Table **3**). Coprostanol, a microbial byproduct of cholesterol degradation [53], has been reported to increase with bean consumption [47]. However, it is not present in canine feces possibly because the canine gut microbiome lacks the organisms responsible for this conversion [54]. By utilizing a non-targeted metabolomic approach, we found that dogs consuming the NB diet had a 4.13 to 11.43 fold increase in fecal excretion of three steroid-like compounds (Table **3**) not found in metabolite databases. This finding provides rationale for an alternate mechanism by which beans may support beneficial lipid metabolism in dogs. At least one of these compounds has also been detected in human stool samples with increased bean intake (unpublished data), and the identification of these metabolites will be useful to expand our knowledge regarding the bean-facilitated excretion of additional lipid molecules.

Urine metabolome analysis from dogs consuming beans showed that excretion of trigonelline (putative), a phytochemical found in beans, was significantly increased. Interestingly, trigonelline, in humans, is more strongly associated with coffee intake [55], but was recently described as a candidate biomarker of bean intake in human plasma [38]. Trigonelline may be a candidate biomarker of NB intake in dogs, as urinary excretion in this study was not confounded by coffee consumption. This metabolite was not detected in the NB diet analysis as the food metabolome was analyzed using GC-MS, and trigonelline cannot be easily derivatized or volatilized using our GC-MS method. Additional metabolites found in urine of dogs consuming NBs included homoeriodictyol chalcone, a metabolite in a flavonoid biosynthesis identified in *Phaseolus vulgaris* (KEGG pathway pvu00941). Unlike the fecal metabolome, there was not a diet associated directional shift in the urinary metabolome. While there were metabolites that changed by both diet and time, the NB diet responsive metabolites were phytochemicals and peptides that may serve as candidate biomarkers for common bean intake.

CONCLUSION

In addition to providing a high quality source of nutrients, a 25 % w/w NB dog diet formulation was shown to have a distinct phytochemical composition compared to a standard, commercial diet formulation. The presence of bean phytochemicals in urine demonstrated the ability to detect candidate biomarkers of bean intake in dogs. Healthy weight companion dogs consuming a navy bean-based diet showed changes in lipid and carbohydrate metabolism that merit further investigation for improving the health and lifespan of companion dogs as well as possible nutritional therapy for dogs with metabolic disorders. These data also support the utility of nutritional metabolomics in companion dogs.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher's web site along with the published article: **Supplemental Appendix A,** LC and GC run conditions for metabolite separation and detection**; Supplemental Table 1,** Metabolite clusters and annotation confidence levels for compounds detected in canine diets, feces, and urine with GC- or LC-MS; **Canine Diet Metabolites,** Spectral library; **Canine Fecal Metabolites,** Spectral library; **Canine Urine Metabolites,** Spectral Library.

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